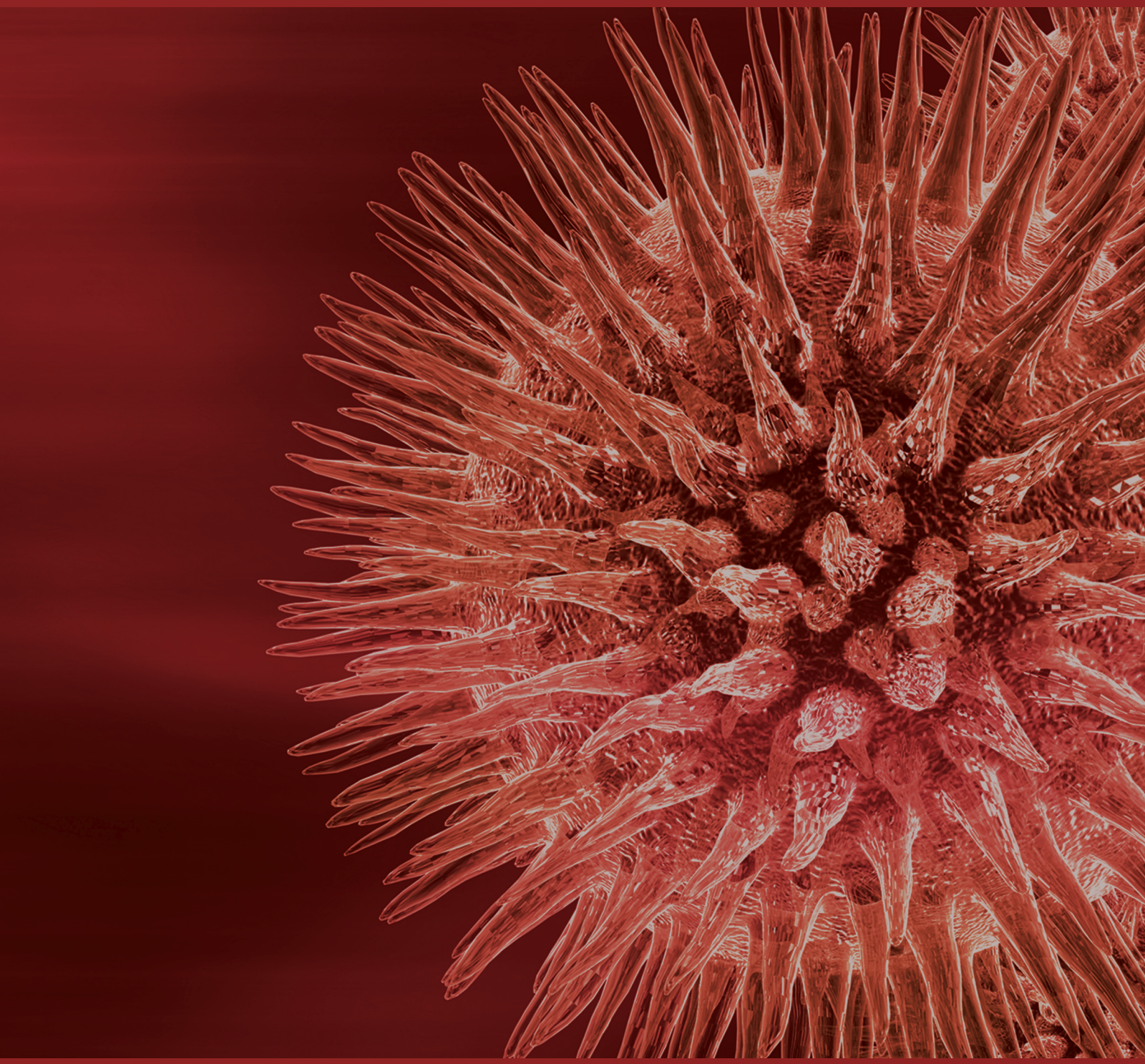
Journal of Biomedicine and Biotechnology

Mercury Toxicity

Guest Editors: João B. T. Rocha, Michael Aschner, José G. Dórea, Sandra Ceccatelli, Marcelo Farina, and Luiz Carlos L. Silveira



Mercury Toxicity

Journal of Biomedicine and Biotechnology

Mercury Toxicity

Guest Editors: João B. T. Rocha, Michael Aschner, José G. Dórea, Sandra Ceccatelli, Marcelo Farina, and Luiz Carlos L. Silveira

Editorial Board

The editorial board of the journal is organized into sections that correspond to the subject areas covered by the journal.

Agricultural Biotechnology

Ahmad Zuhairi Abdullah, Malaysia
Guihua H. Bai, USA
Christopher P. Chanway, Canada
Ravindra N. Chibbar, Canada
Adriana S. Franca, Brazil
Ian Godwin, Australia
Hari B. Krishnan, USA
Carol A. Mallory-Smith, USA
Xiaoling Miao, China
Dennis P. Murr, Canada
Rodomiro Ortiz, Sweden
Encarnación Ruiz, Spain
B. C. Saha, USA
Abdurrahman Saydut, Turkey
Mariam B. Sticklen, USA
Kok Tat Tan, Malaysia
Chiu-Chung Young, Taiwan

Animal Biotechnology

E. S. Chang, USA
Bhanu P. Chowdhary, USA
Noelle E. Cockett, USA
Peter Dovc, Slovenia
Scott C. Fahrenkrug, USA
Dorian J. Garrick, USA
Thomas A. Hoagland, USA
Tosso Leeb, Switzerland
James D. Murray, USA
Anita M. Oberbauer, USA
Jorge A. Piedrahita, USA
Daniel Pomp, USA
Kent M. Reed, USA
Lawrence Reynolds, USA
Lawrence B. Schook, USA
Mari A. Smits, The Netherlands
Leon Spicer, USA
J. Verstegen, USA
Matthew B. Wheeler, USA
Kenneth L. White, USA

Biochemistry

David Ronald Brown, UK
Saulius Butenas, USA
Vittorio Calabrese, Italy
Miguel Castanho, Portugal
Francis J. Castellino, USA
Roberta Chiaraluce, Italy
D. M. Clarke, Canada
Francesca Cutruzzolà, Italy
Paul W. Doetsch, USA
Hicham Fenniri, Canada
Nick V. Grishin, USA
J. Guy Guillemette, Canada
Paul W. Huber, USA
Chen-Hsiung Hung, Taiwan
Maria Jerzykiewicz, Poland
Michael Kalafatis, USA
B. E. Kemp, Australia
Phillip E. Klebba, USA
Wen-Hwa Lee, USA
George Makhatadze, USA
Leonid Medved, USA
Susan A. Rotenberg, USA
Jason Shearer, USA
Andrei Surguchov, USA
John B. Vincent, USA
Y. George Zheng, USA

Bioinformatics

T. Akutsu, Japan
Miguel A. Andrade, Germany
Mark Y. Borodovsky, USA
Rita Casadio, Italy
David Corne, UK
Sorin Draghici, USA
Eugénio Ferreira, Portugal
Stavros J. Hamodrakas, Greece
Paul Harrison, USA
George Karypis, USA
Guohui Lin, Canada
Satoru Miyano, Japan
Zoran Obradovic, USA
Florencio Pazos, Spain
Zhirong Sun, China
Ying Xu, USA
Alexander Zelikovsky, USA
Albert Zomaya, Australia

Biophysics

Miguel Castanho, Portugal
P. Bryant Chase, USA
Kuo-Chen Chou, USA
Rizwan Khan, India
Ali A. Khraibi, Saudi Arabia
Rumiana Koynova, USA
Serdar Kuyucak, Australia
Jianjie Ma, USA
S. B. Petersen, Denmark
Peter Schuck, USA
Claudio M. Soares, Portugal

Cell Biology

Omar Benzakour, France
Sanford I. Bernstein, USA
Phillip I. Bird, Australia
Eric Bouhassira, USA
Mohamed Boutjdir, USA
Chung-Liang Chien, Taiwan
Richard Gomer, USA
Paul J. Higgins, USA
Pavel Hozak, Czech Republic
Xudong Huang, USA
Anton M. Jetten, USA
Seamus J. Martin, Ireland
Manuela Martins-Green, USA
Shoichiro Ono, USA
George Perry, USA
M. Piacentini, Italy
George E. Plopper, USA
Lawrence Rothblum, USA
Michael Sheetz, USA
James L. Sherley, USA
G. S. Stein, USA
Richard Tucker, USA
Thomas van Groen, USA
Andre Van Wijnen, USA
Steve Winder, UK
Chuanyue Wu, USA
Bin-Xian Zhang, USA

Genetics

Adewale Adeyinka, USA
Claude Bagnis, France
J. Birchler, USA
Susan Blanton, USA
Barry J. Byrne, USA
R. Chakraborty, USA
Domenico Coviello, Italy
Sarah H. Elsea, USA
Celina Janion, Poland
J. Spencer Johnston, USA
M. Ilyas Kamboh, USA
Feige Kaplan, Canada
Manfred Kayser, The Netherlands
Brynn Levy, USA
Xiao Jiang Li, USA
Thomas Liehr, Germany
James M. Mason, USA
Mohammed Rachidi, France
Raj S. Ramesar, South Africa
Elliot D. Rosen, USA
Dharambir K. Sanghera, USA
Michael Schmid, Germany
Markus Schuelke, Germany
Wolfgang Arthur Schulz, Germany
Jorge Sequeiros, Portugal
Mouldy Sioud, Norway
Rongjia Zhou, China

Genomics

Vladimir Bajic, Saudi Arabia
Margit Burmeister, USA
Settara Chandrasekharappa, USA
Yataro Daigo, Japan
J. Spencer Johnston, USA
Vladimir Larionov, USA
Thomas Lufkin, Singapore
John L. McGregor, France
John V. Moran, USA
Yasushi Okazaki, Japan
Gopi K. Podila, USA
Momiao Xiong, USA

Immunology

Hassan Alizadeh, USA
Peter Bretscher, Canada
Robert E. Cone, USA
Terry L. Delovitch, Canada
Anthony L. DeVico, USA
Nick Di Girolamo, Australia
Don Mark Estes, USA
Soldano Ferrone, USA
Jeffrey A. Frelinger, USA
John Robert Gordon, Canada
James D. Gorham, USA
Silvia Gregori, Italy
Thomas Griffith, USA
Young S. Hahn, USA
Dorothy E. Lewis, USA
Bradley W. McIntyre, USA
R. Lee Mosley, USA
Marija Mostarica-Stojković, Serbia
Hans Konrad Muller, Australia
Ali Ouaissi, France
Kanury V. S. Rao, India
Yair Reisner, Israel
Harry W. Schroeder, USA
Wilhelm Schwaeble, UK
Nilabh Shastri, USA
Yufang Shi, China
Piet Stinissen, Belgium
Hannes Stockinger, Austria
Graham R. Wallace, UK

Microbial Biotechnology

Suraini Abd-Aziz, Malaysia
Jozef Anné, Belgium
Nuri Azbar, Turkey
Yoav Bashan, Mexico
Marco Bazzicalupo, Italy
Hakan Bermek, Turkey
Nico Boon, Belgium
José Luis Campos, Spain
Yinguang Chen, China
Luca Simone Cocolin, Italy
Peter Coloe, Australia
Daniele Daffonchio, Italy
Han de Winde, The Netherlands
Raf Dewil, Belgium
Jos Domingos Fontana, Brazil
Petros Gikas, Greece
Tom Granstrom, Finland
Ismail Kiran, Turkey
Hongjuan Liu, China
Yanhe Ma, China
Paula Loureiro Paulo, Brazil
Bernd H A Rehm, New Zealand
Alberto Reis, Portugal
Muthuswamy Sathishkumar, Singapore
Ramkrishna Sen, India
Angela Sessitsch, Austria
Ya-Jie Tang, China
Orhan Yenigun, Turkey
Eileen Hao Yu, UK

Microbiology

D. Beighton, UK
Steven R. Blanke, USA
Stanley Brul, The Netherlands
Isaac K. O. Cann, USA
Stephen K. Farrand, USA
Alain Filloux, UK
Gad Frankel, UK
Roy Gross, Germany
Hans-Peter Klenk, Germany
Tanya Parish, UK
Gopi K. Podila, USA
Frederick D. Quinn, USA
Didier A. Raoult, France
Isabel Sá-Correia, Portugal
P. L. C. Small, USA
Michael Thomm, Germany
H. C. van der Mei, The Netherlands
Schwan William, USA

Molecular Biology

Rudi Beyaert, Belgium
Michael Bustin, USA
Douglas Cyr, USA
K. Iatrou, Greece
Lokesh Joshi, Ireland
David W. Litchfield, Canada
Wuyuan Lu, USA
Patrick Matthias, Switzerland
John L. McGregor, France
S. L. Mowbray, Sweden
Elena Orlova, UK
Yeon-Kyun Shin, USA
William S. Trimble, Canada
Lisa Wiesmuller, Germany
Masamitsu Yamaguchi, Japan

Oncology

Colin Cooper, UK
F. M. J. Debruyne, The Netherlands
Nathan Ames Ellis, USA
Dominic Fan, USA
Gary E. Gallick, USA
Daila S. Gridley, USA
Xin-yuan Guan, Hong Kong
Anne Hamburger, USA
Manoor Prakash Hande, Singapore
Beric Henderson, Australia
Daehee Kang, Republic of Korea
Abdul R. Khokhar, USA
Rakesh Kumar, USA
Macus Tien Kuo, USA
Eric W. Lam, UK
Sue-Hwa Lin, USA
Kapil Mehta, USA
Orhan Nalcioglu, USA
P. J. Oefner, Germany
Allal Ouhtit, Oman
Frank Pajonk, USA
Waldemar Priebe, USA
F. C. Schmitt, Portugal
Sonshin Takao, Japan
Ana Maria Tari, USA
Henk G. Van Der Poel, The Netherlands
Haodong Xu, USA
David J. Yang, USA

Pharmacology

Abdel A. Abdel-Rahman, USA
M. Badr, USA
Stelvio M. Bandiera, Canada
Ronald E. Baynes, USA
R. Keith Campbell, USA
Hak-Kim Chan, Australia
Michael D. Coleman, UK
J. Descotes, France
Dobromir Dobrev, Germany
Ayman El-Kadi, Canada
Jeffrey Hughes, USA
Kazim Husain, USA
Farhad Kamali, UK
Michael Kassiou, Australia
Joseph J. McArdle, USA
Mark J. McKeage, New Zealand
Daniel T. Monaghan, USA
T. Narahashi, USA
Kennerly S. Patrick, USA
Vickram Ramkumar, USA
Michael J. Spinella, USA
Quadiri Timour, France
Todd W. Vanderah, USA
Val J. Watts, USA
David J. Waxman, USA

Plant Biotechnology

Prem L. Bhalla, Australia
J. R. Botella, Australia
Elvira Gonzalez De Mejia, USA
Shi-You Ding, USA
Metin Guru, Turkey
H. M. Häggman, Finland
Liwen Jiang, Hong Kong
P. B. Kirti, India
Yong Pyo Lim, Republic of Korea
Gopi K. Podila, USA
Ralf Reski, Germany
Sudhir Sopory, India

Toxicology

Michael Aschner, USA
Juergen Buenger, Germany
Michael L. Cunningham, USA
Laurence D. Fechter, USA
Hartmut Jaeschke, USA
Y. James Kang, USA
M. Firoze Khan, USA
Pascal Kintz, France
Qaisar Mahmood, Pakistan
R. S. Tjeerdema, USA
Kenneth Turteltaub, USA
Brad Upham, USA

Virology

Contents

Hindawi Publishing Corporation
Journal of Biomedicine and Biotechnology
Volume 2012, Article ID 831890, 2 pages
doi:10.1155/2012/831890

Editorial
Mercury Toxicity

João B. T. Rocha,[1] Michael Aschner,[2] José G. Dórea,[3] Sandra Ceccatelli,[4] Marcelo Farina,[5] and Luiz Carlos L. Silveira[6]

[1] *Pós-Graduação em Bioquímica Toxicológica, CCNE, Universidade Federal de Santa Maria, 97105-900 Santa Maria, RS, Brazil*
[2] *Department of Pediatrics, Vanderbilt University Medical Center, 2215-B Garland Avenue 11415 MRB IV Nashville, TN 37232-0414, USA*
[3] *Departamento de Nutrição, Universidade de Brasília, CP 04322 70919-970 Brasília, DF, Brazil*
[4] *Department of Neuroscience, Karolinska Institutet, 17177 Stockholm, Sweden*
[5] *Departamento de Bioquímica, CCB, Universidade Federal de Santa Catarina, 88040-900 Florianópolis, SC, Brazil*
[6] *Núcleo de Medicina Tropical, Universidade Federal do Pará, 66055-240 Belém, PA, Brazil*

Correspondence should be addressed to João B. T. Rocha, jbtrocha@yahoo.com.br

Received 8 August 2012; Accepted 8 August 2012

Mercury (Hg) is one of the most toxic elements in the periodic table. Although Hg is present in nature, it has also been released into the environment for centuries as a result of anthropogenic activities. Nowadays, there are efforts to reduce its anthropogenic use; however, its environmental presence is significant and will persist. We are pleased to present this special issue on mercury toxicity. The objective of collecting research findings in a single issue devoted to the toxicology of mercury was to compile reports on the latest findings on Hg's toxicity from renowned research groups across the world. This special issue affords the opportunity to bring together a wide range of review and research papers devoted both to basic and applied toxicity associated with various exposure scenarios and Hg species (dental material, iatrogenic ethylmercury, fish-methylmercury) along with comprehensive description on experimental models. While human studies demonstrated the noxious effects of these forms of Hg, experimental studies have assisted in defining mechanistic pathways central to Hg's toxicity in various tissues and organ systems.

The volume is dedicated in part to articles that provide new insights on important considerations of subtle effects of exposure to multiple forms of organic mercury (ethylmercury in thimerosal-containing vaccines and methylmercury (MeHg) derived from maternal fish consumption) and neurological outcomes in infants (J. G. Dórea et al.). In addition, hypersensitivity to low-dose Hg exposure from dental amalgam fillings is detailed, showing exquisite sensitivity to amalgam-derived Hg in sensitized individuals (H. McParland and S. Warnakulasuriya). Local effects of amalgam and Hg dental restoration represent the most important nonoccupational exposure to inorganic mercury, while fish consumption represents the most common source of MeHg exposure.

The impacts of exposure to fish-derived MeHg at levels below those considered to pose neurological risk (hair level: 50 μg/g) were explored by Japanese researchers in subjects of the Niigata mercury poisoning (K. Maruyama et al.). Experimental research papers from this issue confirmed and extended observations that exposure of immature rodents to different chemical forms of Hg is associated with differential bodily distribution Hg (M. Blanuša et al.; C.-F. Huang et al.). C.-F. Huang et al. demonstrated that exposure of developing rats to cinnabar (HgS) caused long-lasting neurobehavioral and neurochemical toxic effect, indicating that the use of this millenary component of traditional Chinese medicine continues to represent a toxicological concern. Using an important, yet little explored experimental mouse model, J. P. Bourdineaud and colleagues demonstrated that the ingestion of MeHg-adulterated fish led to higher neurotoxicity in comparison to the ingestion of the "free salt" of methylmercury chloride (MeHgCl). The scarcity of studies on this subject highlights the need for future studies to address these persistent toxicological issues.

The molecular, subcellular, cellular, and systemic toxicity of Hg was also addressed here in this volume. The cardiovascular toxicity of Hg in humans and rodents was reviewed by B. F. Azevedo et al. The impact of Hg exposure on endothelial cell physiology is well established; however, the limit of dietary-derived Hg needed to trigger cardiotoxic effects is still debatable. The negative impact of oral exposure to Hg(II) on reproductive performance of male rats was demonstrated by J. C. Heath and collaborators, highlighting the need for detailed studies to determine the nonobservable adverse effect level (NOAEL) of Hg(II) in the male reproductive system, as well as Hg deposition in target tissues. The comparative renal and hepatic toxicity of Hg(II) and MeHg in fish was addressed by V. Branco et al., demonstrating that both forms of mercury targeted the antioxidant selenoenzyme thioredoxin-reductase (TrxR) and reinforcing the central role of disrupted selenoprotein function in mercurial toxicity. The *in vitro* and *in vivo* targeting of the critical sulfhydryl-containing enzyme, Na^+, K^+-ATPase was reviewed by I. Kade and addressed by T. S. Huang et al., noting divergent effects *in vitro* and *in vivo*. The role of mitochondria and calcium in the neurotoxicity of MeHg was reviewed by D. Roos et al., providing evidence that Ca^{2+}, glutamate, oxidative stress, and mitochondria play a central role in its neurotoxicity. The efficacy of the marine *n*-3 fatty acids, eicosapentaenoic acid (EPA), and docosahexaenoic acid (DHA) in attenuating MeHg-induced toxicity was studied in fish and mammalian cell cultures. O. J. Nøstbakken et al. demonstrated that DHA decreased MeHg uptake into mammalian cells but increased MeHg-induced apoptosis in fish cells.

We hope that the new findings on the subtle effects of combined exposure to iatrogenic ethylmercury (from thimerosal-containing vaccines) and maternal MeHg (from fish consumption), as well as the results of experimental studies and the critical reviews presented herein can shed novel information on mercury's absorption, distribution, metabolism, and excretion, as well as its ill effects at the cellular, molecular, and organismal levels. Understanding of these facets of research is required for derivation on environmental and health policies as well as guidance for the most promising future research venues. Finally, we would like to thank all the reviewers that have contributed their time and insight to this special issue as well as the journal's personnel (particularly Doaa Hassan) for their support and making possible the publication of this special issue.

João B. T. Rocha
Michael Aschner
José G. Dórea
Sandra Ceccatelli
Marcelo Farina
Luiz Carlos L. Silveira

Hindawi Publishing Corporation
Journal of Biomedicine and Biotechnology
Volume 2012, Article ID 924549, 12 pages
doi:10.1155/2012/924549

Review Article

Mercury Toxicity on Sodium Pump and Organoseleniums Intervention: A Paradox

Ige Joseph Kade[1,2]

[1] *Department of Biochemistry, Federal University of Technology, FUTA Road, PMB 704, Akure, Ondo State, Nigeria*
[2] *Departamento de Farmacobiología, Centro de Investigación y de Estudios Avanzados, Sede Sur, Calzada de los Tenorios 235, Colonia Granjas Coapa, DF, 14330 México, Mexico*

Correspondence should be addressed to Ige Joseph Kade, ijkade@yahoo.com

Received 9 April 2012; Accepted 1 July 2012

Academic Editor: João B. T. Rocha

Mercury is an environmental poison, and the damage to living system is generally severe. The severity of mercury poisoning is consequent from the fact that it targets the thiol-containing enzymes, irreversibly oxidizing their critical thiol groups, consequently leading to an inactivation of the enzyme. The Na^+/K^+-ATPase is a sulfhydryl protein that is sensitive to Hg^{2+} assault. On the other hand, organoseleniums are a class of pharmacologically promising compounds with potent antioxidant effects. While Hg^{2+} oxidizes sulfhydryl groups of Na^+/K^+-ATPase under *in vitro* and *in vivo* conditions, the organoselenium compounds inhibit Na^+/K^+-ATPase *in vitro* but enhance its activities under *in vivo* conditions with concomitant increase in the level of endogenous thiols. Paradoxically, it appears that these two thiol oxidants can be used to counteract one another under *in vivo* conditions, and this hypothesis serves as the basis for this paper.

1. Introduction

There are several earlier and more contemporary reviews on the toxicology of mercury and its compounds available to readers that cover a spectrum of findings ranging between types and classifications, sources of mercury exposure to the toxicity of different species and biological outcomes [1–7]. Yearly, data and reviews on mercury and its toxic effects are emerging on an astronomical scale. Hence, the objective of this paper is not to duplicate the earlier reviews but to sensitize readers on intervention strategies that recent authors are pointing to. In this regard, this paper will focus on the toxicity of mercury on a key transmembrane enzyme transporter, Na^+/K^+-ATPase (otherwise known as sodium pump), and the possible intervention of an emerging class of selenium-based compounds—the organoselenium compounds to ameliorate the toxic effect of mercury on this important transmembrane enzyme. Consequently, this paper will present recent findings in the last few years on the observed effect of mercurial compounds on sodium pump and the paradox surrounding the use of organoselenium as possible intervention in mercury toxicity on the activities of sodium pump.

2. Sodium Pump: A Brief Overview

In 1957, Skou described an ATPase preparation from crab nerve membranes that was stimulated by addition of Na^+ and K^+ to the incubation medium [8], and he proposed that this enzymatic activity was associated with the physiological mechanism for active transport of monovalent cations, across the plasma membrane, and eventually this enzymic system was christened Na^+/K^+-ATPase or sodium pump. Na^+/K^+-ATPase transforms chemical energy in ATP to osmotic work and maintains electrochemical Na^+ and K^+ gradients across cell membranes. At rest, Na^+/K^+-ATPase converts 20–30% of the current ATP production in mammals to active Na^+ and K^+ transport in kidney, central nervous system, and other cells of the body where Na^+ and K^+ gradients are required for maintaining membrane potential and volume of animal cells and organelles bounded by flexible lipid

bilayers. Lowering the intracellular Na^+ concentration helps to prevent cell lysis. The electrochemical gradient generated by the unequal exchange of Na^+ for K^+ is used to drive a variety of fundamental physiological processes. The electrical gradient contributes to the resting membrane potential, thereby affecting channel-regulated electrical stimulation of nerve and muscle cells. The Na^+ gradient is the energy source for facilitated transport of ions (e.g., Na^+/Ca^{2+} exchange) and metabolites (e.g., Na^+/glucose cotransport) by carriers. Presumably because the pump is essential for cell viability, Na^+/K^+-ATPase has also been indirectly implicated in the etiology of diseases like essential hypertension and diabetes. Sodium pump is the receptor for cardiac glycosides, like digitalis, used to treat patients with congestive heart failure. Several excellent reviews have described in details the structure and function of the Na^+/K^+-ATPase, and I encourage the readers to consult these references [9–12].

3. Transmembrane Topology

Of importance in this paper is a brief description of the Na^+/K^+-ATPase especially with emphasis on the amino acid composition of the active sites of the pump. Generally, Na^+/K^+-ATPase belongs to the P-type ATPases, and like the H^+/K^+-ATPase, it is composed of majorly two subunits. The larger constituent of the heterodimer is the α-subunit with a molecular mass of about 112000 Da which is responsible for both catalytic and transport function [11]. The α-subunit comprises an acceptor for the γ-phosphate of ATP, phosphorylation of the aspartic residue Asp-369 [13] which leads to formation of the phosphorylated intermediate during the reaction cycle [14]. In addition to the cation binding sites, also the receptor for cardiac glycosides is located on the α-subunit [15]. The smaller constituent is the glycosylated β-subunit with a molecular mass of about 55 000 Da. This subunit was for a long time believed not to participate directly in the catalytic cycle or the binding of cardiac glycosides. Recently, it was demonstrated that the assembly of an (α/β) heterodimer is necessary for a stable and functionally competent configuration of the pump; in particular, the β subunit is needed for the α-subunit to exit from the endoplasmic reticulum and to acquire the correct configuration [16–18]. A third subunit with a molecular mass of about 12000 Da has been detected as a part of the native enzyme assembly [19]. However, the importance of this so-called γ-subunit for neither ATPase or phosphatase activity nor ion transport characteristics has been demonstrated so far [20]. All these subunits have been cloned and sequenced from a variety of tissues and animal species [11].

4. Binding Sites of the Na^+/K^+-ATPase

By definition as P-type ATPase, the sodium pump molecule is temporarily phosphorylated during the reaction cycle. Demonstration that an aspartate residue accepts the γ-phosphate from ATP during its hydrolysis was provided by Post and Kume [14]. Localization of the phosphorylation site has been achieved by means of controlled tryptic digestion [21]. After sequence analysis of the sheep kidney enzyme, Asp-369 has been identified as the target for catalytic phosphorylation [13]. The consensus sequence Cys-Ser-Asp-376-Lys-Thr-GIy-Thr-Leu-Thr around the phosphorylation site of the α-subunit is highly conserved among α-subunit isoforms and even with respect to other P-type ATPases like H^+, K^+-ATPase, sarcoplasmic reticulum Ca^{2+}-ATPase, and the yeast H^+-ATPase [22]. Mutations of Asp-376 α-subunit to Asn, Glu, or Thr resulted in loss of enzyme function [23]. Seven amino acid residues Asp-376, Lys-487, Lys-507, Cys-663, Asp-716, Asp-720, and Lys-725 are suggested to participate in the formation of the ATP-binding pocket [24, 25].

With respect to the cationic binding sites, the role of transmembrane glutamic acid residues in cation binding was investigated by the technique of site-directed mutagenesis. Mutations of Glu-955 and Glu-956 of rat α-subunit (corresponding to Glu-959 and Glu-960 of the *Torpedo* pump) to glutamine or aspartic acid revealed no differences in cation stimulation of ATPase activity compared to the wild type if measured in open membrane fragments. This has been taken as evidence that the carboxyl groups of these glutamic acid residues are not essential for cation binding or occlusion. In addition to these glutamic amino acid residues, Pro-333 and Leu-337 (Pro-328 and Leu-332 of the α-subunit) of the Pro-Glu-334-Gly-Leu-Leu range have been suggested to be important for the Na^+ and K^+ affinities [26]. It has been discussed that particularly the proline is involved in optimizing cation binding to the glutamic acid residue Glu-334 in the E_2 conformation [27]. A diagrammatic representation of the operation of the sodium pump is presented in Figure 1.

5. Interaction of Mercurial Compounds with Sodium Pump

Excellent findings and reviews have shown that Hg^{2+} has very strong affinity for thiols on proteins. And considering the topology of Na^+/K^+-ATPase, the ATP and cationic binding sites are potential targets for Hg^{2+} assaults. For example, the nucleotide binding site contains SH group that favors the binding of Hg^{2+}. Herein, it suffices to say that since inorganic mercury salts and other nonorganic forms of mercury only poorly penetrate the cerebral microvascular endothelial cells comprising the blood-brain barrier (BBB), their neurotoxicity may be predicted to result from interference with this transport enzyme in other tissues. In addition, it is of note that specific binding of Hg^{2+} to ouabain-sensitive Na^+/K^+-ATPase of rat liver plasma membrane was demonstrated and observed that the binding of mercury to the enzyme also causes significant inhibition of the enzyme, which is greater than its ouabain sensitivity. Also it has been established that in the cytosol Hg^{2+} binding to reduced glutathione (GSH) is stimulated by GSH-S-transferase (GST). It is proposed that the transport of Hg^{2+} inside the cell takes place by increased dissociation of Hg^{2+} from the membrane due to greater avidity of Hg^{2+} towards cytosolar GSH binding. The GSH-Hg complex enters the nucleus where it dissociates to bind the metal response element (MRE) of the metallothionein (MT) gene to induce MT transcription. In fact, a schematic

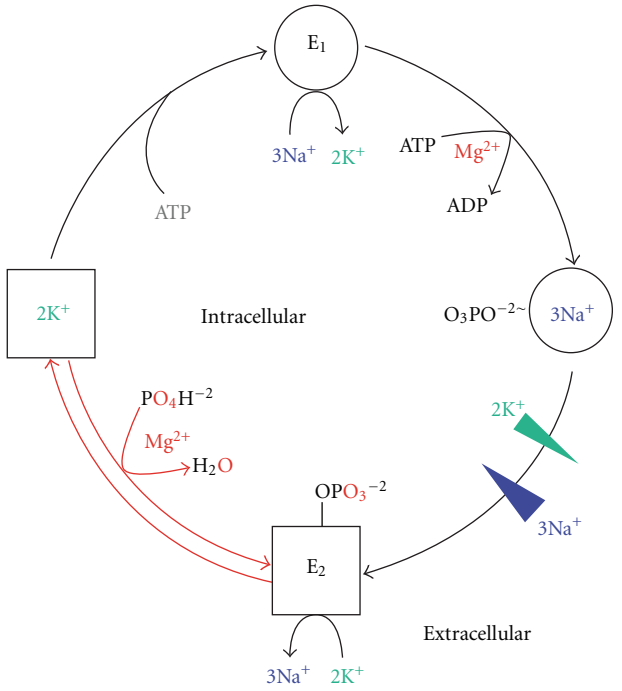
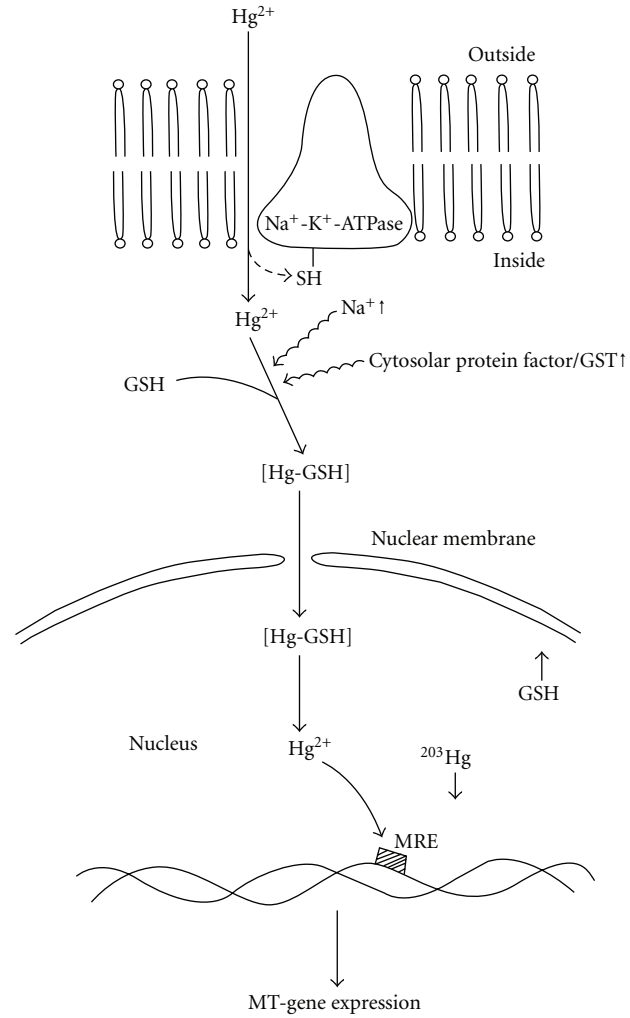


Figure 1: Reaction cycle. The minimal steps in the catalysis-transport cycle of sodium pump are shown schematically. Black arrows indicate the normal, clockwise direction of the reaction sequence. Oxygen isotope (red) exchange may occur in the reversible step colored red. The cycle represents the sidedness, as well as repetition, of the reactions. For example, the 3 Na^+ (blue) that displace 2 K^+ (green) intracellularly are subsequently displaced by 2 K^+ extracellularly, generating the inward Na^+ (larger) and outward K^+ (smaller) gradients indicated by wedge shapes. A circle and a square symbolize the limiting protein conformations (E_1 and E_2, resp.) with enclosed ions indicating occlusion. Different colors denote ATP acting as an effector (gray) or substrate (black) [9].



Scheme 1: The model proposes a mechanism of signal transduction by inorganic mercury in the rat hepatocyte, inducing metallothionein synthesis. Hg first binds to the Na^+-K^+-ATPase at the inner surface of the membrane and then dissociates to bind to GSH which is preferred due to higher affinity of the metal for GSH. The Hg-GSH complex enters the nucleus through the 9 nm aqueous channel of the nuclear membrane where Hg induces the expression of the metallothionein gene by binding to the metal responsive element [28].

illustration of the possible differential avidity of Hg^{2+} towards different thiols in cellular compartment is illustrated in the excellent work described by Bhattacharya and his coworkers (Scheme 1, [28]). At this point, information will be abstracted from available findings by various authors with respect to binding and subsequent toxicity of Hg^{2+} on the activity of the sodium pump under *in vitro* and *in vivo* conditions.

6. Mercury Toxicity on Sodium Pump *In Vitro*

In 1986, Kramer et al. [29] observed that among other heavy metals, Hg^{2+} ranked the highest in their inhibition of the Na^+/K^+-ATPase. Therefore, this finding showed that mercury toxicity on sodium pump is of significance and deserved critical attention. In that paper [29], they observed that the degree of inhibition of sodium pump by heavy metals is in the order: $Hg^{2+} > Pb^{2+} > Cd^{2+} > Ur^{2+} > Cu^{2+} > Zn^{2+} > Mn^{2+} > Ba^{2+} > Ni^{2+}Sr^{2+}$. Enzyme kinetic studies showed that Hg^{2+} and other heavy metals such as Pb^{2+}, and Cd^{2+} competitively, whereas Cu^{2+} noncompetitively, inhibited the enzyme. Similarly, in the same year, Magour [30] showed that mercurial compounds inhibited the brain synaptosomal Na^+/K^+-ATPase *in vitro*. In the paper [30], he observed that $HgCl_2$ is a more powerful inhibitor than methylmercury (MeHg). But in contrast to Kramer et al., they reported that both mercurials exhibited a noncompetitive type of inhibition. He observed however that the nonessential lipids do not play a significant role in the inhibitory effect of MeHg and that the potency of these mercurials to inhibit brain synaptosomal Na^+/K^+-ATPase largely depends on their capacity to block sulfhydryl groups. In the following year, Magour and his collaborators [31] revealed that the nonionic detergent Lubrol potentiated the inhibitory effect of mercurial compounds on the Na^+/K^+-ATPase, and they concluded that Lubrol removes the bulk lipids present

outside the catalytic center of the enzyme. Consequently, they concluded that the enzyme was more sensitive to the inhibition by both mercurials. However, in my opinion, this argument is not plausible since such lipid moiety that possibly shields the sodium pump as proposed by Magour and his associates can equally serve as a hindrance for the entry of the substrates (Na^+ and K^+) of the sodium pump as well as interaction of Mg^{2+} with the ATP. It is reasonable to conclude that Lubrol could possibly alter the fluid mosaic bilayer structure anchoring the sodium pump consequently leading to an altered three-dimensional structure of the transmembrane pump under consideration. Apparently, the emerging transmembrane protein structure could be such that the partially embedded thiols on the protein are more exposed and consequently affording a more direct interaction of Hg^{2+} with these exposed thiols, thus leading to a potentiated inhibitory effect of Hg^{2+} on the sodium pump. Alternatively, experimental evidence in our laboratory (data not yet published) has revealed that prooxidants that induce lipid peroxidation can also cause a diminished activity of the pump. With this preliminary observation, it is rational to conclude that lubrol may per se have a direct inhibitory effect on the pump by disrupting the lipids anchoring the sodium pump ultimately leading to a loss of activity which was potentiated by Hg^{2+}. Furthermore, in that same year that Magour and his associates evaluated the effect of lubrol on the pump, Ahammad Sahib and his coworkers [32] further observed that dithiothreitol (DTT) and the monothiols, glutathione (GSH), and cysteine (CYS) can protect against inhibition of sodium pump caused by mercury MeHg. Both monothiols and dithiols have the same ability in regenerating sulfhydryl (-SH) groups or chelating the metals. In addition, they also reported that Hg^{2+} inhibited K^+-p-nitrophenyl phosphatase (K^+-PNPPase), the component enzyme catalyzing the K^+-dependent dephosphorylation in the overall Na^+-K^+-ATPase reaction. They partially concluded that the mechanism of inhibition of the pump by mercurials involves binding to this phosphatase. Interestingly, they also found that the inhibition imposed by Hg^{2+} on the activity of K^+-PNPPase could be reversed by dithiothreitol (DTT), glutathione (GSH), and cysteine (CYS) suggesting the presence of sulfhydryl groups at the binding sites. Binding of ouabain, a cardiac glycoside and inhibitor of both phosphorylation and dephosphorylation, to brain fraction was significantly decreased by MeHg, and this inhibition was reversed by the three thiol compounds, suggesting presence of -SH group(s) in the ouabain receptor site. They concluded that the critical conformational property of enzyme common to both kinase (E_1) and phosphatase (E_2) is susceptible to MeHg and that MeHg decreased the catalytic velocity of dephosphorylation of the enzyme-phosphoryl complex and inhibits K^+-PNPPase by binding at two different sites [33]. Interestingly, Kumar et al. [34] also reported that mercury binds specifically to thiol groups present in the platelet membrane Na^+/K^+-ATPase, consequently inhibiting the enzyme, and induces changes in platelet function, namely, platelet aggregation by interfering with the sodium pump. In the work of Wang and Horisberger [35], they observed that although Hg^{2+} reacts with sulfhydryl groups on proteins to form mercaptides, in Xenopus laevis oocytes expressing wild-type and mutant forms of Na^+/K^+-ATPase, the pump was inhibited with first-order kinetics. Furthermore, they observed that Hg^{2+} binding to C_{113} of the first transmembrane segment of the alpha subunit from the extracellular side is one of the mechanisms by which mercury inhibits Na^+/K^+-ATPase. More recently, in my research group, we observed that Hg^{2+} significantly inhibited the transmembrane enzyme in a concentration-dependent manner. In addition, Hg^{2+} exerts its inhibitory effect on the activity of the enzyme by interacting with groups at the ATP, Na^+, and K^+ binding sites which was prevented but not reversed by the monothiol CYS. Hence, we speculated that the small molecular volume of Hg^{2+} in comparison with the substrates (ATP, Na^+, and K^+) of sodium pump, its possibly high reactivity, and strong affinity for thiols may account for its high toxicity towards the membrane bound ouabain-sensitive electrogenic pump [36]. However, the monothiol GSH also has the same protective effect as CYS. One worrisome finding is the fact that GSH at 4 mM markedly inhibited the activity of the pump. However, since GSH is differentially distributed in various organelles (1–15 mM), we speculate that there is a probable differential sensitivity of the sodium pump in various organelles towards the antioxidant tripeptide suggesting the possibility of a strong dynamics in the regulation of GSH in the various compartments of the organelles. We partially concluded that GSH may possibly be a potential candidate as a preintervention remedy in cases of mammalian exposure to inorganic mercury [37]. It should be emphasized that the inhibition of sodium pump by Hg^{2+} in the absence of ATP as observed in our group [36, 37] elicits a complex and puzzling chemistry. This puzzle is consequent from the fact that the binding of Hg^{2+} to a vicinal thiol not directly participating in ATP binding possibly impose steric hindrance to the overall three-dimensional structure of the pump consequently leading to loss of activity.

7. Mercury Toxicity on Sodium Pump *In Vivo*

Although *in vitro* data clearly showed that Hg^{2+} toxicity can be related to its inhibition of the Na^+/K^+-ATPase, it is desirable to also discuss the effect of Hg on the activities of the sodium pump under *in vivo* conditions. Herein, the focus will shift to a discussion of some findings by authors on the effect of Hg^{2+} on the activities of Na^+/K^+-ATPase under *in vivo* conditions. About a decade ago, Chuu et al. [38] treated mice with MeHg and HgS and observed that the analysis of auditory brainstem response (ABR) indicated that significant elevation of the physiological hearing threshold as well as significant prolongation of interwave latency was observed for MeHg- (2.0 and 0.2 mg/kg per day) or HgS- (1.0 g/kg per day, but not 0.1 g/kg per day) treated mice. Furthermore, both MeHg- and HgS-treated animals demonstrated a significant prolongation of interwave latency that increased with an increasing mean blood-Hg level. The otoneurotoxicity of MeHg (2.0 mg/kg per day) persisted to at least 11 weeks subsequent to the cessation of its administration. The toxic effect of HgS, however, disappeared completely 5 weeks

subsequent to the cessation of its administration. These results suggest that there is a correlation between the Hg^{2+}-elicited hearing dysfunction and the availability of mercury in brain tissue. However, of particular importance to this paper is the relationship of these physiological abnormalities to the activities of the sodium pump. They observed that the inhibition of Na^+/K^+-ATPase activity was accompanied with overproduction of nitric oxide in the brainstem and is consistent with an analysis of the physiological hearing threshold and latencies of ABR waveform at all time points throughout the experimental process. Consequently, they proposed that high-dose HgS or MeHg intoxication is associated with a decrease in functional Na^+/K^+-ATPase activity in the brainstem of affected animals, this presumably arising via excessive nitric oxide production, and suggesting that brainstem damage may play a role in mercury-induced hearing loss. Similarly, while studying the neurobehavioral effect of MetHg, HgS, and cinnabar (HgS-based Chinese medicine), they also observed that MeHg and cinnabar prominently and irreversibly caused a decrease in body weight, prolongation of latency for escape from electric shock, a decrease in the percentage for the conditioned avoidance response (CAR) to electric shock, impairment of spontaneous locomotion and inhibition of Na^+/K^+-ATPase activity of the cerebral cortex. In contrast, these authors also found that HgS reversibly inhibited spontaneous locomotion, and Na^+/K^+-ATPase activity. It was noted that HgS significantly decreased the latency of escape from electric shock during the administration period, which lasted for 33 weeks after discontinuous administration [39]. In fact, further study [40] showed that cinnabar and HgS evoked a significant inhibition of the enzymatic Na^+/K^+-ATPase activity of cerebellum, and this inhibition was associated with increase in the amount of cerebellar nitric oxide (NO) production. Hence, they partly concluded that the increased Hg^{2+} contents in the cerebellum following oral administration of HgS and cinnabar were responsible, at least in part, for the detrimental neurotoxic effect, decreasing Na^+/K^+-ATPase activity and increasing NO production within the cerebellum. About half a decade ago, Chuu et al. [41] also found that MeHg reversibly decreased both of motor nerve conduction velocity (MNCV) and tail flick response, whereas, irreversibly inhibited all of the motor equilibrium performance, recovery of compound muscle action potentials (CMAPs) following exhaustic tetanic stimuli, and Na^+/K^+-ATPase activity of the isolated sciatic nerve. These toxic effects of MeHg were found to correlation well with that of Hg^{2+} contents of various tissues (blood, cerebral cortex, liver, and kidney) in rats. In this paper, it was reported that neurotoxic effects produced by HgS were estimated to be about 1000 of those induced by MeHg. These authors have consistently reported that HgS, the major component of cinnabar as well as other mercurials such as MeHg decreased Na^+/K^+-ATPase activities, and they associated this decrease to production of NO radicals subsequently, suggesting that Hg^{2+} toxicity on the Na^+/K^+-ATPase may be an indirect effect. In fact in some of these papers, increase in lipid peroxidation has been associated with increase in NO and decrease in Na^+/K^+-ATPase [42–46]. In a closely related study [47], Chanez and his colleagues investigated the effects of thimerosal and mercuric chloride on Na^+/K^+-ATPase activity in total brain homogenate, synaptosomes and myelin at weaning, and the reversal effect of serotonin on mercury-inhibited Na^+/K^+-ATPase activity. They reported that the toxicity in terms of inhibition of Na^+/K^+-ATPase activity was greater with mercuric chloride than with thimerosal. In addition, synaptosomes and principally myelin were more sensitive to the metal salts than total homogenate. In addition, they found that serotonin stimulated Na^+/K^+-ATPase activity in total brain homogenate and synaptosomes but inhibited the enzyme in the myelin fraction. Furthermore, serotonin (1 mM) added to total homogenate pretreated with the mercury salts produced variable reversal effects. In the synaptosomal fraction, reverse effect was noted with serotonin. In myelin fraction, added serotonin increased inhibition caused by thimerosal. On the other hand, Verma and his associates [48] studied the effect of sublethal concentrations of $HgCl_2$ Na^+-K^+, Mg^{2+}, and total ATPase activities in brain, gills, kidney and liver of Notopterus notopterus after 30 days exposure. Their findings indicated that Na^+-K^+-ATPase were inhibited maximally and significantly in brain and minimally and insignificantly in liver. Mg^{2+} ATPase was inhibited maximally and significantly (P less than 0.01) in brain and minimally and insignificantly in kidney. The relative inhibition of total, Na^+-K^+ and Mg^{2+} ATPases for the tissues studied was brain > gill > kidney > liver. At the concentration (1/25 fraction) the enzyme activity returned to the normal range.

In the work of Klonne and Johnson [49], they investigate whether DTT, a sulfhydryl-reducing agent, protected renal cortical sulfhydryl status in general, or the activity of various renal enzymes (Mg^{2+} and Na^+/K^+-ATPases, alkaline phosphatase, and glutathione peroxidase) in particular. Additionally, the occurrence of conjugated dienes was used to assess the degree of lipid peroxidation. It was found that $HgCl_2$ produced significant decreases in renal cortical protein-bound sulfhydryl concentration, alkaline phosphatase activity, and Na^+-K^+-ATPase activity within 2.5 h of administration, with no effect observed on glutathione peroxidase activity or the levels of conjugated dienes in rat renal cortex. Furthermore, administration of DTT 60 min after mercury neither provided protection from inhibition nor promoted restoration of the affected enzymes or sulfhydryl status. Consequently, they concluded that the partial protection of renal function offered by DTT in the early stages of mercury toxicity does not result from maintaining the integrity of renal cortical sulfhydryl status or the activity of the enzymes investigated. Furthermore, the early stages of mercury toxicity did not appear to be related to lipid peroxidation. However, using routine histochemical staining in conjunction with light and electron microscopy to evaluate the changes in the Na^+-K^+-ATPase activity in cerebral cortical microvessels of rats who received a single intraperitoneal injection of 6 mg/kg $HgCl_2$, Szumańska and his collaborators [50] observed that at 1 h after $HgCl_2$ administration, light microscopy revealed uniform reduction of the Na^+-K^+-ATPase reaction in all cortical layers. Electron microscopy confirmed that the enzyme reaction is to be very weak to completely absent in both the luminal and abluminal endothelial cell membranes, and the luminal plasmalemma showed invaginations and

pinocytic vesicles indicative of changes in its transport functions. The enzyme inhibition coincided with, and was likely to contribute to, profound perivascular swelling, involving mainly the astrocytic endfeet. The enzyme activity showed a partial recovery 18 h after $HgCl_2$ treatment, mainly in cortical layers II and III. After 5 days, the recovery of the enzyme activity appeared complete as observed by light and electron microscopy. They also reported that the recovery of the microvascular Na^+-K^+-ATPase coincided with the appearance of a strongly positive Na^+-K^+-ATPase reaction in the adjacent astrocytic processes and with the diminution of perivascular swelling. Using genetically hypertensive rats models, Anner and his collaborates [51] investigated putative Na^+-K^+-ATPase alterations associated with the disease. Na^+-K^+-ATPase of two strains of spontaneously hypertensive rats, the Milan hypertensive strain (MHS) and the spontaneously hypertensive rat (SHR), were characterized in comparison with enzymes isolated from their matched normotensive controls; the sensitivity to Na ions and the shape and span of the inhibition curves for ouabain and mercury of the isolated Na^+/K^+-ATPases were compared. They found that no functional changes between the purified "normotensive" and "hypertensive" Na^+-K^+-ATPase from brain and kidney were detected ruling out drastic structural alterations of the transport system in these two organs of diseased animals. Mondal et al. [52] demonstrated that $HgCl_2$ treatment enhanced a remarkably high rate of progesterone synthesis accompanied by a low rate of conversion to 17 beta-estradiol in the oocyte of *Channa punctatus*. On depuration, however, there was a reversal of the steroidogenic scenario with a low progesterone and high estradiol level. They observed that the accumulation of progesterone was positively correlated with the significant increase in 3 beta-hydroxysteroid dehydrogenase activity in the Hg-treated fish. Thus, it was clear that at the early stage of intoxication Hg^{2+} does play a role in the induction of 3 beta-hydroxysteroid dehydrogenase in the oocyte of fish at the spawning stage. The induction of this enzyme was found to be mediated by specific binding of Hg^{2+} to the plasma membrane Na^+-K^+-ATPase and increase in the specific messenger RNA translating 3 beta-hydroxysteroid dehydrogenase. It is concluded that inorganic mercury is able to initiate translatable messenger RNA synthesis in fish oocyte at a low degree of intoxication.

8. Organoseleniums: Promising Intervention in Mercury Toxicity

Excellent reports and reviews have shown that selenium is a promising candidate in the management of mercury toxicity. In recent years, organoselenium compounds have been given considerable attention. These compounds have been well documented as potent antioxidants [53–62] and have a wide range of application in the management of oxidative stress-related diseases such as diabetes [63], cancer [64–66] and atherosclerosis [67] and other oxidative stress-related diseases [68]. Interestingly, since mercury toxicity has been linked to oxidative stress [69–71], consequently organoselenium compounds may be potential agents that can remedy mercury toxicity. Indeed, while the literature has an enormous volume of data on inorganic selenium, only few data have been available on the potential candidacy of organoselenium compounds in ameliorating mercury toxicity. Herein, some key findings on the effect of organoselenium compounds are described.

Although not the first reported organoselenium compound, diphenyl diselenide, a member of the diorganyl diselenide has shown promise as a candidate in the management of degenerative diseases [53–68] including mercury toxicity [69–71]. Diphenyl diselenide (1 mg/kg) has been shown to confer protection against MeHg-induced hepatic, renal, and cerebellum lipid peroxidation and prevented the reduction in hepatic NPSH levels. Of particular importance, diphenyl diselenide decreased the deposition of Hg^{2+} in cerebrum, cerebellum, kidney, and liver. This indicates that DPDS can protect against some toxic effects of MeHg in mice [62]. Furthermore, DPDS protected against the observed reduction in some haematological immunological alterations induced by mercury in mice [73]. In combination with N-acetyl cysteine, (NAC), DPDS was reported to be partially effective in protecting against the effects of mercury. In combination with 2,3-Dimercapto-1-propanesulfonic acid (DMPS), DPDS was reported to be effective in restoring the increment in urea concentration caused by mercury [74]. In the work of Meinerz et al. [75], they found that coincubation with DPDS (100 μM) completely prevented the disruption of mitochondrial activity as well as the increase in TBARS levels caused by MeHg. The compound 3′3-ditrifluoromethyldiphenyl diselenide provided a partial but significant protection against methylmercury-induced mitochondrial dysfunction (45.4 ± 5.8% inhibition of the methylmercury effect). These authors also found that hydrogen peroxide as a vector during methylmercury toxicity and that thiol peroxidase activity of organoselenium compounds accounts for their protective actions against methylmercury-induced oxidative stress. They conclude that DPDS and potentially other organoselenium compounds may represent important molecules in the search for an improved therapy against the deleterious effects of methylmercury as well as other mercury compounds. On the other hand, Brandão et al. [76] showed that for mice treated with a daily dose of $HgCl_2$ (4.6 mg kg (-1), subcutaneously) for three consecutive days followed by treatment with DPDS (31.2 mg kg (-1), subcutaneously), they observed that the combination of Hg^{2+} and DPDS exposure caused a decrease in renal GST and Na^+/K^+-ATPase activities and an increase in renal ascorbic acid and TBARS concentrations when compared with the Hg^{2+} group. DPDS potentiated the increase in plasma urea caused by Hg^{2+}. Similarly, combination of Hg^{2+} and DPDS exposure caused a reduction in plasma protein levels and an increase in hemoglobin and hematocrit contents when compared with the $HgCl_2$ group. There was a significant reduction in hepatic CAT activity and an increase in TBARS levels in mice exposed to Hg^{2+} and DPDS when compared with the Hg^{2+} group. The results demonstrated that DPDS did not modify mercury levels in mice. In conclusion, DPDS potentiated damage caused by Hg^{2+} affecting mainly the renal tissue.

Farina and his collaborates [77] studied the effects of MeHg exposure (subcutaneous injections of methylmercury chloride: 2 mg/kg) on the hepatic levels of thiobarbituric acid reactive substances (TBARS) and nonprotein thiols (NPSH), and on liver glutathione peroxidase (GSHPx) activity, as well as the possible antagonist effect of ebselen (another potent organoselenium compound; (10 mg/kg, subcutaneously)) against MeHg effects during the postnatal period. They observed that ebselen abolished the MeHg-induced inhibition on liver GSHPx activity, but did not prevent the oxidative effects of MeHg on liver lipids and NPSH. MeHg affects the *in vitro* interaction between ebselen and GSH, and this phenomenon seems to be responsible for its inhibitory effect toward thiol-peroxidase activity. Additionally, ebselen presents pro-oxidative effects on rat liver, pointing to thiol depletion as a molecular mechanism related to ebselen-induced hepatotoxicity [77]. However, it has been observed that while Hg^{2+} inhibited renal ALA-D activity, increased TBARS level in kidney, and reduced the hepatic content of nonprotein thiol groups, but organoselenium compounds did not prevent such effects. However, under *in vitro* conditions, renal and hepatic ALA-D activity was inhibited by Hg^{2+} and ebselen. Also Hg^{2+} significantly increased TBARS production in renal and hepatic tissue preparations *in vitro*, and this effect was completely or partially prevented by organoselenium compounds. They concluded that it appears that organoselenium compounds could not prevent mercury toxicity *in vivo*. Consequently, they related the protective effect of ebselen and other organoselenium compounds tested against mercury-induced increase of TBARS production *in vitro* to an antioxidant action rather than to mercury binding [78, 79]. Also Yin et al. [80] found that although MeHg treatment significantly decreased astrocytic [3*H*]-glutamine uptake at all time points and concentrations, ebselen fully reversed MeHg's (1 μM) effect on [3*H*] -glutamine uptake at 1 min. At higher MeHg concentrations, ebselen partially reversed the MeHg-induced astrocytic inhibition of [3*H*] -glutamine uptake. In addition, ebselen inhibited MeHg-induced phosphorylation of ERK ($P < 0.05$) and blocked MeHg-induced activation of caspase-3. These results are consistent with the hypothesis that MeHg exerts its toxic effects via oxidative stress and that the phosphorylation of ERK and the dissipation of the astrocytic mitochondrial membrane potential are involved in MeHg toxicity. In addition, the protective effects elicited by ebselen reinforce the idea that organic selenocompounds represent promising strategies to counteract MeHg-induced neurotoxicity [80].

9. Organoselenium and Na^+/K^+-ATPase

So far, we have seen that Hg^{2+} exerts its inhibitory effect on the sodium pump by oxidizing critical thiols on the enzyme. In like manner, available data shows that organoseleniums also inhibit the sodium pump by oxidizing thiols on the sodium pump. The data presented thereafter illustrates this fact. For example, while investigating the toxic effects of some organoselenium compounds: m-trifluoromethyl-diphenyl diselenide (m-CF(3)-C(6)H(4)Se)(2), p-chloro-diphenyl diselenide (p-Cl-C(6)H(4)Se)(2), and p-methoxyl-diphenyl diselenide (p-CH(3)O-C(6)H(4)Se)(2), Brüning et al. [81] observed that these organoseleniums inhibited $Na(^+)$, $K(^+)$-ATPase activity and that the enzyme is more sensitive to (p-Cl-C(6)H(4)Se)(2), and (m-CF(3)-C(6)H(4)Se)(2) (IC(50) 6 microM) than (p-CH(3)O-C(6)H(4)Se)(2) and (PhSe)(2) (IC(50) 45 and 31 microM, resp.). Furthermore, they observed that the inhibition was reversed by the thiol-protecting agents such as dithiothreitol DTT. Furthermore, they concluded that the effect of diselenides on Na^+/K^+-ATPase is dependent on their substitutions in the aromatic ring and that oxidation of critical thiols on the enzyme is one mechanisms by which these organoselenium compounds inhibit Na^+/K^+-ATPase activities involves the oxidation of thiol groups. Earlier, Borges et al., [82] observed that besides the diroganyl diselenides, other classes or organochalcogens such as ebselen and telluride compounds evoke similar inhibitory pattern as reported in [81]. They concluded that cerebral Na^+/K^+-ATPase is a potential molecular target for the toxic effect of organochalcogens, and the inhibition may occur through a change in the crucial thiol groups of this enzyme.

The above data were obtained under *in vitro* conditions, and clearly it shows that organoselenium compounds are potential target of the sodium pump. However, it is not clear whether the observed *in vitro* effect will be replicated *in vivo*. Findings on the effect of organoseleniums on the activities of the sodium pump consistently show that organoselenium compound significantly improves the activities of the pump under *in vivo* conditions. Some specific data are presented to drive home this fact. Barbosa and her collaborates observed that when organoselenium was administered to diabetic rats, they attenuated both hyperglycemic conditions and oxidative stress indices that were high in the diabetic rats. Interestingly, the low activities of cerebral sodium pump observed in diabetic rats were significantly improved upon after treatment with DPDS [83, 84]. In a related study, I equally observed that although STZ evokes a significant diminution on the antioxidant status and activity of Na^+/K^+-ATPase, DPDS was able to markedly restore the observed imbalance in cerebral antioxidant status and also relieve the inhibition of Na^+/K^+-ATPase caused by streptozotocin [85].

More recently, a comparison of the effect of two organoselenium compounds, DPDS and dicholesteroyl diselenide (DCDS), shows that mice administered these compounds at doses of 0.5 mmol kg^{-1} body weight in soya bean oil for four consecutive days exhibited no alteration in the activities of thiol-containing enzymes such as delta aminolevulinic acid dehydratase (ALA-D), Na^+/K^+-ATPase, and isoforms of lactate dehydrogenase (LDH) and catalase further indicating that cerebral Na^+/K^+-ATPase may not be a molecular target of organodiselenides toxicity [86]. Consequently, the pharmacological and toxicological chemistry of organoselenium compounds is complex and multifactorial and is dependent on delicate equations [86].

In a related experiment, authors have reported that subchronic administration of DPDS (300 micromole/kg body weight once a day for 14 days) significantly increased aspartate aminotransferase (AST) and alanine aminotransferase

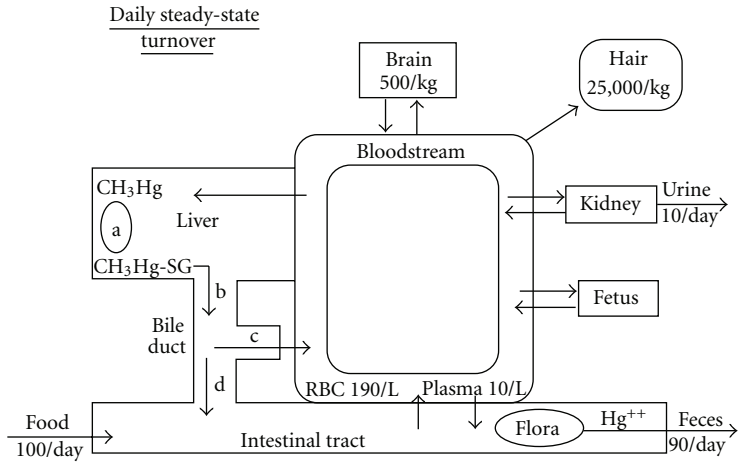


Figure 2: A diagram of the enterohepatic recirculation of methylmercury and its movement to maternal brain, kidney, and hair and to fetal tissues. (a) Methylmercury combines with reduced glutathione to form a complex, (b) which is secreted into bile. This glutathione is hydrolyzed to its constituent amino acids, releasing the methylmercury-cysteine complex. The latter, in part, is reabsorbed in the gallbladder into the bloodstream (c) and, in part, secreted into the intestinal tract along with any unhydrolysed glutathione complex (d). Once in the intestinal tract, it is in part reabsorbed into the portal circulation as the cysteine complex and in part demethylated by intestinal microflora. Most of the inorganic mercury produced in this way is excreted in the feces. The numbers quoted in each body compartment are the relative concentrations of methylmercury and the intakes and excretion rates [72].

(ALT) activities in plasma but did not alter lactate dehydrogenase (LDH) activity, urea and creatinine levels in plasma, and renal Na^+/K^+-ATPase [87]. Conversely, chronic administration of DPDS (500 mg/kg) increased the levels of lipid peroxidation and catalase activity as well as decreased delta-ALA-D (delta-aminolevulinate dehydratase) and Na^+/K^+-ATPase activity in the brain of rat pups [88, 89].

10. Organoselenium and Mercury Toxicity on Na^+/K^+-ATPase: A Paradox

From the foregoing, it is apparent that both mercury and organoselenium compounds inhibit Na^+/K^+-ATPase under *in vitro* conditions, and the mechanism involved in this inhibition mainly involves the oxidation of critical thiols on the pump. However, under *in vivo* conditions, Hg^{2+} inhibits the pump, whereas under *in vivo* and even different pathological conditions, organoselenium improves the activity of the pump that had been hitherto inhibited by the pathologies. This is a paradox, and the mechanisms for the observed protection offered by organoselenium under these conditions are yet to be completely elucidated. However, available data on the protection offered by organoselenium on the pump under consideration are scanty. One striking observation from my lab [66, 85, 86] and my collaborators [83, 84] as case studies is the fact that irrespective of the route of administration, organoseleniums elicit an increase in thiol contents in tissues of rats or mice. This effect of organoselenium points to the fact that the use of organoseleniums holds promise as a preventive measure in the management of mercury toxicity. It has been reported that inhibition of the sodium pump under conditions of oxidative stress *in vivo* can be prevented or reversed by thiols [49, 90] showing that oxidation of the critical thiols in the pump is responsible for its inactivity. Similarly, mercury has also been documented to oxidize these critical thiols on this transmembrane pump [49]. It is rational therefore to speculate that preadministration of organoseleniums may be a first line of defense in occupationally exposed individuals such as those in mines and factories where contact with mercury is generally unavoidable. Interestingly, mercury has been demonstrated to bind thiols such as glutathione with such efficiency, and such thiols have been employed in the management of mercury toxicity. For example, while they are not the first to make this observation, however, as a reference, the work of Khan [91] and his collaborators will underscore this point. In their paper, they observed that decrease in GSH level was dependant on mercuric chloride concentration and time of incubation *in vivo*. They suggested that the decrease in the concentration of reduced state glutathione may be due to the interaction of reduced state glutathione (GSH) and mercuric chloride to form oxidized glutathione (GSSG) or mercuric-glutathione complex. This change in GSH metabolic status provides information regarding the role of GSH in detoxification of mercuric chloride. Their argument is well illustrated in Figure 2.

Consequently, this paper may serve as a drive towards the synthesis as well as biological testing of these emerging selenocompounds on the activities of the sodium pump. Few available data show that this is feasible [53, 54]. Consider for example, a paper by Hassan et al., [92] where he and his collaborators reported on the antioxidant and toxicological profile of an imine (-N) containing organoselenium compound that did not inhibit Na^+/K^+-ATPase activities. The interaction of mercury with sodium pump may be critical in medical assessment and intervention of mercury toxicity.

For example, due to the inaccessibility of human nerve tissue for direct biochemical evaluation, there appears to be a need to identify peripheral markers which will reflect toxicity to the central nervous system by relatively noninvasive means. The erythrocytes Na^+/K^+-ATPase have been postulated to be a possible marker of mercury toxicity even though the first experimental data is not pointing in this direction especially under acute exposure [93].

Acknowledgment

The author is grateful for the financial support of CNPQ-INCT for excitotoxicity and neuroprotection.

References

[1] W. J. Crinnion, "Environmental medicine, part three: long-term effects of chronic low- dose mercury exposure," *Alternative Medicine Review*, vol. 5, no. 3, pp. 209–223, 2000.

[2] T. Y. K. Chan, "Inorganic mercury poisoning associated with skin-lightening cosmetic products," *Clinical Toxicology*, vol. 49, pp. 886–891, 2011.

[3] R. A. Bernhoft, "Mercury toxicity and treatment: a review of the literature," *Journal of Environmental and Public Health*, vol. 2012, Article ID 460508, 10 pages, 2012.

[4] L. C. Masur, "A review of the use of mercury in historic and current ritualistic and spiritual practices," *Alternative Medicine Review*, vol. 16, no. 4, pp. 314–320, 2011.

[5] P. Li, X. Feng, and G. Qiu, "Methylmercury exposure and health effects from rice and fish consumption: a review," *International Journal of Environmental Research and Public Health*, vol. 7, no. 6, pp. 2666–2691, 2010.

[6] D. A. Geier, P. G. King, L. K. Sykes, and M. R. Geier, "A comprehensive review of mercury provoked autism," *Indian Journal of Medical Research*, vol. 128, no. 4, pp. 383–411, 2008.

[7] S. T. Schultz, "Does thimerosal or other mercury exposure increase the risk for autism? A review of current literature," *Acta Neurobiologiae Experimentalis*, vol. 70, no. 2, pp. 187–195, 2010.

[8] J. C. Skou, "The influence of some cations on an adenosine triphosphatase from peripheral nerves," *Biochimica et Biophysica Acta*, vol. 23, pp. 394–401, 1957.

[9] L. D. Faller, "Mechanistic studies of sodium pump," *Archives of Biochemistry and Biophysics*, vol. 476, no. 1, pp. 12–21, 2008.

[10] P. L. Jorgensen and P. A. Pedersen, "Structure-function relationships of Na^+, K^+, ATP, or Mg^{2+} binding and energy transduction in Na,K-ATPase," *Biochimica et Biophysica Acta*, vol. 1505, no. 1, pp. 57–74, 2001.

[11] L. A. Vasilets and W. Schwarz, "Structure-function relationships of cation binding in the Na^+/K^+-ATPase," *Biochimica et Biophysica Acta*, vol. 1154, no. 2, pp. 201–222, 1993.

[12] J. D. Robinson and P. R. Pratap, "Indicators of conformational changes in the Na^+/K^+-ATPase and their interpretation," *Biochimica et Biophysica Acta*, vol. 1154, no. 1, pp. 83–104, 1993.

[13] G. E. Shull, A. Schwartz, and J. B. Lingrel, "Amino-acid sequence of the catalytic subunit of the (Na^+- K^+)ATPase deduced from a complementary DNA," *Nature*, vol. 316, no. 6030, pp. 691–695, 1985.

[14] R. L. Post and S. Kume, "Evidence for an aspartyl phosphate residue at the active site of sodium and potassium ion transport adenosine triphosphatase," *Journal of Biological Chemistry*, vol. 248, no. 20, pp. 6993–7000, 1973.

[15] E. M. Price, D. A. Rice, and J. B. Lingrel, "Structure-function studies of Na,K-ATPase. Site-directed mutagenesis of the border residues from the H1-H2 extracellular domain of the α subunit," *Journal of Biological Chemistry*, vol. 265, no. 12, pp. 6638–6641, 1990.

[16] K. Geering, I. Theulaz, F. Verrey, M. T. Hauptle, and B. C. Rossier, "A role for the β-subunit in the expression of functional Na^+-K^+-ATPase in Xenopus oocytes," *American Journal of Physiology*, vol. 257, no. 5, pp. C851–C858, 1989.

[17] M. Kawamura and S. Noguchi, "Possible role of the β-subunit in the expression of the sodium pump," in *The Sodium Pump: Structure, Mechanism, and Regulation*, J. H. Kaplan and P. DeWeer, Eds., pp. 45–62, The Rockefeller University Press, New York, NY, USA, 1991.

[18] A. A. McDonough, K. Geering, and R. A. Farley, "The sodium pump needs its β subunit," *FASEB Journal*, vol. 4, no. 6, pp. 1598–1605, 1990.

[19] W. E. Harris and W. L. Stahl, "Origin of the γ polypeptide of the Na^+/K^+-ATPase," *Biochimica et Biophysica Acta*, vol. 942, no. 2, pp. 236–244, 1988.

[20] R. W. Mercer, D. Biemesderfer, D. P. Bliss, J. H. Collins, and B. Forbush, "Molecular cloning and immunological characterization of the γ-subunit of the Na, K-ATPase," in *The Sodium Pump: Recent Developments*, P. DeWeer and J. Kaplan, Eds., pp. 37–41, Rockefeller University Press, New York, 1991.

[21] J. Castro and R. A. Farley, "Proteolytic fragmentation of the catalytic subunit of the sodium and potassium adenosine triphosphatase. Alignment of tryptic and chymotryptic fragments and location of sites labeled with ATP and iodoacetate," *Journal of Biological Chemistry*, vol. 254, no. 7, pp. 2221–2228, 1979.

[22] P. L. Jorgensen and J. P. Andersen, "Structural basis for E1-E2 conformational transitions in Na,K-pump and Ca-pump proteins," *Journal of Membrane Biology*, vol. 103, no. 2, pp. 95–120, 1988.

[23] M. Ohtsubo, S. Noguchi, K. Takeda, M. Morohashi, and M. Kawamura, "Site-directed mutagenesis of Asp-376, the catalytic phosphorylation site, and Lys-507, the putative ATP-binding site, of the α-subunit of Torpedo californica Na^+/K^+-ATPase," *Biochimica et Biophysica Acta*, vol. 1021, no. 2, pp. 157–160, 1990.

[24] C. H. Pedemonte and J. H. Kaplan, "Chemical modification as an approach to elucidation of sodium pump structure-function relations," *American Journal of Physiology*, vol. 258, no. 1, pp. C1–C23, 1990.

[25] K. N. Dzhandzhugazyan, S. V. Lutsenko, and N. N. Modyanov, "Target-residues of the active site affinity modification are different in E1 and E2 forms," in *The Na^+, K^+-Pump. Part A: Molecular Aspects*, J. C. Skou, J. G. Norby, A. B. Maunsbach, and M. Esmann, Eds., pp. 181–188, Alan R. Liss, New York, NY, USA, 1988.

[26] B. Vilsen, "Functional consequences of alterations to Pro328 and Leu332 located in the 4th transmembrane segment of the α-subunit of the rat kidney N^+,K^+-ATPase," *FEBS Letters*, vol. 314, no. 3, pp. 301–307, 1992.

[27] J. P. Andersen and B. Vilsen, "Structural basis for the E1/E1P-E2/E2P conformation changes in the sarcoplasmic reticulum Ca^{2+}-ATPase studied by site-specific mutagenesis," *Acta Physiologica Scandinavica, Supplement*, vol. 146, no. 607, pp. 151–159, 1992.

[28] S. Bhattacharya, S. Bose, B. Mukhopadhyay et al., "Specific binding of inorganic mercury to Na^+-K^+-ATPase in rat liver plasma membrane and signal transduction," *BioMetals*, vol. 10, no. 3, pp. 157–162, 1997.

[29] H. J. Kramer, H. C. Gonick, and E. Lu, "*In vitro* inhibition of Na-K-ATPase by trace metals: relation to renal and cardiovascular damage," *Nephron*, vol. 44, no. 4, pp. 329–336, 1986.

[30] S. Magour, "Studies on the inhibition of brain synaptosomal Na^+/K^+-ATPase by mercury chloride and methyl mercury chloride," *Archives of Toxicology*, vol. 9, pp. 393–396, 1986.

[31] S. Magour, H. Maser, and H. Greim, "The effect of mercury chloride and methyl mercury on brain microsomal Na^+-K^+-ATPase after partial delipidisation with Lubrol," *Pharmacology and Toxicology*, vol. 60, no. 3, pp. 184–186, 1987.

[32] K. I. Ahammadsahib, R. Ramamurthi, and D. Dusaiah, "Mechanism of inhibition of rat brain (Na^+-K^+)-stimulated adenosine triphosphatase reaction by cadmium and methyl mercury," *Journal of Biochemical Toxicology*, vol. 2, pp. 169–180, 1987.

[33] K. I. Ahammad Sahib, K. S. Moorthy, and D. Desaiah, "Effects of methyl mercury and cadmium on the kinetics of substrate activation of (K^+)-paranitrophenyl phosphatase," *Journal of Applied Toxicology*, vol. 7, no. 3, pp. 221–226, 1987.

[34] S. V. Kumar, S. Maitra, and S. Bhattacharya, "*In vitro* binding of inorganic mercury to the plasma membrane of rat platelet affects Na^+-K^+-ATPase activity and platelet aggregation," *BioMetals*, vol. 15, no. 1, pp. 51–57, 2002.

[35] X. Wang and J. D. Horisberger, "Mercury binding site on Na^+/K^+-ATPase: a cysteine in the first transmembrane segment," *Molecular Pharmacology*, vol. 50, no. 3, pp. 687–691, 1996.

[36] T. I. Omotayo, J. B. T. Rocha, E. O. Ibukun, and I. J. Kade, "Inorganic mercury interacts with thiols at the nucleotide and cationic binding sites of the ouabain-sensitive cerebral electrogenic sodium pump," *Neurochemistry International*, vol. 58, no. 7, pp. 776–784, 2011.

[37] I. J. Kade, T. I. Omotayo, and J. B. T. Rocha, "Threshold protective effect of glutathione on inorganic mercury inhibition of cerebral sodium pump," *Biochemistry-An Indian Journal*, vol. 6, no. 2, pp. 65–70, 2012.

[38] J. J. Chuu, C. J. Hsu, and S. Y. Lin-Shiau, "Abnormal auditory brainstem responses for mice treated with mercurial compounds: involvement of excessive nitric oxide," *Toxicology*, vol. 162, no. 1, pp. 11–22, 2001.

[39] J. J. Chuu, S. H. Liu, and S. Y. Lin-Shiau, "Effects of methyl mercury, mercuric sulfide and cinnabar on active avoidance responses, Na^+/K^+-ATPase activities and tissue mercury contents in rats," *Proceedings of the National Science Council, Republic of China B*, vol. 25, no. 2, pp. 128–136, 2001.

[40] Y. H. Young, J. J. Chuu, S. H. Liu, and S. Y. Lin-Shiau, "Neurotoxic mechanism of cinnabar and mercuric sulfide on the vestibulo-ocular reflex system of guinea pigs," *Toxicological Sciences*, vol. 67, no. 2, pp. 256–263, 2002.

[41] J. J. Chuu, S. H. Liu, and S. Y. Lin-Shiau, "Differential neurotoxic effects of methylmercury and mercuric sulfide in rats," *Toxicology Letters*, vol. 169, no. 2, pp. 109–120, 2007.

[42] C. F. Huang, S. H. Liu, C. J. Hsu, and S. Y. Lin-Shiau, "Neurotoxicological effects of low-dose methylmercury and mercuric chloride in developing offspring mice," *Toxicology Letters*, vol. 201, pp. 196–204, 2011.

[43] C. F. Huang, C. J. Hsu, S. H. Liu, and S. Y. Lin-Shiau, "Ototoxicity induced by cinnabar (a naturally occurring HgS) in mice through oxidative stress and down-regulated Na^+/K^+-ATPase activities," *NeuroToxicology*, vol. 29, no. 3, pp. 386–396, 2008.

[44] C. F. Huang, C. J. Hsu, S. H. Liu, and S. Y. Lin-Shiau, "Neurotoxicological mechanism of methylmercury induced by low-dose and long-term exposure in mice: oxidative stress and down-regulated Na^+/K^+-ATPase involved," *Toxicology Letters*, vol. 176, no. 3, pp. 188–197, 2008.

[45] J. J. Chuu, Z. N. Huang, H. H. Yu, L. H. Chang, and S. Y. Lin-Shiau, "Attenuation by methyl mercury and mercuric sulfide of pentobarbital induced hypnotic tolerance in mice through inhibition of ATPase activities and nitric oxide production in cerebral cortex," *Archives of Toxicology*, vol. 82, no. 6, pp. 343–353, 2008.

[46] C. F. Huang, S. H. Liu, and S. Y. Lin-Shiau, "Neurotoxicological effects of cinnabar (a Chinese mineral medicine, HgS) in mice," *Toxicology and Applied Pharmacology*, vol. 224, no. 2, pp. 192–201, 2007.

[47] C. Chanez, M. A. Flexor, and J. M. Bourre, "Effect of organic and inorganic mercuric salts on Na^+K^+ATPase in different cerebral fractions in control in intrauterine growth-retarded rats: alterations induced by serotonin," *NeuroToxicology*, vol. 10, no. 4, pp. 699–706, 1989.

[48] S. R. Verma, M. Jain, and I. P. Tonk, "In vivo effect of mercuric chloride on tissue ATPases of Notopterus notopterus," *Toxicology Letters*, vol. 16, no. 3-4, pp. 305–309, 1983.

[49] D. R. Klonne and D. R. Johnson, "Enzyme activity and sulfhydryl status in rat renal cortex following mercuric chloride and dithiothreitol administration," *Toxicology Letters*, vol. 42, no. 2, pp. 199–205, 1988.

[50] G. Szumańska, R. Gadamski, and J. Albrecht, "Changes of the Na/K ATPase activity in the cerebral cortical microvessels of rat after single intraperitoneal administration of mercuric chloride: histochemical demonstration with light and electron microscopy," *Acta Neuropathologica*, vol. 86, no. 1, pp. 65–70, 1993.

[51] B. M. Anner, E. Imesch, and M. Moosmayer, "Normal sensitivity of Na^+/K^+-ATPase isolated from brain and kidney of spontaneously hypertensive rats to sodium, ouabain or mercury," *Biochimica et Biophysica Acta*, vol. 1270, no. 1, pp. 95–99, 1995.

[52] S. Mondal, B. Mukhopadhyay, and S. Bhattacharya, "Inorganic mercury binding to fish oocyte plasma membrane induces steroidogenesis and translatable messenger RNA synthesis," *BioMetals*, vol. 10, no. 4, pp. 285–290, 1997.

[53] C. W. Nogueira, G. Zeni, and J. B. T. Rocha, "Organoselenium and organotellurium compounds: toxicology and pharmacology," *Chemical Reviews*, vol. 104, no. 12, pp. 6255–6285, 2004.

[54] C. W. Nogueira and J. B. T. Rocha, "Toxicology and pharmacology of selenium: emphasis on synthetic organoselenium compounds," *Archives of Toxicology*, vol. 85, no. 11, pp. 1313–1359, 2011.

[55] A. S. Prestes, S. T. Stefanello, S. M. Salman et al., "Antioxidant activity of b-selenoamines and their capacity to mimic different enzymes," *Molecular and Cellular Biochemistry*, vol. 365, no. 1-2, pp. 85–92, 2012.

[56] C. A. Prauchner, A. S. Prestes, and J. B. T. Rocha, "Effects of diphenyl diselenide on oxidative stress induced by sepsis in rats," *Pathology*, vol. 207, pp. 554–558, 2011.

[57] A. S. Freitas and J. B. T. Rocha, "Diphenyl diselenide and analogs are substrates of cerebral rat thioredoxin reductase: a pathway for their neuroprotective effects," *Neuroscience Letters*, vol. 503, pp. 1–5, 2011.

[58] M. Ibrahim, M. Prigol, W. Hassan, C. W. Nogueira, and J. B. T. Rocha, "Protective effect of binaphthyl diselenide, a synthetic organoselenium compound, on 2-nitropropane-induced hepatotoxicity in rats," *Cell Biochemistry and Function*, vol. 28, no. 4, pp. 258–265, 2010.

[59] M. Ibrahim, C. Luchese, S. Pinton et al., "Involvement of catalase in the protective effect of binaphthyl diselenide against

renal damage induced by glycerol," *Experimental and Toxicologic Pathology*, vol. 63, no. 4, pp. 331–335, 2011.

[60] C. L. D. Corte, R. Fachinetto, R. Puntel et al., "Chronic treatment with fluphenazine alters parameters of oxidative stress in liver and kidney of rats," *Basic and Clinical Pharmacology and Toxicology*, vol. 105, no. 1, pp. 51–57, 2009.

[61] D. H. Roos, R. L. Puntel, M. M. Santos et al., "Guanosine and synthetic organoselenium compounds modulate methylmercury-induced oxidative stress in rat brain cortical slices: involvement of oxidative stress and glutamatergic system," *Toxicology in Vitro*, vol. 23, no. 2, pp. 302–307, 2009.

[62] A. S. de Freitas, V. R. Funck, M. D. S. Rotta et al., "Diphenyl diselenide, a simple organoselenium compound, decreases methylmercury-induced cerebral, hepatic and renal oxidative stress and mercury deposition in adult mice," *Brain Research Bulletin*, vol. 79, no. 1, pp. 77–84, 2009.

[63] M. Soriano-García, "Organoselenium compounds as potential therapeutic and chemopreventive agents: a review," *Current Medicinal Chemistry*, vol. 11, no. 12, pp. 1657–1669, 2004.

[64] R. Naithani, "Organoselenium compounds in cancer chemoprevention," *Mini-Reviews in Medicinal Chemistry*, vol. 8, no. 7, pp. 657–668, 2008.

[65] K. El-Bayoumy and R. Sinha, "Mechanisms of mammary cancer chemoprevention by organoselenium compounds," *Mutation Research*, vol. 551, no. 1-2, pp. 181–197, 2004.

[66] N. B. de Vargas Barbosa, C. W. Nogueira, T. N. Guecheva, M. de Lourdes Bellinaso, and J. B. T. Rocha, "Diphenyl diselenide supplementation delays the development of N-nitroso-N-methylurea-induced mammary tumors," *Archives of Toxicology*, vol. 82, no. 9, pp. 655–663, 2008.

[67] A. F. D. Bem, M. Farina, R. D. L. Portella et al., "Diphenyl diselenide, a simple glutathione peroxidase mimetic, inhibits human LDL oxidation *in vitro*," *Atherosclerosis*, vol. 201, no. 1, pp. 92–100, 2008.

[68] F. C. Meotti, E. C. Stangherlin, G. Zeni, C. W. Nogueira, and J. B. T. Rocha, "Protective role of aryl and alkyl diselenides on lipid peroxidation," *Environmental Research*, vol. 94, no. 3, pp. 276–282, 2004.

[69] M. Farina, M. Aschner, and J. B. T. Rocha, "Oxidative stress in MeHg-induced neurotoxicity," *Toxicology and Applied Pharmacology*, vol. 256, no. 3, pp. 405–417, 2011.

[70] M. Farina, J. B. T. Rocha, and M. Aschner, "Mechanisms of methylmercury-induced neurotoxicity: evidence from experimental studies," *Life Sciences*, vol. 89, no. 15–16, pp. 555–563, 2011.

[71] M. Aschner, T. Syversen, D. O. Souza, J. B. T. Rocha, and M. Farina, "Involvement of glutamate and reactive oxygen species in methylmercury neurotoxicity," *Brazilian Journal of Medical and Biological Research*, vol. 40, no. 3, pp. 285–291, 2007.

[72] T. W. Clarkson and L. Magos, "The toxicology of mercury and its chemical compounds," *Critical Reviews in Toxicology*, vol. 36, no. 8, pp. 609–662, 2006.

[73] R. Brandão, L. P. Borges, R. de Oliveira, J. B. T. Rocha, and C. W. Nogueira, "Diphenyl diselenide protects against hematological and immunological alterations induced by mercury in mice," *Journal of Biochemical and Molecular Toxicology*, vol. 22, no. 5, pp. 311–319, 2008.

[74] R. Brandão, F. W. Santos, M. Farina et al., "Antioxidants and metallothionein levels in mercury-treated mice," *Cell Biology and Toxicology*, vol. 22, no. 6, pp. 429–438, 2006.

[75] D. F. Meinerz, M. T. de Paula, B. Comparsi et al., "Protective effects of organoselenium compounds against methylmercury-induced oxidative stress in mouse brain mitochondrial-enriched fractions," *Brazilian Journal of Medical and Biological Research*, vol. 44, pp. 1156–1163, 2011.

[76] R. Brandão, R. N. Moresco, L. P. Bellé et al., "Diphenyl diselenide potentiates nephrotoxicity induced by mercuric chloride in mice," *Journal of Applied Toxicology*, vol. 31, pp. 773–782, 2011.

[77] M. Farina, F. A. A. Soares, G. Zeni, D. O. Souza, and J. B. T. Rocha, "Additive pro-oxidative effects of methylmercury and ebselen in liver from suckling rat pups," *Toxicology Letters*, vol. 146, no. 3, pp. 227–235, 2004.

[78] J. Perottoni, O. E. D. Rodrigues, M. W. Paixão et al., "Renal and hepatic ALA-D activity and selected oxidative stress parameters of rats exposed to inorganic mercury and organoselenium compounds," *Food and Chemical Toxicology*, vol. 42, no. 1, pp. 17–28, 2004.

[79] J. Perottoni, L. P. Lobato, A. Silveira, J. B. T. Rocha, and T. Emanuelli, "Effects of mercury and selenite on δ-aminolevulinate dehydratase activity and on selected oxidative stress parameters in rats," *Environmental Research*, vol. 95, no. 2, pp. 166–173, 2004.

[80] Z. Yin, E. Lee, M. Ni et al., "Methylmercury-induced alterations in astrocyte functions are attenuated by ebselen," *NeuroToxicology*, vol. 32, no. 3, pp. 291–299, 2011.

[81] C. A. Brüning, M. Prigol, D. A. Barancelli, C. W. Nogueira, and G. Zeni, "Disubstituted diaryl diselenides inhibit δ-ALA-D and Na^+, K^+-ATPase activities in rat brain homogenates *in vitro*," *Molecular and Cellular Biochemistry*, vol. 332, no. 1-2, pp. 17–24, 2009.

[82] V. C. Borges, J. B. T. Rocha, and C. W. Nogueira, "Effect of diphenyl diselenide, diphenyl ditelluride and ebselen on cerebral Na^+, K^+-ATPase activity in rats," *Toxicology*, vol. 215, no. 3, pp. 191–197, 2005.

[83] N. B. V. Barbosa, J. B. T. Rocha, D. C. Wondracek, J. Perottoni, G. Zeni, and C. W. Nogueira, "Diphenyl diselenide reduces temporarily hyperglycemia: possible relationship with oxidative stress," *Chemico-Biological Interactions*, vol. 163, no. 3, pp. 230–238, 2006.

[84] N. B. V. Barbosa, J. B. T. Rocha, J. C. M. Soares et al., "Dietary diphenyl diselenide reduces the STZ-induced toxicity," *Food and Chemical Toxicology*, vol. 46, no. 1, pp. 186–194, 2008.

[85] I. J. Kade, C. W. Nogueira, and J. B. T. Rocha, "Diphenyl diselenide and streptozotocin did not alter cerebral glutamatergic and cholinergic systems but modulate antioxidant status and sodium pump in diabetic rats," *Brain Research*, vol. 1284, pp. 202–211, 2009.

[86] I. J. Kade and J. B. T. Rocha, "Comparative study on the influence of subcutaneous administration of diphenyl and dicholesteroyl diselenides on sulphydryl proteins and antioxidant parameters in mice," *Journal of Applied Toxicology*, vol. 30, no. 7, pp. 688–693, 2010.

[87] F. C. Meotti, V. C. Borges, J. Perottoni, and C. W. Nogueira, "Toxicological evaluation of subchronic exposure to diphenyl diselenide in rats," *Journal of Applied Toxicology*, vol. 28, no. 5, pp. 638–644, 2008.

[88] M. Prigol, E. A. Wilhelm, C. C. Schneider, J. B. T. Rocha, C. W. Nogueira, and G. Zeni, "Involvement of oxidative stress in seizures induced by diphenyl diselenide in rat pups," *Brain Research*, vol. 1147, no. 1, pp. 226–232, 2007.

[89] M. Prigol, E. A. Wilhelm, E. C. Stangherlin, D. A. Barancelli, C. W. Nogueira, and G. Zeni, "Diphenyl diselenide-induced seizures in rat pups: possible interaction with glutamatergic system," *Neurochemical Research*, vol. 33, no. 6, pp. 996–1004, 2008.

[90] O. P. Mishra, M. Delivoria-Papadopoulos, G. Cahillane, and L. C. Wagerle, "Lipid peroxidation as the mechanism of modification of the affinity of the Na^+, K^+-ATPase active sites

for ATP, K^+, Na^+, and strophanthidin *in vitro*," *Neurochemical Research*, vol. 14, no. 9, pp. 845–851, 1989.

[91] H. Khan, M. F. Khan, S. U. Jan, M. Mukhtiar, N. Ullah, and N. Anwar, "Role of Glutathione in protection against mercury induced poisoning," *Pakistan Journal of Pharmaceutical Science*, vol. 25, no. 2, pp. 395–400, 2012.

[92] W. Hassan, S. Pinton, J. T. D. Rocha et al., "Hydroxyl containing seleno-imine compound exhibits improved anti-oxidant potential and does not inhibit thiol-containing enzymes," *Chemico-Biological Interactions*, vol. 190, no. 1, pp. 35–44, 2011.

[93] W. E. Maier and L. G. Costa, "Na^+/K^+-ATPase in rat brain and erythrocytes as a possible target and marker, respectively, for neurotoxicity: studies with chlordecone, organotins and mercury compounds," *Toxicology Letters*, vol. 51, no. 2, pp. 175–188, 1990.

Hindawi Publishing Corporation
Journal of Biomedicine and Biotechnology
Volume 2012, Article ID 248764, 15 pages
doi:10.1155/2012/248764

Review Article

Role of Calcium and Mitochondria in MeHg-Mediated Cytotoxicity

Daniel Roos,[1] Rodrigo Seeger,[1] Robson Puntel,[2] and Nilda Vargas Barbosa[1]

[1] *Departamento de Química, CCNE, Programa de Pós-Graduação em Bioquímica Toxicológica, Universidade Federal de Santa Maria, 97105.900 Santa Maria, RS, Brazil*
[2] *Universidade Federal do Pampa-Campus Uruguaiana, BR-472 Km 7, 97500-970 Uruguaiana, RS, Brazil*

Correspondence should be addressed to Robson Puntel, robson_puntel@yahoo.com.br
and Nilda Vargas Barbosa, nvbarbosa@yahoo.com.br

Received 6 April 2012; Revised 12 June 2012; Accepted 14 June 2012

Academic Editor: Marcelo Farina

Methylmercury (MeHg) mediated cytotoxicity is associated with loss of intracellular calcium (Ca^{2+}) homeostasis. The imbalance in Ca^{2+} physiology is believed to be associated with dysregulation of Ca^{2+} intracellular stores and/or increased permeability of the biomembranes to this ion. In this paper we summarize the contribution of glutamate dyshomeostasis in intracellular Ca^{2+} overload and highlight the mitochondrial dysfunctions induced by MeHg via Ca^{2+} overload. Mitochondrial disturbances elicited by Ca^{2+} may involve several molecular events (i.e., alterations in the activity of the mitochondrial electron transport chain complexes, mitochondrial proton gradient dissipation, mitochondrial permeability transition pore (MPTP) opening, thiol depletion, failure of energy metabolism, reactive oxygen species overproduction) that could culminate in cell death. Here we will focus on the role of oxidative stress in these phenomena. Additionally, possible antioxidant therapies that could be effective in the treatment of MeHg intoxication are briefly discussed.

1. Introduction

Mercury is one of the most studied heavy metal due to its wide distribution in nature. In the environment, humans and animals can be exposed to different chemical forms of mercury, including elemental mercury vapor (Hg^0), inorganic mercurous (Hg^{2++}), mercuric (Hg^{++}), and organic mercuric compounds as ethylmercury, methylmercury, and dimethylmercury [1, 2]. All forms of mercury can be toxic and the extent of the toxic effects varies depending on the dose, chemical form, and level of exposure. Among the organic forms, methylmercury (MeHg) is the most frequently encountered in the environment. It is formed mainly as the result of methylation of inorganic (mercuric) forms of mercury by microorganisms in aquatic milieu, where it can pass up through the aquatic food chain and bioaccumulates in fish and sea mammals [1] (Figure 1). At present, sea food consumption represents the main human exposure route for MeHg and the brain is the main target organ for its toxicity. Neurological symptoms induced by MeHg intoxication can include cerebellar ataxia, paresthesia, dysarthria, mnemic deficits, memory impairment, and sensory disorders [3, 4]. MeHg has become a ubiquitous pollutant since outbreak of environmental disasters that occurred in Japan (1950s) and Iraq (1970s) due the consumption of MeHg-contaminated fish and seed grain, respectively [5, 6]. Although MeHg is known to affect adult central nervous system (CNS), these catastrophic episodes revealed the particular sensitivity of immature brain to high concentration of MeHg. Epidemiological evidence also shows that acute or chronic prenatal exposure to low MeHg levels from maternal consumption of fish can cause neurological deficits in children. Cerebral palsy, mental retardation, deafness, and blindness are some of abnormalities caused by fetal and neonatal MeHg exposure [3, 4, 7, 8]. Despite these observations, there is evidence that the fetal brain is more susceptible than infantile brain to MeHg toxicity. Differences among gestation stage, exposure duration, and efficacy of antioxidant systems in developing brain might be determinant factors in the age-dependent neuronal vulnerability to MeHg [4, 8–10].

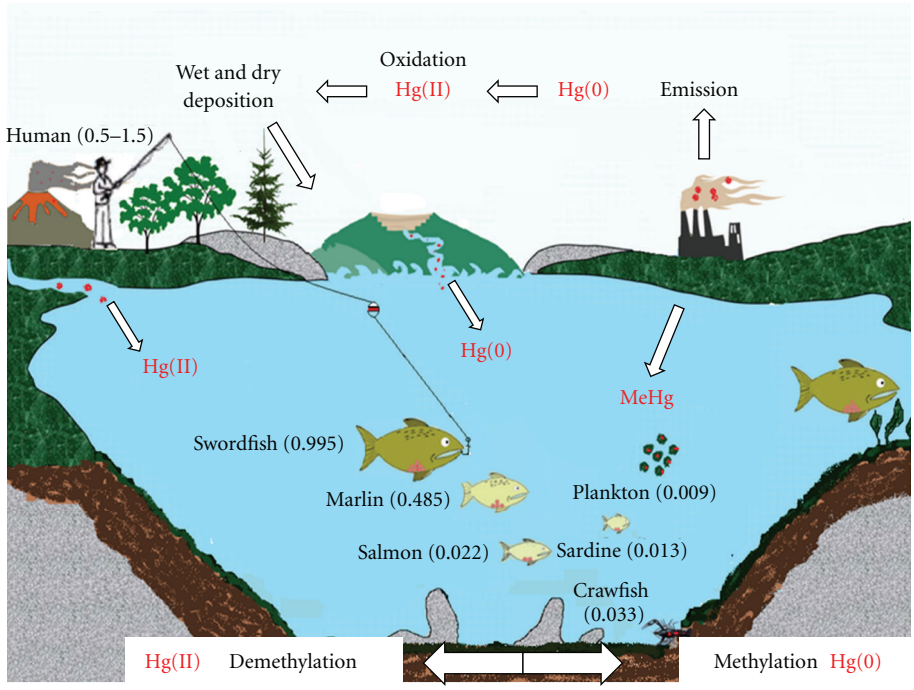


Figure 1: Cycle of mercury and its bioaccumulation in aquatic food chain. The values of mercury levels in the plankton and fish are represented as ppm. All data presented in this figure were obtained from (FDA and EPA).

As a consequence of high affinity of MeHg for –SH groups, the molecular interaction between MeHg and sulfhydryl-containing molecules as L-cysteine, glutathione (GSH), hemoglobin, and albumin has been implicated in the mechanisms involving transport, uptake, and accumulation of MeHg into living tissues [11]. In this regard, several studies have demonstrated that the cellular uptake of MeHg is markedly increased when it is present as Cys-MeHg conjugate [12, 13] once that this complex by mimicking structurally the amino acid methionine is a substrate for the neutral amino acid carrier L-type [11, 14] (Figure 2).

Different mechanism and molecular targets have been proposed to be involved in MeHg neurotoxicity. Thiol depletion (especially glutathione), glutamate dyshomeostasis, calcium dysregulation, oxidative stress, cytoskeletal disruption, and mitochondrial dysfunctions are among the detrimental effects known to render neurons vulnerability to MeHg toxicity [15, 16] (Figure 3).

One of the most widely documented effects caused by MeHg on the CNS is associated with glutamate-mediated excitotoxicity, which can be linked to or followed by intracellular Ca^{2+} overload. In this way, there are a number of experimental findings from *in vivo* and *in vitro* studies pointing that the inhibition of glutamate uptake by astrocytes, the increase in spontaneous release of glutamate from presynaptic terminals, and the inhibition of vesicular glutamate uptake are critical phenomena linked to MeHg-mediated excitotoxicity [15, 17, 18] (Figure 4). In this scenario, the excitatory amino acid receptors (N-methyl D-aspartate (NMDA) and non-NMDA-types) mediated pathways have been indicated as the main routes responsible by Ca^{2+} entry into cells following MeHg exposure [19, 20]. In accordance, a recent study using mouse spinal motor neurons in culture demonstrated that the excitatory amino acid receptor blockers MK-801 and AP-5 both NMDA receptor-operated ion channel blockers and the CNQX, a non-NMDA receptor blocker, were effective in delaying the development of increased Ca^{2+} after MeHg exposure [20]. In addition to glutamatergic receptors, there is evidence that voltage-dependent Ca^{2+} channels also contribute substantially to calcium influx after exposure to MeHg in neurons [20].

Regarding to a possible redox modulation of NMDA-type glutamate receptors directly by MeHg, there are no available data in the literature. However, it is important to mention here the study of Tang and Aizenman [21] showing that the alkylation of the NMDA redox site by the sulfhydryl alkylating agent N-ethylmaleimide (NEM) potentiated the response of receptor and renders NMDA receptors unresponsive to oxidation by Ellman's reagent 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB). Considering that the MeHg is a molecule as small as NEM and has high affinity by –SH groups, it is plausible to suppose that part of effects induced by MeHg on glutamate dyshomeostasis may be a consequence of its interaction with the redox modulatory site of the NMDA receptor. Taken together, the events mentioned above cause sustained elevation of intracellular Ca^{2+} that may trigger cell death pathways by many different mechanisms [20]. In these circumstances is pointed the close relationship existent between Ca^{2+} overload and mitochondria dysfunctions, which plays a crucial role in regulating cellular injury induced by MeHg (Figure 4).

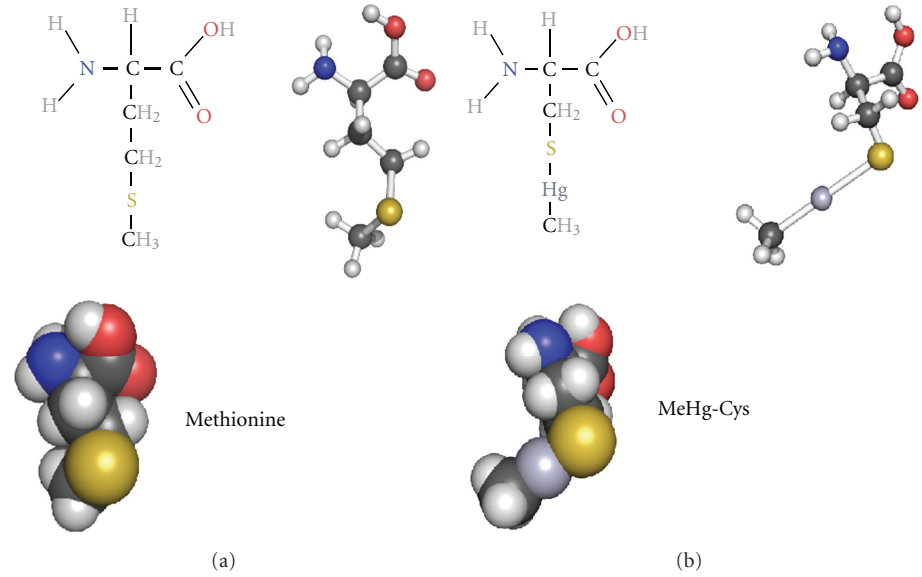


Figure 2: Schematic representation of the structures and space-filled models of methionine (a) and MeHg-Cys complex (b). Note the similarities in chemical structure between the MeHg-Cys conjugate and the amino acid methionine. The geometry-optimized using Universal Force Field (UFF). The representations (ball and stick, Vander Waals spheres) were obtained using the program PyMOL (Molecular Graphics System, Version 1.5.0.1, Schrödinger LLC).

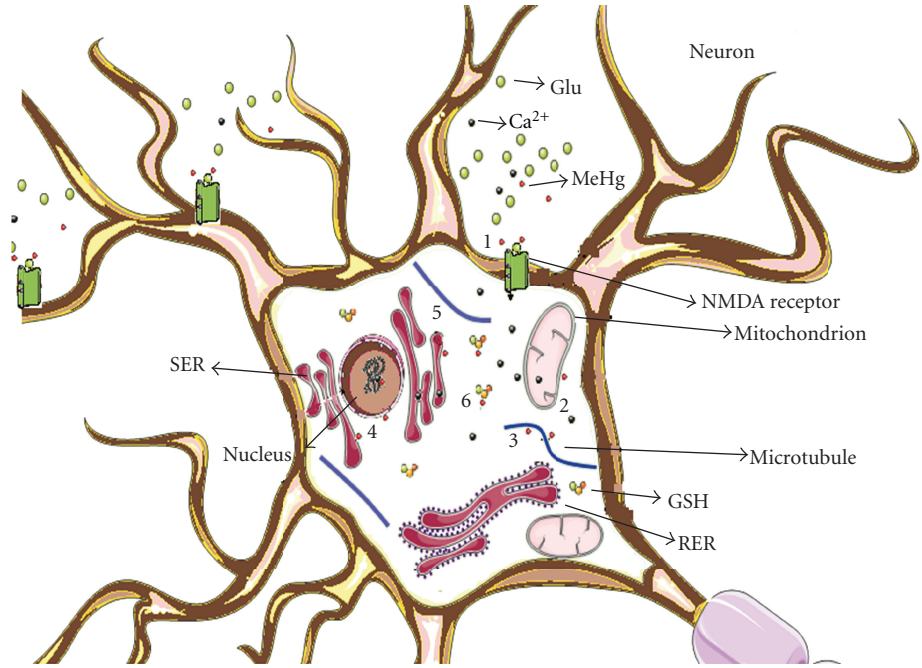


Figure 3: Schematic representation of possible mechanisms and cellular targets involved in the neurotoxicity MeHg-induced: (1) glutamate dyshomeostasis and Ca^{2+} intracellular dysregulation; (2) mitochondrial dysfunction; (3) cytoskeletal disruption; (4) DNA damage; (5) SER dysfunction; (6) thiol depletion (especially glutathione). This scheme is merely representative and the scale of structures does not represent the real size.

2. Calcium Homeostasis and Mitochondria

The concentration of Ca^{2+} in the cytosol is tightly regulated in all cells because calcium is a key element in metabolic and intracellular signaling regulation. Conversely, Ca^{2+} is also a relevant marker of numerous pathological processes when it is present at high, nonphysiological concentrations, notably neurological disorders [22]. The Ca^{2+} transport across neuronal cells membranes can occur by a variety of different mechanisms. In general, under resting conditions

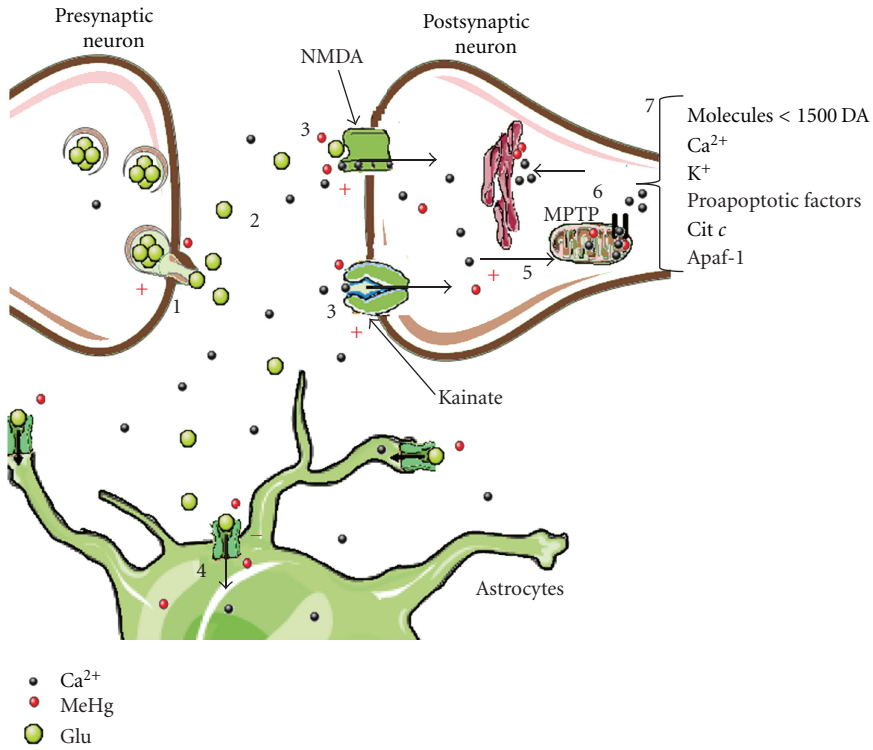


Figure 4: MeHg as mediator of neuronal toxicity via Ca^{2+}-mediated excitotoxicity: (1) glutamate (Glu) release from presynaptic neuron induced by MeHg; (2) increase of Glu into synaptic cleft; (3) Ca^{2+} influx via NMDA and Kainate receptors; (4) MeHg binds at Glu transporters in astrocytes; (5) mitochondria buffering the excess of Ca^{2+} intracellular; (6) MeHg causing mitochondrial and SER damage; (7) MTPT opening with release of pro-apoptotic factors induced by MeHg alone and/or excess of $[Ca^{2+}]_m$.

the intracellular Ca^{2+} levels ($[Ca^{2+}]_i$) are maintained at very low concentrations (in the nM range) when compared to the extracellular levels ($[Ca^{2+}]_e$) (in the mM range) [20, 22, 23]. On the order hand, in the region of transmitter release from active zones, at nerve terminals, the $[Ca^{2+}]_i$ can be many times greater, reaching, at least transiently, mM levels [20, 23–29].

During normal neuronal activity the entry of Ca^{2+} into neurons occurs very fast and without energy expenditure, since it takes place in favor of the concentration gradient across the plasma membrane. However, the restoration of $[Ca^{2+}]_i$ (following a transient increase) is much slower and depends on energy expenditure (either directly or indirectly through electrogenic "pumping" activities) [23, 25]. Therefore, the restoration of $[Ca^{2+}]_i$ homeostasis is extremely "expensive", being the "cost" directly proportional to the cell activity (i.e., highly active cells such as neurons expend a lot of cellular energy in order to restore the Ca^{2+} homeostasis). In this condition, some organelles can act as "Ca^{2+} buffers" by sequestering the excess of Ca^{2+}; however, this "storage" of Ca^{2+} also requires cost of cellular ATP equivalents [23, 30, 31] (Figure 5).

It is now well established that sustained elevations of $[Ca^{2+}]_i$ can cause neuronal degeneration and cell death by activating biochemical cascades that result in either necrotic or apoptotic processes [30–39]. However, the exact molecular mechanisms by which continuous increases in $[Ca^{2+}]_i$ elicit neuronal cell death pathways are not fully understood. It is believed that these may include the activation of degradative enzymes, such as phospholipases, proteases and endonucleases, perturbation of cytoskeletal organization, and, primarily mitochondrial dysfunctions [22, 30, 33].

Considering the importance of maintaining appropriate intracellular concentrations of Ca^{2+} for proper cellular function and the delicate balance between the physiological and toxicological effects of $[Ca^{2+}]_i$, in addition to mechanisms for Ca^{2+} removal from the cell, there are also critic mechanisms for the intracellular storage of Ca^{2+} in the cells. The most important cellular Ca^{2+} stores are the organelles mitochondria and smooth endoplasmic reticulum (SER). Mitochondria present low affinity and high capacity to Ca^{2+}, whereas the SER is a high-affinity and low-capacity Ca^{2+} pool [40]. So, while the SER moves Ca^{2+} from the cytosol into the SER lumen under low cytosolic $[Ca^{2+}]_i$, the mitochondria requires a more powerful stimulus, being necessary a local extra-mitochondrial Ca^{2+} concentration of approximately 0.5 mM, for neuronal mitochondria to take up Ca^{2+} actively [31, 41–43] (Figure 5). Disruption of Ca^{2+} regulation in either of these stores can compromise the neuronal function and survival [44] (Figure 5).

With particular emphasis in mitochondria, it is well recognized that at physiological concentration, Ca^{2+} is a powerful regulator of organelle metabolic activity, which acts primarily promoting ATP synthesis by stimulating crucial

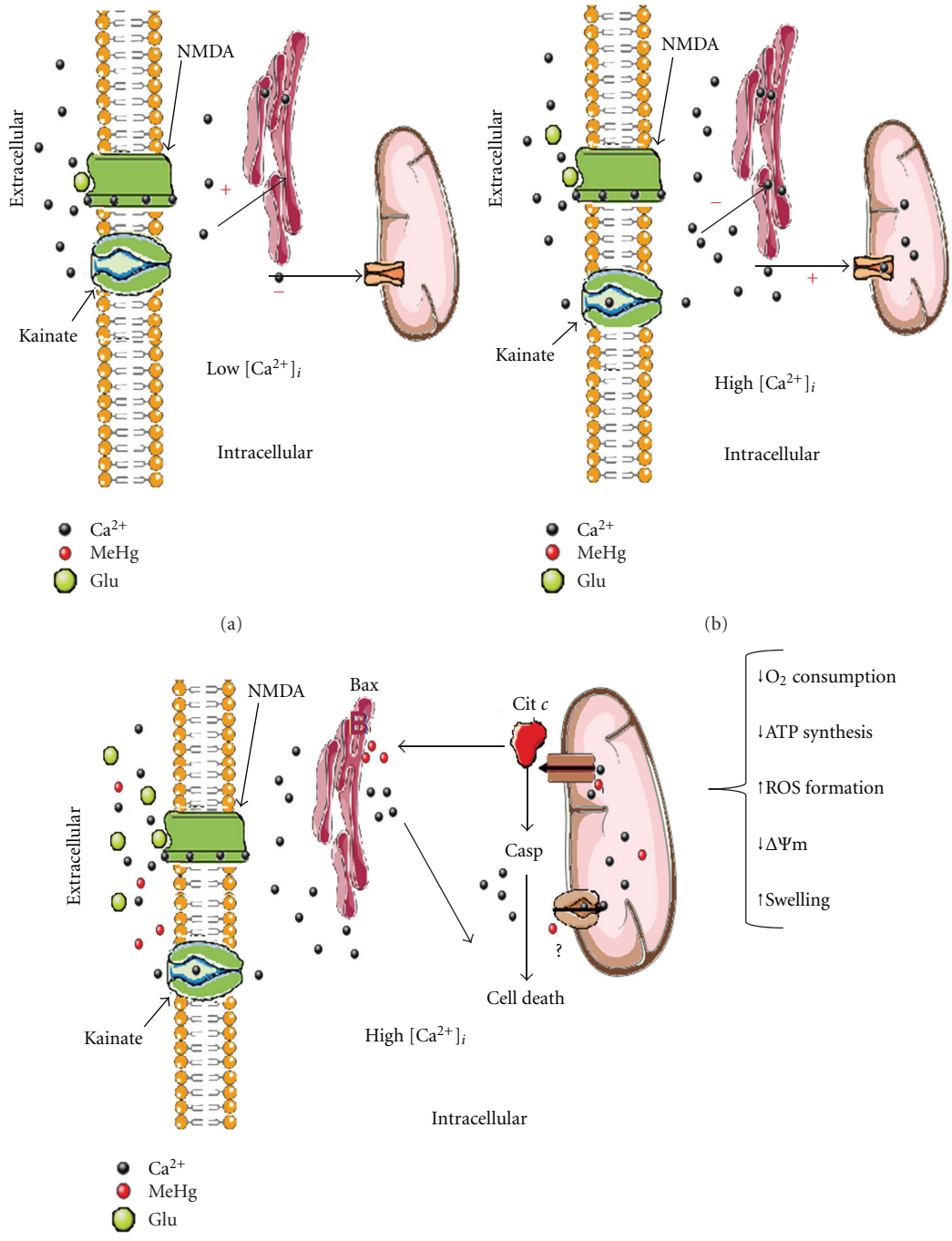


Figure 5: Schematic representation on the role of mitochondria and SER as intracellular Ca^{2+}stores and on the cellular death induced by MeHg via Ca^{2+} dyshomeostasis. (a) Under low cytosolic $[Ca^{2+}]_i$ the SER preferentially moves Ca^{2+} from cytosol due its high affinity and low capacity to stores Ca^{2+}; whereas (b) the mitochondria by presenting low affinity and high capacity to stores Ca^{2+} moves it under high cytosolic $[Ca^{2+}]_i$; (c) the disruption of Ca^{2+} regulation produced by MeHg in either of these stores can lead to release of neuronal proapoptotic factors that may trigger cell death pathways. The scheme presented here is merely representative, and the scale of the different cellular structures does not represent the real size.

enzymes of Krebs cycle (pyruvate, ketoglutarate, and isocitrate dehydrogenases). On the order hand, mitochondrial changes that occur in most instances of cell death (apoptosis and necrosis) require an elevated influx of Ca^{2+} into matrix [30–38] (Figure 5).

It has been suggested that the outer membrane voltage-dependent anion channel (VDAC), a ruthenium red (RuRed-) sensitive Ca^{2+} channel, serves to regulate Ca^{2+} entry to mito-chondrial intermembrane space (Figure 6). Furthermore, mitochondria take up a large quantity of

Ca^{2+} across the inner membrane mainly via the activity of the mitochondrial calcium uniporter channel (MCU) [45] (Figure 6). This uptake is driven by the membrane potential ($\Delta\Psi m$), and consequently the net movement of charge due to Ca^{2+} uptake collapses $\Delta\Psi m$ [45]. Although there is a general consensus that MCU is dominantly responsible for the Ca^{2+} influx into mitochondrial matrix, further studies have identified other pathways related to mitochondrial Ca^{2+} influx, including the mitochondrial ryanodine receptor (mRyR), mitochondrial uncoupling proteins, Letm1 (Ca^{2+}/H^{+} antiporter), and rapid mode of mitochondrial (RaM) [45] (Figure 6). It is also important to highlight here the ability of mitochondria in releasing Ca^{2+}. Under normal physiological conditions, Ca^{2+} efflux from mitochondria is mediated primarily by mitochondrial Na^{+}/Ca^{2+} exchanger. Indeed, Ca^{2+} efflux can also occur through uniporter channel if $\Delta\Psi m$ is collapsed. Interestingly, another Ca^{2+} efflux pathway is the classical mitochondrial permeability transition pore (MPTP), which is a large voltage-dependent channel formed by a set of mitochondrial proteins located in the inner membrane, mitochondrial matrix as well as on the outer membrane (Figure 6). Existing predominantly in the closed position, the pore transient opening under normal conditions may serve as a physiological means of releasing excess of metabolites, inorganic anions, and ions, especially Ca^{2+} [46–50]. Indeed, MPTP permits the passage of several apoptotic mediators from mitochondria and is also a primary target of others, especially the Bcl-2 family proteins [51–55]. In this circumstances, it is important to emphasize the key role of mitochondrial Ca^{2+} overload and oxidative stress in MPTP opening.

There is a general conception that Ca^{2+} overload results in mitochondrial dysfunction, which trigger apoptosis by many different and connected pathways. In fact, a variety of molecular events involved in apoptotic stimulation focus on mitochondria. Mitochondrial changes as exacerbated ROS formation, dissipation of the $\Delta\Psi m$, altered redox potential, MPTP opening, release of apoptotic mediators (cytochrome c, Apaf-1 and apoptosis inducing factor (AIF)), and participation of proapoptotic Bcl-2 family proteins are among the diverse signals associated to apoptosis induction by intrinsic or mitochondrial pathways [16, 51–55] (Figure 5). Indeed, ATP synthesis failure associated with mitochondrial dysfunctions can lead to abnormal cellular homeostasis causing swelling and cellular disruption, which eventually lead to necrotic death.

2.1. MeHg Disturbs Intracellular Calcium Homeostasis: Effects on Mitochondrial Function. A number of reports have indicated that MeHg, at low micromolar concentrations, disrupts Ca^{2+} homeostasis and causes elevations in $[Ca^{2+}]_i$ in different cells types, ranging from neurons and neuroblastoma cells to T lymphocytes and Purkinje cells [56–61]. The Ca^{2+} involvement on MeHg neurotoxicity is supported by several data, including the findings showing that the BAPTA (a Ca^{2+} chelator) is able to protect granule cells from MeHg-induced mortality after short periods of exposure. The regulation of other divalent cations besides Ca^{2+}, such as Zn^{2+} may be also

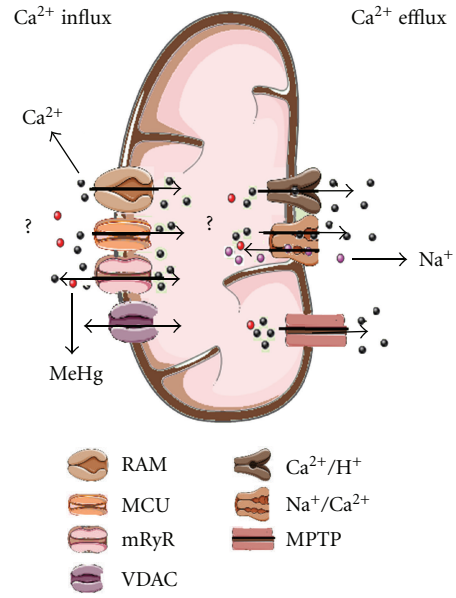


Figure 6: Representation of different channels responsible for Ca^{2+} influx/efflux in mitochondria and the possible interaction of MeHg with these channels. VDAC: outer membrane voltage-dependent anion channel; RAM: rapid uptake model; MCU: inner membrane calcium uniporter channel; mRyR: mitochondrial ryanodine receptor; Ca^{2+}/H^{+} antiporter; Na^{+}/Ca^{2+} exchanger; MPTP: mitochondrial permeability transition pore. The scale of structures represented in this scheme does not represent the real size.

disrupted during MeHg exposure; nevertheless, here we will focus on the effect of MeHg in Ca^{2+} homeostasis [62].

As previously mentioned, it has been postulated that the effects of MeHg on neuronal Ca^{2+} homeostasis, at least in part, involve the excitatory amino acids receptors (either NMDA and non-NMDA receptors), since that channel blockers as ω-conotoxin GVIA and nifedipine delay MeHg-induced elevation in $[Ca^{2+}]_i$ levels [19, 20]. In accordance, several studies have demonstrated that the glutamate overactivation of NMDA receptor induced by MeHg inhibtion of glutamate uptake can raise intracellular Ca^{2+} influx and ROS overproduction [16]. In addition, experimental evidence also supports the participation of L- and N-type Ca^{2+} channels on MeHg neurotoxicity given that blockers of voltage-dependent Ca^{2+} channels prevent the appearance of neurological disorders in rats exposed to MeHg [63, 64]. In this sense, a recent work suggested that canonical transient receptor potential channels (TRPC), Ca^{2+}-permeable cationic channels, could be important for the mechanisms of cytotoxicity and neuronal impairment mediated by MeHg. In fact, in this study was observed that MeHg that potently activates TRPC5 as well as TRPC4 channels via binding to the two extracellular cysteine residues near the channel pore, whereas silence of TRPC5 gene with small interfering RNA (siRNA) or blockade of TRPC channel activity with pharmacological tools alleviates MeHg-related cytotoxicity [65]. Notwithstanding, some findings have shown that MeHg toxicity may be triggered by elevating $[Ca^{2+}]_i$ through activation of phosphatidylcholine-specific phospholipase C (PC-PLC), which could contribute

to the entry of extracellular Ca^{2+} as well as to the opening of transmembrane Ca^{2+} channels from intracellular Ca^{2+} store [66, 67].

Numerous lines of evidence indicate that the MeHg-mediated neuronal cells death is associated with a severe loss of $[Ca^{2+}]_i$ homeostasis [58, 59, 68]. In neurons and NG108-15 neuroblastoma cells, MeHg causes a characteristic biphasic increase in $[Ca^{2+}]_i$ that consists of an initial release of Ca^{2+} from one or more intracellular stores into the cytosol ("first-phase"), and a secondary influx of $[Ca^{2+}]_e$ ("second-phase") [56, 58]. However, it is necessary to consider that the elevation of $[Ca^{2+}]_i$ occurs at much lower MeHg concentrations in cerebellar granule cells than in NG108-15 cells, which could explain, at least in part, the different sensitivity of granule cells to the toxic effects of MeHg as compared to other neuronal cells [56, 58]. In analogy, there is evidence that elevated $[Ca^{2+}]_i$ has a important role in dimethylmercury (di-MeHg-) induced cell death. Of note, Ca^{2+} deposits (as calcospherites, a spherical mass of calcium salts and organic matter) were found in cerebellar slices of rats treated with di-MeHg. The cerebellar accumulation of calcospherites was directly proportional to exposure time, and the atrophy of the granule cell layer was evident at later time points [69]. Moreover, it has been observed that adjacent Purkinje cells did not show any sign of cell loss or death, nor accumulate Ca^{2+} [69]. These findings suggest that the presence or absence of Ca^{2+} deposits within the cerebellum seems to be directly correlated with sensitivity or resistance of cells to MeHg neurotoxicity. Noteworthy, it is important to mention here that this study was done with di-MeHg which is much more toxic to humans than mono-MeHg [68]. This is important to avoid confusion between mono-MeHg and di-MeHg, particularly in view of the extreme toxicological effects of di-MeHg to humans (even at low concentrations). Other important critical aspect here is the involvement of cerebellum in MeHg toxicity. Although this seems to be the case for humans (and cats), it is difficult to find in rodent literature "a clear picture" that mono-MeHg targets preferentially cerebellum than other brain structure. It is possible that cerebellum of rats be more sensitive to di-MeHg, which has been little used (possibly because of its chemical instability and toxicity).

Important early studies by Yoshino et al. [70] showed that MeHg is able to accumulate rapidly in mitochondria. In this way, subsequent works indicated a spectrum of mitochondrial effects, either directly or indirectly via Ca^{2+} overload, of MeHg both *in vivo* and *in vitro*, including alterations in complex III of the mitochondrial electron transport chain (ETC), depression of respiration and ATP production, swelling of the mitochondrial matrix, and loss of $\Delta\Psi m$ with subsequent release of cytochrome c [71–80]. The loss of $\Delta\Psi m$ seems to result from MPTP opening, which can be prevented by treatment with the pore-blocking agent cyclosporin A (CsA) or bongkrekic acid [81–84]. In several studies CsA, but not FK506, that is similar to CsA but without MPTP inhibitory activity, provides a marked degree of neuroprotection against MeHg, supporting the involvement of MPTP in the MeHg neurotoxicity [73, 85–87]. Other experimental evidence shows that mitochondria contributes to the MeHg-induced first-phase $[Ca^{2+}]_i$ increase and subsequent cell death through opening of the MPTP [56].

To note, mitochondria have been considered the principal source of intracellular Ca^{2+} release in culture of cerebellar granule cells during MeHg exposure [73]. It has been documented that MeHg affects mitochondrial Ca^{2+} ($[Ca^{2+}]_m$) regulation through at least two potential mechanisms. The first involves its direct interaction with mitochondrial proteins, which is thought to alter the ability of mitochondria in functioning normally. Corroborating this idea, there is evidence that MeHg inhibits mitochondrial nucleic acid synthesis and interferes with the electron transport chain via direct interaction with respiratory proteins [79] as well as causes loss of inner mitochondrial membrane potential in isolated mitochondria from different tissues, including neuronal cells [48, 71, 73, 85, 88, 89]. The second mechanism is associated with an indirect inhibition of mitochondrial function via excessive uptake of Ca^{2+} into the mitochondria. The elevated $[Ca^{2+}]_m$ may depolarize the inner mitochondrial membrane, inhibit the tricarboxylic acid cycle and the mitochondrial ATPase, dissipate the mitochondrial proton gradient, and promote MPTP opening [88–94]. Of particular importance, $[Ca^{2+}]_m$ can be increased after Ca^{2+} release from SER. Experiments in NG108-15 neuroblastoma cells and cerebellar granule cells show that MeHg causes Ca^{2+} release from SER, primarily through IP3 receptor, which is buffered by mitochondria (tentatively to maintain $[Ca^{2+}]_i$ homeostasis) [95, 96]. Corroborating these findings, an elegant study performed by Budd and Nicholls [97] demonstrated that mitochondria can store and release large amounts of Ca^{2+} under both physiological and pathological conditions in granule neurons [96–98]. Taken together, these data suggest that the excessive uptake of $[Ca^{2+}]_m$ after release of Ca^{2+} from the SER is a potential signal for the MPTP opening and $[Ca^{2+}]_m$ release from the mitochondrial lumen into the cytosol in cerebellar granule cells exposed to MeHg. These observations are also consistent with the hypothesis that both Ca^{2+} ionophore A23187 and MeHg cause a rapid and massive increase in mitochondrial Ca^{2+} influx, which secondarily triggers the overproduction of ROS that facilitates MPTP induction, loss of the electron transport chain activity, and the ensuing mitochondrial death [98]. Thus, an uncontrolled release of Ca^{2+} from the mitochondria may occur during oxidative stress, a condition resulting from the imbalance between the production of free radicals and the counteraction by the cellular antioxidant defenses [49, 99].

Based on the evidence addressed previously, mitochondria must be considered critical in the regulation of neuronal cell death induced by MeHg via Ca^{2+} dyshomeostasis and/or ROS generation (under oxidative stress conditions) [48–50]. In this sense, literature data have pointed that ROS formation is not the cause of mitochondrial dysregulations after MeHg exposure but rather a secondary event that reflects MeHg-induced elevations in mitochondrial Ca^{2+} levels [48, 53]. In agreement, an *in vitro* study revealed that the treatment of striatal synaptosomes with the antioxidant Trolox was effective in reducing ROS levels but failed on restoring mitochondrial damage induced by MeHg [100]. Similarly,

in a recent work using rat cortical slices was evidenced that the flavonoids quercitrin and quercetin reduced significantly mitochondria MeHg-generated ROS production, which was dependent upon an increase in intracellular Ca^{2+} levels [74, 101].

2.2. MeHg-Induced ROS Formation and Mitochondrial Dysfunction: Effect of Antioxidants. As already cited before, mitochondrial dysfunctions elicited by MeHg may lead to a mitochondrial burst of ROS production. ROS are important mediators of damage to cell structures, including lipids and membranes, as well as proteins and nucleic acids [102, 103]. In mitochondria, ROS may impair energy metabolism by inducing oxidative structural changes and the ensuing loss of activity in a number of mitochondrial enzymes that play critical roles in ATP production [42]. Besides, the direct action of ROS on mitochondrial membrane lipids and proteins results in the activation of apoptotic cascades by MPTP opening dependent or independent mechanisms [51, 53, 54].

The detrimental effects of ROS are balanced by the antioxidant action of nonenzymatic and enzymatic systems [104]. Indeed to ROS overproduction, *in vivo* and *in vitro* experimental observations have shown that the toxic effects of MeHg usually are accompanied by significant deficits in antioxidant defenses. MeHg can cause a decrease in the endogenous nonenzymatic antioxidants as well as an inhibition of the antioxidant enzymes [104–110]. Thus, it is reasonable to suppose that the cellular/mitochondrial ROS production may be directly or indirectly triggered by MeHg. In fact, the high affinity of MeHg by –SH groups might decrease GSH content and consequently facilitates ROS formation [11]. On the order hand, MeHg also could exacerbate ROS formation by increasing $[Ca^{2+}]_i$. From a molecular point of view, as highlighted in Figure 7, these factors (GSH depletion, ROS and Ca^{2+}) exist as a pyramidal network, where they may act independently or through a complex interaction to activate the cascade of events involved in cell injury mediated by MeHg. With this in mind, the following sections discuss two possible antioxidant therapies (natural and synthetic) that could be effective in ameliorating the symptoms of MeHg intoxication by interfering with these factors.

2.2.1. Natural Antioxidants against MeHg-Induced Toxicity. Despite the massive efforts in the search for new drugs that counteract mercurial toxicity, there are no effective treatments available that completely abolish its toxic effects. In general, the available antidotal strategies to treat mercury poisonings are largely based on chelating therapies. The use of sulfhydryl-enriched chelators is based on the high affinity of MeHg to –SH groups, leading to mercury elimination from tissues predominantly via renal excretion. However, these drugs are of limited use, because of their adverse side effects [111]. Moreover, it has been proposed that chelating therapies are ineffective in poisonings with organic forms of mercury [112], although this issue remains controversial [113, 114]. Nevertheless, there is general agreement that metal chelators are unable to completely

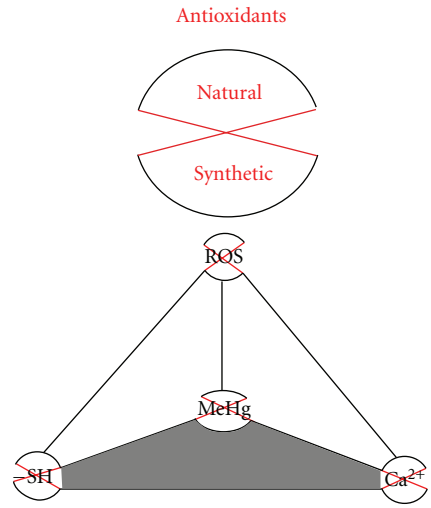


Figure 7: Pyramidal network involved in MeHg neurotoxicity: Arrows denote the interlinked possible pathways by which MeHg may cause cellular damage and the protective effect of natural and synthetic antioxidants against MeHg neurotoxicity by blocking oxidative events triggered via –SH depletion, ROS formation, and Ca^{2+} dyshomeostasis. For full details, see the text.

eliminate mercurials' body burden and by inference, toxicity. Thus, the use of compounds with antioxidant properties and no apparent side effects could represent an efficient coadjuvant strategy to counteract MeHg toxicity. Therefore, various purified phytochemicals or plant extracts have been shown to confer some protection against MeHg-mediated excitotoxicity and oxidative stress *in vivo* and *in vitro* [106, 115]. Possible mechanisms involved in the mitigation of MeHg effects by phytochemicals may include the reduction of ROS production, activation of enzymatic antioxidant systems, restoration of the mitochondrial membrane potential, and modulation of cell signaling pathways [116]. Dietary phytochemicals may also affect additional endpoints such as MeHg bioavailability and pharmacokinetics [117, 118]. In particular, natural products (such as flavonoids) whose antioxidant properties have been well described [119–121] could represent important therapeutic choices. In this sense, it has been characterized in both *in vitro* and *in vivo* experimental models that flavonoids exert beneficial effects by preventing or reducing free radical production, blocking Ca^{2+} influx into cells, chelating iron, and exerting anti-inflammatory action [100, 121, 122] (Figure 7). Indeed, such class of compounds has displayed neuroprotective effects in several experimental models of neurodegeneration [123–125]. Of particular significance, the current literature shows that plant extracts containing flavonoid provide protection against MeHg-induced neurotoxicity in mice [115, 121]. The protective effect of flavonoids has been correlated with their capacity in detoxifying the H_2O_2 generated in the presence of mercurials [71]. In addition to a direct interaction with H_2O_2, it is possible that flavonoids may also reduce H_2O_2 production via inhibition of Ca^{2+} influx into cells or mitochondria [105] and/or by forming redox inactive complexes with iron, rendering this prooxidant unavailable for Fenton reaction.

A recent *in vitro* study using the flavonoids quercetin, rutin, and quercitrin showed that lipid peroxidation and ROS generation in both mitochondria-enriched fractions and cortical brain slices exposed to MeHg were significantly reduced by quercetin and quercitrin [74]. Likewise, further findings demonstrated that mangiferin was effective in offering protection to human neuroblastoma cells against the toxic effects of MeHg. The protective effect of this flavonoid was attributed to its antigenotoxic, antiapoptotic and antilipid peroxidative potential plausibly because of its free radical scavenging ability, which reduced the oxidative stress and in turn facilitated the downregulation of mitochondrial apoptotic signalling pathways [126–128]. Corroborating these experimental observations, a current study performed by Franco et al. showed that the flavonoids myricetin, myricitrin, and rutin are able in reducing mitochondrial dysfunctions in mouse brain mitochondrial-enriched fractions treated with MeHg *in vitro*; being that the myricetin displayed higher protective effect against MeHg-induced mitochondrial toxicity when compared to myricitrin and rutin [129]. Additionally, only the myricetin was able to inhibit completely ROS formation and lipid peroxidation MeHg induced [129]. The scavenger property of this flavonoid was considered, at least in part, responsible for its protective effects against mitochondrial dysfunction induced by MeHg.

From a molecular point of view, the antioxidant activities of flavonoids can be influenced by their chemical structure [130, 131], and there are several molecular characteristics that confer the ability of a given flavonoid to promptly donate electrons and reduce reactive species. Basically, polyphenolic flavonoids possess a diphenylpropane (C6C3C6) skeleton [130, 131]. The presence of hydroxyl groups linked to phenolic rings correlates with their capability to donate electrons [132]. The positions and, more importantly, the amounts of hydroxyl groups present in the polyphenolic skeleton increase their ability to neutralize reactive species [130, 131]. The removal of this functional group from flavonoids has been reported to impair their antioxidant potency [130–132]. Lack of saturation at the C-ring is another structural property that confers antioxidant ability to flavonoids. Similarly, the blockade of the hydroxyl group in the C-ring through glycosylation has also been reported to decrease the antioxidant ability of this class of compounds [130–133].

Despite the potential use of natural products (specially flavonoids) to counteract the MeHg-induced cell damage via mitochondria-mediated ROS generation, more *in vivo* experiments are needed to validate the efficacy of flavonoids in attenuating MeHg-mediated injuries, particularly in view of the recently published synergistic neurotoxic action of quercetin and MeHg in adult mice [101].

2.3. Synthetic Antioxidants against MeHg-Induced Toxicity. Similar to natural compounds, numerous synthetic compounds exhibit potent antioxidant properties that could be effective in reducing the oxidative damage elicited by MeHg. However, the focus of this section will be in some selenium (Se) compounds, since it has long been hypothesized that

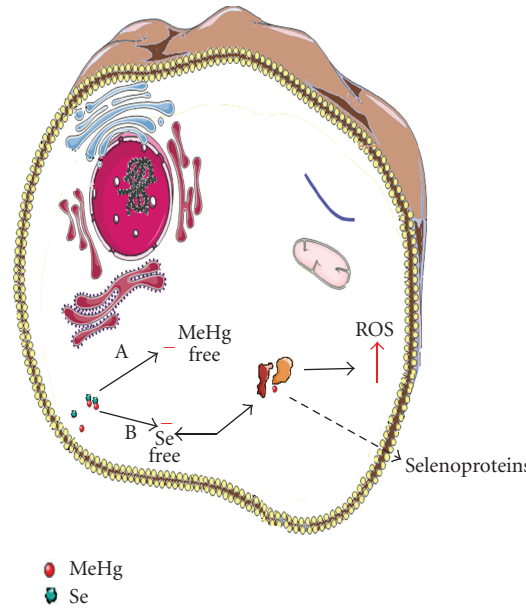


Figure 8: Possible role of Selenium (Se) on MeHg toxicity: (A) MeHg toxicity reduced by formation of complex MeHg-Se; (B) MeHg toxicity increased due Se depletion by formation of complex MeHg-Se: ↓ Selenoprotein activities and synthesis and ↑ ROS.

Se may protect against the harmful effects of mercury, particularly organic MeHg [134].

Most of beneficial effects exhibited by Se in biological systems are connected to activity of enzymes glutathione peroxidases family (GPx1–4), thioredoxin reductase family (TrxR1-2), thioredoxin glutathione reductase (TGR), and iodothyronine deiodinase isoforms (DIO1–3), which present this element as a structural component [135].

Regarding to mercury toxicity, it has been pointed that the strong interaction between mercury and Se is an important mechanism related to neuroprotection offered by selenium compounds [135, 136] (Figure 8). From this point of view, the Se's protective role is associated with the formation of stable complex(es) resulting of the reaction between mercurials and selonohydryl/selenol groups (–SeH) formed from de selenium compounds. However, it is important to mention here that little is known about the toxicokinetics of these complexes in biological systems. Furthermore, some studies argue that Se sequestration due to mercury binding to Se could compromise Se's biological functions and availability, mainly by impairing selenoenzyme activities and synthesis [135] (Figure 8).

The use of synthetic compounds containing selenium has received growing interest as therapeutic strategies in the treatment of mercury intoxication symptoms, including the detrimental effects of MeHg in CNS [135–137]. Of particular importance are the GPx- and TRxR-like activities displayed by ebselen and diphenyl diselenide, two simple synthetic organoselenium compounds that have been considered potent antioxidant and neuroprotective agents in different experimental models [136, 138]. Current literature findings show that these compounds may be reduced by mammalian

(TrxR) forming selenolate intermediates that are potent nucleophiles and can readily react with electrophilic species, including ROS [138]. This implies that these compounds could efficiently attenuate the oxidative damage caused by MeHg. Corroborating this hypothesis, a recent study revealed that diphenyl diselenide reduced cerebral oxidative stress as well as Hg deposition in the liver, kidneys, and brain of adult mice exposed to MeHg [13]. In this work the authors assume that the effectiveness exhibited by compound is derived from the formation of a stable complex (MeHg-Selenol) due interaction of MeHg with a "selenol intermediate" [13].

Based on the observations previously cited, it is reasonable to suppose that ebselen and diphenyl diselenide might diminish the neurotoxic action of MeHg by increasing the excretion of MeHg from the body due their abilities in forming inert complexes with MeHg [13]. Regarding to the involvement of glutamatergic system on MeHg neurotoxicity, there is *in vitro* evidence that both organoselenium compounds as well as MK-801 were effective on blocking ROS formation caused by MeHg in cortical rat brain slices by maintaining the hydrogen peroxide status at low physiological levels and/or through a direct modulation of the NMDA receptor redox site [110]. Likewise, the treatment of cerebral cortex slices from young rats with ebselen restored the glutamate uptake and cell viability changed by MeHg exposure [139]. Differently, in this experimental protocol diphenyl diselenide did not modify these parameters [139].

In addition, data from *ex vivo* experiments show that glutamate release from brain synaptosomal preparations and glutamate uptake by brain cortical slices is increased in rat pups exposed to MeHg during the suckling period and that these effects were prevented by ebselen [140]. Equally important, there is evidence that ebselen was effective in reversing the inhibition induced by oral exposure to MeHg on glutamate uptake of brain cortical slices of adult mice as well as in restoring the activity of enzymes glutathione peroxidase and catalase activity, which were modified by MeHg. These protective effects of ebselen were related to its ability to detoxify H_2O_2 [140].

Of particular importance, recently an elegant study demonstrated the potential ability of ebselen and diphenyl diselenide in preventing neuronal dysfunction caused by MeHg via cytoskeletal proteins disruption. In this work MeHg exposure induced hyperphosphorylation of the high molecular weight neurofilaments subunit from slices of cerebral cortex of young rats, and both selenium compounds ebselen and diphenyl diselenide were efficient in reversing these alterations [141].

As stated before, there is numerous experimental evidence suggesting that MeHg exposure may induce damage in mitochondria from different organs and tissue [74], and that this effect could be associated to ROS overproduction and/or increased intracellular calcium levels [74, 142]. However, to the best of our knowledge, there are no reports on the role of organoselenium compounds in isolated mitochondria challenged by MeHg. Thus, although the organic selenium forms cited here may represent an interesting class of compounds as therapeutic agents against MeHg intoxication, further research to evaluate the efficacy and safety of such compounds will be needed.

3. Conclusion

Despite the large number of studies on MeHg toxicity, the understanding about the metabolism and toxicokinetics of MeHg as well as the molecular mechanisms involved in its neurotoxic effects remains to be fully elucidated. Meanwhile, taken together the data discussed in this paper collaborate for a better understanding of the multifactorial mechanisms (glutamate and calcium dyshomeostasis, as well as mitochondrial dysfunction) involved on neurotoxicity produced by MeHg and point the possible interlinked pathways by which MeHg might cause neuronal damage, with special emphasis on mitochondrial disturbances, since the mitochondria seem to be a key organelle implicated in the detrimental effects triggered by MeHg both *in vivo* and *in vitro*. In this regard, experimental studies show that both synthetic and natural antioxidants may be considered promising molecules in counteracting the toxic effects elicited by MeHg. Thus, although experimental research has been highly instrumental in shedding novel information on aspects involved in MeHg-induced neurotoxicity, further studies on the precise temporal relationship between the listed phenomena, as well as potential therapeutic/antidotal strategies to counteract the mitochondrial dysfunction produced by MeHg are warranted.

Acknowledgments

The authors would like to thank the colleagues/coauthors who contributed to several studies referenced in this paper. The financial support by FINEP Research Grant "Rede Instituto Brasileiro de Neurociência (IBN-Net)" no. 01.06.0842-00, FAPERGS/Pronex, CAPES/SAUX, CAPES/NOVOS TALENTOS, VITAE Fundation, INCT-CNPq-Excitotoxicity and Neuroprotection, and CNPq is gratefully acknowledged. Nilda Vargas Barbosa is the recipient of CNPq fellowships.

References

[1] T. W. Clarkson, "The three modern faces of mercury," *Environmental Health Perspectives*, vol. 110, no. 1, pp. 11–23, 2002.

[2] R. A. Bernhoft, "Mercury toxicity and treatment: a review of the literature," *Journal of Environmental and Public Health*, vol. 2012, Article ID 460508, 10 pages, 2012.

[3] E. Oken and D. C. Bellinger, "Fish consumption, methylmercury and child neurodevelopment," *Current Opinion in Pediatrics*, vol. 20, no. 2, pp. 178–183, 2008.

[4] G. J. Myers, S. W. Thurston, A. T. Pearson et al., "Postnatal exposure to methyl mercury from fish consumption: a review and new data from the Seychelles Child Development Study," *NeuroToxicology*, vol. 30, no. 3, pp. 338–349, 2009.

[5] F. Bakir, S. F. Damluji, and I. Amin Zaki, "Methylmercury poisoning in Iraq: an interuniversity report," *Science*, vol. 181, no. 4096, pp. 230–241, 1973.

[6] M. Harada, "Congenital Minamata disease: intrauterine methylmercury poisoning," *Teratology*, vol. 18, no. 2, pp. 285–288, 1978.

[7] A. Spurgeon, "Prenatal methylmercury exposure and developmental outcomes: review of the evidence and discussion of future directions," *Environmental Health Perspectives*, vol. 114, no. 2, pp. 307–312, 2006.

[8] S. Bose-O'Reilly, K. M. McCarty, N. Steckling, and B. Lettmeier, "Mercury exposure and children's health," *Current Problems in Pediatric and Adolescent Health Care*, vol. 40, no. 8, pp. 186–215, 2010.

[9] J. Stringari, A. K. C. Nunes, J. L. Franco et al., "Prenatal methylmercury exposure hampers glutathione antioxidant system ontogenesis and causes long-lasting oxidative stress in the mouse brain," *Toxicology and Applied Pharmacology*, vol. 227, no. 1, pp. 147–154, 2008.

[10] A. Dreiem, C. C. Gertz, and R. F. Seegal, "The effects of methylmercury on mitochondrial function and reactive oxygen species formation in rat striatal synaptosomes are age-dependent," *Toxicological Sciences*, vol. 87, no. 1, pp. 156–162, 2005.

[11] Z. Yin, H. Jiang, T. Syversen, J. B. T. Rocha, M. Farina, and M. Aschner, "The methylmercury-L-cysteine conjugate is a substrate for the L-type large neutral amino acid transporter," *Journal of Neurochemistry*, vol. 107, no. 4, pp. 1083–1090, 2008.

[12] N. Ballatori, "Transport of toxic metals by molecular mimicry," *Environmental Health Perspectives*, vol. 110, no. 5, pp. 689–694, 2002.

[13] D. H. Roos, R. L. Puntel, T. H. Lugokenski et al., "Complex methylmercury-cysteine alters mercury accumulation in different tissues of mice," *Basic and Clinical Pharmacology and Toxicology*, vol. 107, no. 4, pp. 789–792, 2010.

[14] D. H. Roos, R. L. Puntel, M. Farina et al., "Modulation of methylmercury uptake by methionine: prevention of mitochondrial dysfunction in rat liver slices by a mimicry mechanism," *Toxicology and Applied Pharmacology*, vol. 252, no. 1, pp. 28–35, 2011.

[15] M. Aschner, T. Syversen, D. O. Souza, J. B. T. Rocha, and M. Farina, "Involvement of glutamate and reactive oxygen species in methylmercury neurotoxicity," *Brazilian Journal of Medical and Biological Research*, vol. 40, no. 3, pp. 285–291, 2007.

[16] M. Farina, M. Aschner, and J. B. T. Rocha, "Oxidative stress in MeHg-induced neurotoxicity," *Toxicology and Applied Pharmacology*, vol. 256, no. 3, pp. 405–417, 2011.

[17] G. Shanker and M. Aschner, "Identification and characterization of uptake systems for cystine and cysteine in cultured astrocytes and neurons: evidence for methylmercury-targeted disruption of astrocyte transport," *Journal of Neuroscience Research*, vol. 66, no. 5, pp. 998–1002, 2001.

[18] G. Shanker and M. Aschner, "Methylmercury-induced reactive oxygen species formation in neonatal cerebral astrocytic cultures is attenuated by antioxidants," *Molecular Brain Research*, vol. 110, no. 1, pp. 85–91, 2003.

[19] Y. Yuan and W. D. Atchison, "Methylmercury-induced increase of intracellular Ca^{2+} increases spontaneous synaptic current frequency in rat cerebellar slices," *Molecular Pharmacology*, vol. 71, no. 4, pp. 1109–1121, 2007.

[20] G. Ramanathan and W. D. Atchison, "Ca^{2+} entry pathways in mouse spinal motor neurons in culture following in vitro exposure to methylmercury," *NeuroToxicology*, vol. 32, no. 6, pp. 742–750, 2011.

[21] L. H. Tang and E. Aizenman, "The modulation of *N*-methyl-*D*-aspartate receptors by redox and alkylating reagents in rat cortical neurones *in vitro*," *Journal of Physiology*, vol. 465, pp. 303–323, 1993.

[22] G. E. N. Kass and S. Orrenius, "Calcium signaling and cytotoxicity," *Environmental Health Perspectives*, vol. 107, no. 1, pp. 25–35, 1999.

[23] T. L. Limke, S. R. Heidemann, and W. D. Atchison, "Disruption of intraneuronal divalent cation regulation by methylmercury: are specific targets involved in altered neuronal development and cytotoxicity in methylmercury poisoning?" *NeuroToxicology*, vol. 25, no. 5, pp. 741–760, 2004.

[24] M. R. Bennett, L. Farnell, and W. G. Gibson, "The probability of quantal secretion near a single calcium channel of an active zone," *Biophysical Journal*, vol. 78, no. 5, pp. 2201–2221, 2000.

[25] M. Bortolozzi, A. Lelli, and F. Mammano, "Calcium microdomains at presynaptic active zones of vertebrate hair cells unmasked by stochastic deconvolution," *Cell Calcium*, vol. 44, no. 2, pp. 158–168, 2008.

[26] R. Llinas, M. Sugimori, and R. B. Silver, "Presynaptic calcium concentration microdomains and transmitter release," *Journal of Physiology Paris*, vol. 86, no. 1–3, pp. 135–138, 1992.

[27] R. Lliná, Msugimori, and R. B. Silver, "The concept of calcium concentration microdomains in synaptic transmission," *Neuropharmacology*, vol. 34, no. 11, pp. 1443–1451, 1995.

[28] R. Llinas, M. Sugimori, and R. B. Silver, "Time resolved calcium microdomains and synaptic transmission," *Journal of Physiology Paris*, vol. 89, no. 2, pp. 77–81, 1995.

[29] R. R. Llinás, M. Sugimori, and R. B. Silver, "Localization of calcium concentration microdomains at the active zone in the squid giant synapse," *Advances in Second Messenger and Phosphoprotein Research*, vol. 29, pp. 133–137, 1994.

[30] H. Kawamata and G. Manfredi, "Mitochondrial dysfunction and intracellular calcium dysregulation in ALS," *Mechanisms of Ageing and Development*, vol. 131, no. 7-8, pp. 517–526, 2010.

[31] R. Malli and W. F. Graier, "Mitochondrial Ca^{2+} channels: great unknowns with important functions," *FEBS Letters*, vol. 584, no. 10, pp. 1942–1947, 2010.

[32] A. Andreyev and G. Fiskum, "Calcium induced release of mitochondrial cytochrome *c* by different mechanisms selective for brain *versus* liver," *Cell Death and Differentiation*, vol. 6, no. 9, pp. 825–832, 1999.

[33] I. Bogeski, R. Kappl, C. Kummerow, R. Gulaboski, M. Hoth, and B. A. Niemeyer, "Redox regulation of calcium ion channels: chemical and physiological aspects," *Cell Calcium*, vol. 50, no. 5, pp. 407–423, 2011.

[34] M. R. Duchen, "Mitochondria and calcium: from cell signalling to cell death," *Journal of Physiology*, vol. 529, no. 1, pp. 57–68, 2000.

[35] T. E. Gunter and S. S. Sheu, "Characteristics and possible functions of mitochondrial Ca^{2+} transport mechanisms," *Biochimica et Biophysica Acta*, vol. 1787, no. 11, pp. 1291–1308, 2009.

[36] F. Ichas and J. P. Mazat, "From calcium signaling to cell death: two conformations for the mitochondrial permeability transition pore. Switching from low- to high-conductance state," *Biochimica et Biophysica Acta*, vol. 1366, no. 1-2, pp. 33–50, 1998.

[37] I. I. Kruman and M. P. Mattson, "Pivotal role of mitochondrial calcium uptake in neural cell apoptosis and necrosis," *Journal of Neurochemistry*, vol. 72, no. 2, pp. 529–540, 1999.

[38] J. J. Lemasters, A. L. Nieminen, T. Qian et al., "The mitochondrial permeability transition in cell death: a common mechanism in necrosis, apoptosis and autophagy," *Biochimica et Biophysica Acta*, vol. 1366, no. 1-2, pp. 177–196, 1998.

[39] M. P. Mattson, "Apoptosis in neurodegenerative disorders," *Nature Reviews Molecular Cell Biology*, vol. 1, no. 2, pp. 120–129, 2000.

[40] A. P. Somlyo, M. Bond, and A. V. Somlyo, "Calcium content of mitochondria and endoplasmic reticulum in liver frozen rapidly *in vivo*," *Nature*, vol. 314, no. 6012, pp. 622–625, 1985.

[41] D. G. Nicholls, "The regulation of extramitochondrial free calcium ion concentration by rat liver mitochondria," *Biochemical Journal*, vol. 176, no. 2, pp. 463–474, 1978.

[42] D. G. Nicholls and I. D. Scott, "The regulation of brain mitochondrial calcium-ion transport. The role of ATP in the discrimination between kinetic and membrane-potential-dependent calcium-ion efflux mechanisms," *Biochemical Journal*, vol. 186, no. 3, pp. 833–839, 1980.

[43] S. Von Stockum, E. Basso, V. Petronilli, P. Sabatelli, M. A. Forte, and P. Bernardi, "Properties of Ca $^{2+}$ transport in mitochondria of *Drosophila melanogaster*," *The Journal of Biological Chemistry*, vol. 286, no. 48, pp. 41163–41170, 2011.

[44] M. P. Mattson, F. M. LaFerla, S. L. Chan, M. A. Leissring, P. N. Shepel, and J. D. Geiger, "Calcium signaling in the ER: its role in neuronal plasticity and neurodegenerative disorders," *Trends in Neurosciences*, vol. 23, no. 5, pp. 222–229, 2000.

[45] S. Pan, S. Y. Ryu, and S. S. Sheu, "Distinctive characteristics and functions of multiple mitochondrial Ca^{2+} influx mechanisms," *Science China Life Sciences*, vol. 54, no. 8, pp. 763–769, 2011.

[46] R. Assaly, A. D. De Tassigny, S. Paradis, S. Jacquin, A. Berdeaux, and D. Morin, "Oxidative stress, mitochondrial permeability transition pore opening and cell death during hypoxia-reoxygenation in adult cardiomyocytes," *European Journal of Pharmacology*, vol. 675, no. 1–3, pp. 6–14, 2012.

[47] A. P. Halestrap and P. Pasdois, "The role of the mitochondrial permeability transition pore in heart disease," *Biochimica et Biophysica Acta*, vol. 1787, no. 11, pp. 1402–1415, 2009.

[48] R. A. Haworth and D. R. Hunter, "The Ca^{2+}-induced membrane transition in mitochondria. II. Nature of the Ca^{2+} trigger site," *Archives of Biochemistry and Biophysics*, vol. 195, no. 2, pp. 460–467, 1979.

[49] D. R. Hunter and R. A. Haworth, "The Ca^{2+}-induced membrane transition in mitochondria. The protective mechanisms," *Archives of Biochemistry and Biophysics*, vol. 195, no. 2, pp. 453–459, 1979.

[50] D. R. Hunter and R. A. Haworth, "The Ca^{2+}-induced membrane transition in mitochondria. III. Transitional Ca^{2+} release," *Archives of Biochemistry and Biophysics*, vol. 195, no. 2, pp. 468–477, 1979.

[51] M. Polunas, A. Halladay, R. B. Tjalkens, M. A. Philbert, H. Lowndes, and K. Reuhl, "Role of oxidative stress and the mitochondrial permeability transition in methylmercury cytotoxicity," *NeuroToxicology*, vol. 32, no. 5, pp. 526–534, 2011.

[52] L. Ravagnan, T. Roumier, and G. Kroemer, "Mitochondria, the killer organelles and their weapons," *Journal of Cellular Physiology*, vol. 192, no. 2, pp. 131–137, 2002.

[53] J. Jordán, V. Ceña, and J. H. M. Prehn, "Mitochondrial control of neuron death and its role in neurodegenerative disorders," *Journal of Physiology and Biochemistry*, vol. 59, no. 2, pp. 129–142, 2003.

[54] J. D. Robertson and S. Orrenius, "Molecular mechanisms of apoptosis induced by cytotoxic chemicals," *Critical Reviews in Toxicology*, vol. 30, no. 5, pp. 609–627, 2000.

[55] M. W. Ward, D. Kögel, and J. H. M. Prehn, "Neuronal apoptosis: BH3-only proteins the real killers," *Journal of Bioenergetics and Biomembranes*, vol. 36, no. 4, pp. 295–298, 2004.

[56] M. F. Hare, K. M. McGinnis, and W. D. Atchison, "Methylmercury increases intracellular concentrations of Ca++ and heavy metals in NG108-15 cells," *Journal of Pharmacology and Experimental Therapeutics*, vol. 266, no. 3, pp. 1626–1635, 1993.

[57] X. Tan, C. Tang, A. F. Castoldi, L. Manzo, and L. G. Costa, "Effects of inorganic and organic mercury on intracellular calcium levels in rat lymphocytes," *Journal of Toxicology and Environmental Health*, vol. 38, no. 2, pp. 159–170, 1993.

[58] M. Sue Marty and W. D. Atchison, "Pathways mediating Ca^{2+} entry in rat cerebellar granule cells following *in vitro* exposure to methyl mercury," *Toxicology and Applied Pharmacology*, vol. 147, no. 2, pp. 319–330, 1997.

[59] M. Sue Marty and W. D. Atchison, "Elevations of intracellular Ca^{2+} as a probable contributor to decreased viability in cerebellar granule cells following acute exposure to methylmercury," *Toxicology and Applied Pharmacology*, vol. 150, no. 1, pp. 98–105, 1998.

[60] E. Okazaki, Y. Oyama, L. Chikahisa, T. Nagano, N. Katayama, and M. Sakamoto, "Fluorescent estimation on cytotoxicity of methylmercury in dissociated rat cerebellar neurons: its comparison with ionomycin," *Environmental Toxicology and Pharmacology*, vol. 3, no. 4, pp. 237–244, 1997.

[61] Y. Oyama, F. Tomiyoshi, S. Ueno, K. Furukawa, and L. Chikahisa, "Methylmercury-induced augmentation of oxidative metabolism in cerebellar neurons dissociated from the rats: its dependence on intracellular Ca^{2+}," *Brain Research*, vol. 660, no. 1, pp. 154–157, 1994.

[62] M. F. Denny and W. D. Atchison, "Mercurial-induced alterations in neuronal divalent cation homeostasis," *NeuroToxicology*, vol. 17, no. 1, pp. 47–62, 1996.

[63] M. Sakamoto, N. Ikegami, and A. Nakano, "Protective effects of Ca^{2+} channel blockers against methyl mercury toxicity," *Pharmacology and Toxicology*, vol. 78, no. 3, pp. 193–199, 1996.

[64] A. F. Castoldi, T. Coccini, S. Ceccatelli, and L. Manzo, "Neurotoxicity and molecular effects of methylmercury," *Brain Research Bulletin*, vol. 55, no. 2, pp. 197–203, 2001.

[65] S.-Z. Xu, B. Zeng, N. Daskoulidou, G.-L. Chen, S. L. Atkin, and B. Lukhele, "Activation of TRPC cationic channels by mercurial compounds confers the cytotoxicity of mercury exposure," *Toxicological Sciences*, vol. 125, no. 1, Article ID kfr268, pp. 56–68, 2012.

[66] M. Tepel, S. Löwe, J. R. Nofer, G. Assmann, H. Schlüter, and W. Zidek, "Diadenosine polyphosphates regulate cytosolic calcium in human fibroblast cells by interaction with P(2x) purinoceptors coupled to phospholipase C," *Biochimica et Biophysica Acta*, vol. 1312, no. 2, pp. 145–150, 1996.

[67] N. Macrez-Leprêtre, J. L. Morel, and J. Mironneau, "Effects of phospholipase C inhibitors on Ca^{2+} channel stimulation and Ca^{2+} release from intracellular stores evoked by α1A- and α2A-adrenoceptors in rat portal vein myocytes," *Biochemical and Biophysical Research Communications*, vol. 218, no. 1, pp. 30–34, 1996.

[68] J. K. Fahrion, Y. Komuro, Y. Li et al., "Rescue of neuronal migration deficits in a mouse model of fetal Minamata disease by increasing neuronal Ca $^{2+}$ spike frequency," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 109, no. 13, pp. 5057–5062, 2012.

[69] F. Mori, K. Tanji, and K. Wakabayashi, "Widespread calcium deposits, as detected using the alizarin red S technique, in the nervous system of rats treated with dimethyl mercury," *Neuropathology*, vol. 20, no. 3, pp. 210–215, 2000.

[70] Y. Yoshino, T. Mozai, and K. Nakao, "Distribution of mercury in the brain and its subcellular units in experimental organic mercury poisoning," *Journal of Neurochemistry*, vol. 13, pp. 397–406, 1966.

[71] S. C. Bondy and M. McKee, "Disruption of the potential across the synaptosomal plasma membrane and mitochondria by neurotoxic agents," *Toxicology Letters*, vol. 58, no. 1, pp. 13–21, 1991.

[72] M. F. Hare and W. D. Atchison, "Comparative action of methylmercury and divalent inorganic mercury on nerve terminal and intraterminal mitochondrial membrane potentials," *Journal of Pharmacology and Experimental Therapeutics*, vol. 261, no. 1, pp. 166–172, 1992.

[73] T. L. Limke and W. D. Atchison, "Acute exposure to methylmercury opens the mitochondrial permeability transition pore in rat cerebellar granule cells," *Toxicology and Applied Pharmacology*, vol. 178, no. 1, pp. 52–61, 2002.

[74] C. Wagner, A. P. Vargas, D. H. Roos et al., "Comparative study of quercetin and its two glycoside derivatives quercitrin and rutin against methylmercury (MeHg)-induced ROS production in rat brain slices," *Archives of Toxicology*, vol. 84, no. 2, pp. 89–97, 2010.

[75] S. Yee and B. H. Choi, "Oxidative stress in neurotoxic effects of methylmercury poisoning," *NeuroToxicology*, vol. 17, no. 1, pp. 17–26, 1996.

[76] B. J. Shenker, T. L. Guo, O. Insug, and I. M. Shapiro, "Induction of apoptosis in human T-cells by methyl mercury: temporal relationship between mitochondrial dysfunction and loss of reductive reserve," *Toxicology and Applied Pharmacology*, vol. 157, no. 1, pp. 23–35, 1999.

[77] B. J. Shenker, L. Pankoski, A. Zekavat, and I. M. Shapiro, "Mercury-induced apoptosis in human lymphocytes: caspase activation is linked to redox status," *Antioxidants and Redox Signaling*, vol. 4, no. 3, pp. 379–389, 2002.

[78] N. Sone, M. K. Larsstuvold, and Y. Kagawa, "Effect of methyl mercury on phosphorylation, transport, and oxidation in mammalian mitochondria," *Journal of Biochemistry*, vol. 82, no. 3, pp. 859–868, 1977.

[79] M. A. Verity, W. J. Brown, and M. Cheung, "Organic mercurial encephalopathy: *in vivo* and *in vitro* effects of methyl mercury on synaptosomal respiration," *Journal of Neurochemistry*, vol. 25, no. 6, pp. 759–766, 1975.

[80] B. A. Fowler and J. S. Woods, "Ultrastructural and biochemical changes in renal mitochondria during chronic oral methyl mercury exposure. The relationship to renal function," *Experimental and Molecular Pathology*, vol. 27, no. 3, pp. 403–412, 1977.

[81] P. Bernardi, K. M. Broekemeier, and D. R. Pfeiffer, "Recent progress on regulation of the mitochondrial permeability transition pore; A cyclosporin-sensitive pore in the inner mitochondrial membrane," *Journal of Bioenergetics and Biomembranes*, vol. 26, no. 5, pp. 509–517, 1994.

[82] A. P. Halestrap, "What is the mitochondrial permeability transition pore?" *Journal of Molecular and Cellular Cardiology*, vol. 46, no. 6, pp. 821–831, 2009.

[83] J. J. Lemasters, T. P. Theruvath, Z. Zhong, and A. L. Nieminen, "Mitochondrial calcium and the permeability transition in cell death," *Biochimica et Biophysica Acta*, vol. 1787, no. 11, pp. 1395–1401, 2009.

[84] A. L. Nieminen, T. G. Petrie, J. J. Lemasters, and W. R. Selman, "Cyclosporin A delays mitochondrial depolarization induced by N-methyl-D-aspartate in cortical neurons: evidence of the mitochondrial permeability transition," *Neuroscience*, vol. 75, no. 4, pp. 993–997, 1996.

[85] T. L. Limke, J. K. L. Otero-Montañez, and W. D. Atchison, "Evidence for interactions between intracellular calcium stores during methylmercury-induced intracellular calcium dysregulation in rat cerebellar granule neurons," *Journal of Pharmacology and Experimental Therapeutics*, vol. 304, no. 3, pp. 949–958, 2003.

[86] P. G. Sullivan, A. G. Rabchevsky, P. C. Waldmeier, and J. E. Springer, "Mitochondrial permeability transition in CNS trauma: cause or effect of neuronal cell death?" *Journal of Neuroscience Research*, vol. 79, no. 1-2, pp. 231–239, 2005.

[87] H. Friberg, M. Ferrand-Drake, F. Bengtsson, A. P. Halestrap, and T. Wieloch, "Cyclosporin A, but not FK 506, protects mitochondria and neurons against hypoglycemic damage and implicates the mitochondrial permeability transition in cell death," *Journal of Neuroscience*, vol. 18, no. 14, pp. 5151–5159, 1998.

[88] L. M. Loew, W. Carrington, R. A. Tuft, and F. S. Fay, "Physiological cytosolic Ca^{2+} transients evoke concurrent mitochondrial depolarizations," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 91, no. 26, pp. 12579–12583, 1994.

[89] O. Insug, S. Datar, C. J. Koch, I. M. Shapiro, and B. J. Shenker, "Mercuric compounds inhibit human monocyte function by inducing apoptosis: evidence for formation of reactive oxygen species, development of mitochondrial membrane permeability transition and loss of reductive reserve," *Toxicology*, vol. 124, no. 3, pp. 211–224, 1997.

[90] P. Bernardi, P. Veronese, and V. Petronilli, "Modulation of the mitochondrial cyclosporin A-sensitive permeability transition pore. I. Evidence for two separate Me^{2+} binding sites with opposing effects on the pore open probability," *The Journal of Biological Chemistry*, vol. 268, no. 2, pp. 1005–1010, 1993.

[91] V. Petronilli, C. Cola, and P. Bernardi, "Modulation of the mitochondrial cyclosporin A-sensitive permeability transition pore. II. The minimal requirements for pore induction underscore a key role for transmembrane electrical potential, matrix pH, and matrix Ca^{2+}," *The Journal of Biological Chemistry*, vol. 268, no. 2, pp. 1011–1016, 1993.

[92] P. B. Simpson and J. T. Russell, "Mitochondrial Ca^{2+} uptake and release influence metabotropic and ionotropic cytosolic Ca^{2+} responses in rat oligodendrocyte progenitors," *Journal of Physiology*, vol. 508, no. 2, pp. 413–426, 1998.

[93] K. K. Gunter and T. E. Gunter, "Transport of calcium by mitochondria," *Journal of Bioenergetics and Biomembranes*, vol. 26, no. 5, pp. 471–485, 1994.

[94] A. M. Byrne, "Contribution of increased mitochondrial free Ca^{2+} to the mitochondrial permeability transition induced by tert-butylhydroperoxide in rat hepatocytes," *Hepatology*, vol. 29, no. 5, pp. 1523–1531, 1999.

[95] T. L. Limke, J. J. Bearss, and W. D. Atchison, "Acute exposure to methylmercury causes Ca^{2+} dysregulation and neuronal death in rat cerebellar granule cells through an M3 muscarinic receptor-linked pathway," *Toxicological Sciences*, vol. 80, no. 1, pp. 60–68, 2004.

[96] M. F. Hare and W. D. Atchison, "Methylmercury mobilizes Ca++ from intracellular stores sensitive to inositol 1,4,5-trisphosphate in NG108-15 cells," *Journal of Pharmacology and Experimental Therapeutics*, vol. 272, no. 3, pp. 1016–1023, 1995.

[97] S. L. Budd and D. G. Nicholls, "A reevaluation of the role of mitochondria in neuronal Ca^{2+} homeostasis," *Journal of Neurochemistry*, vol. 66, no. 1, pp. 403–411, 1996.

[98] M. R. Duchen, "Mitochondria and Ca^{2+} in cell physiology and pathophysiology," *Cell Calcium*, vol. 28, no. 5-6, pp. 339–348, 2000.

[99] S. Ceccatelli, E. Daré, and M. Moors, "Methylmercury-induced neurotoxicity and apoptosis," *Chemico-Biological Interactions*, vol. 188, no. 2, pp. 301–308, 2010.

[100] P. Pietta, P. Simonetti, C. Gardana, and P. Mauri, "Trolox equivalent antioxidant capacity (TEAC) of Ginkgo biloba flavonol and Camellia sinensis catechin metabolites," *Journal of Pharmaceutical and Biomedical Analysis*, vol. 23, no. 1, pp. 223–226, 2000.

[101] R. d. P. Martins, H. d. C. Braga, A. P. da Silva et al., "Synergistic neurotoxicity induced by methylmercury and quercetin in mice," *Food and Chemical Toxicology*, vol. 47, no. 3, pp. 645–649, 2009.

[102] A. Dreiem and R. F. Seegal, "Methylmercury-induced changes in mitochondrial function in striatal synaptosomes are calcium-dependent and ROS-independent," *NeuroToxicology*, vol. 28, no. 4, pp. 720–726, 2007.

[103] M. Valko, H. Morris, and M. T. D. Cronin, "Metals, toxicity and oxidative stress," *Current Medicinal Chemistry*, vol. 12, no. 10, pp. 1161–1208, 2005.

[104] Q. Cai, R. O. Rahn, and R. Zhang, "Dietary flavonoids, quercetin, luteolin and genistein, reduce oxidative DNA damage and lipid peroxidation and quench free radicals," *Cancer Letters*, vol. 119, no. 1, pp. 99–107, 1997.

[105] J. L. Franco, H. C. Braga, J. Stringari et al., "Mercurial-induced hydrogen peroxide generation in mouse brain mitochondria: protective effects of quercetin," *Chemical Research in Toxicology*, vol. 20, no. 12, pp. 1919–1926, 2007.

[106] G. M. R. D. S. Lucena, J. L. Franco, C. M. Ribas et al., "Cipura paludosa extract prevents methyl mercury-induced neurotoxicity in mice," *Basic and Clinical Pharmacology and Toxicology*, vol. 101, no. 2, pp. 127–131, 2007.

[107] M. Farina, F. Campos, I. Vendrell et al., "Probucol increases glutathione peroxidase-1 activity and displays long-lasting protection against methylmercury toxicity in cerebellar granule cells," *Toxicological Sciences*, vol. 112, no. 2, pp. 416–426, 2009.

[108] M. Farina, F. A. A. Soares, G. Zeni, D. O. Souza, and J. B. T. Rocha, "Additive pro-oxidative effects of methylmercury and ebselen in liver from suckling rat pups," *Toxicology Letters*, vol. 146, no. 3, pp. 227–235, 2004.

[109] J. Y. Chang and P. F. Tsai, "Prevention of methylmercury-induced mitochondrial depolarization, glutathione depletion and cell death by 15-deoxy-delta-12,14-prostaglandin J2," *NeuroToxicology*, vol. 29, no. 6, pp. 1054–1061, 2008.

[110] D. H. Roos, R. L. Puntel, M. M. Santos et al., "Guanosine and synthetic organoselenium compounds modulate methylmercury-induced oxidative stress in rat brain cortical slices: involvement of oxidative stress and glutamatergic system," *Toxicology in Vitro*, vol. 23, no. 2, pp. 302–307, 2009.

[111] P. B. Tchounwou, W. K. Ayensu, N. Ninashvili, and D. Sutton, "Environmental exposure to mercury and its toxicopathologic implications for public health," *Environmental Toxicology*, vol. 18, no. 3, pp. 149–175, 2003.

[112] T. W. Clarkson, L. Magos, and G. J. Myers, "The toxicology of mercury-current exposures and clinical manifestations," *The New England Journal of Medicine*, vol. 349, no. 18, pp. 1731–1737, 2003.

[113] M. C. Carvalho, J. L. Franco, H. Ghizoni et al., "Effects of 2,3-dimercapto-1-propanesulfonic acid (DMPS) on methylmercury-induced locomotor deficits and cerebellar toxicity in mice," *Toxicology*, vol. 239, no. 3, pp. 195–203, 2007.

[114] S. D. Pingree, P. L. Simmonds, and J. S. Woods, "Effects of 2,3-dimercapto-1-propanesulfonic acid (DMPS) on tissue and urine mercury levels following prolonged methylmercury exposure in rats," *Toxicological Sciences*, vol. 61, no. 2, pp. 224–233, 2001.

[115] M. Farina, J. L. Franco, C. M. Ribas et al., "Protective effects of Polygala paniculata extract against methylmercury-induced neurotoxicity in mice," *Journal of Pharmacy and Pharmacology*, vol. 57, no. 11, pp. 1503–1508, 2005.

[116] M. R. Campos-Esparza, M. V. Sánchez-Gómez, and C. Matute, "Molecular mechanisms of neuroprotection by two natural antioxidant polyphenols," *Cell Calcium*, vol. 45, no. 4, pp. 358–368, 2009.

[117] C. J. S. Passos, D. Mergler, M. Fillion et al., "Epidemiologic confirmation that fruit consumption influences mercury exposure in riparian communities in the Brazilian Amazon," *Environmental Research*, vol. 105, no. 2, pp. 183–193, 2007.

[118] B. D. Laird, C. Shade, N. Gantner, H. M. Chan, and S. D. Siciliano, "Bioaccessibility of mercury from traditional northern country foods measured using an *in vitro* gastrointestinal model is independent of mercury concentration," *Science of the Total Environment*, vol. 407, no. 23, pp. 6003–6008, 2009.

[119] C. A. Rice-Evans, N. J. Miller, and G. Paganga, "Structure-antioxidant activity relationships of flavonoids and phenolic acids," *Free Radical Biology and Medicine*, vol. 20, no. 7, pp. 933–956, 1996.

[120] C. Rice-Evans, "Flavonoid antioxidants," *Current Medicinal Chemistry*, vol. 8, no. 7, pp. 797–807, 2001.

[121] P. G. Pietta, "Flavonoids as antioxidants," *Journal of Natural Products*, vol. 63, no. 7, pp. 1035–1042, 2000.

[122] F. C. Meotti, R. Fachinetto, L. C. Maffi et al., "Antinociceptive action of myricitrin: involvement of the K+ and Ca^{2+} channels," *European Journal of Pharmacology*, vol. 567, no. 3, pp. 198–205, 2007.

[123] N. A. Kelsey, H. M. Wilkins, and D. A. Linseman, "Nutraceutical antioxidants as novel neuroprotective agents," *Molecules*, vol. 15, no. 11, pp. 7792–7814, 2010.

[124] J. Dai and R. J. Mumper, "Plant phenolics: extraction, analysis and their antioxidant and anticancer properties," *Molecules*, vol. 15, no. 10, pp. 7313–7352, 2010.

[125] K. H. Lee and E. M. Choi, "Myricetin, a naturally occurring flavonoid, prevents 2-deoxy-d-ribose induced dysfunction and oxidative damage in osteoblastic MC3T3-E1 cells," *European Journal of Pharmacology*, vol. 591, no. 1–3, pp. 1–6, 2008.

[126] R. J. Williams, J. P. E. Spencer, and C. Rice-Evans, "Flavonoids: antioxidants or signalling molecules?" *Free Radical Biology and Medicine*, vol. 36, no. 7, pp. 838–849, 2004.

[127] Y. Shimmyo, T. Kihara, A. Akaike, T. Niidome, and H. Sugimoto, "Multifunction of myricetin on Aβ: neuroprotection via a conformational change of Aβ and reduction of Aβ via the interference of secretases," *Journal of Neuroscience Research*, vol. 86, no. 2, pp. 368–377, 2008.

[128] S. Das, B. Nageshwar Rao, and B. S. Satish Rao, "Mangiferin attenuates methylmercury induced cytotoxicity against IMR-32, human neuroblastoma cells by the inhibition of oxidative stress and free radical scavenging potential," *Chemico-Biological Interactions*, vol. 193, no. 2, pp. 129–140, 2011.

[129] J. L. Franco, T. Posser, F. Missau et al., "Structure-activity relationship of flavonoids derived from medicinal plants in preventing methylmercury-induced mitochondrial dysfunction," *Environmental Toxicology and Pharmacology*, vol. 30, no. 3, pp. 272–278, 2010.

[130] C. A. Rice-Evans and N. J. Miller, "Antioxidant activities of flavonoids as bioactive components of food," *Biochemical Society Transactions*, vol. 24, no. 3, pp. 790–795, 1996.

[131] J. B. Harborne, "Nature, distribution and function of plant flavonoids," *Progress in Clinical and Biological Research*, vol. 213, pp. 15–24, 1986.

[132] F. Shahidi and P. K. Wanasundara, "Phenolic antioxidants," *Critical Reviews in Food Science and Nutrition*, vol. 32, no. 1, pp. 67–103, 1992.

[133] A. Saija, M. Scalese, M. Lanza, D. Marzullo, F. Bonina, and F. Castelli, "Flavonoids as antioxidant agents: importance of their interaction with biomembranes," *Free Radical Biology and Medicine*, vol. 19, no. 4, pp. 481–486, 1995.

[134] H. E. Ganther, C. Goudie, M. L. Sunde et al., "Selenium: relation to decreased toxicity of methylmercury added to diets containing tuna," *Science*, vol. 175, no. 4026, pp. 1122–1124, 1972.

[135] N. V. C. Ralston and L. J. Raymond, "Dietary selenium's protective effects against methylmercury toxicity," *Toxicology*, vol. 278, no. 1, pp. 112–123, 2010.

[136] C. W. Nogueira and J. B. T. Rocha, "Toxicology and pharmacology of selenium: emphasis on synthetic organoselenium compounds," *Archives of Toxicology*, vol. 85, no. 11, pp. 1313–1359, 2011.

[137] D. F. Meinerz, M. D. Paula, B. Comparsi et al., "Protective effects of organoselenium compounds against methylmercury-induced oxidative stress in mouse brain mitochondrial-enriched fractions," *Brazilian Journal of Medical and Biological Research*, vol. 44, no. 11, pp. 1156–1163, 2011.

[138] A. S. De Freitas and J. B. T. Rocha, "Diphenyl diselenide and analogs are substrates of cerebral rat thioredoxin reductase: a pathway for their neuroprotective effects," *Neuroscience Letters*, vol. 503, no. 1, pp. 1–5, 2011.

[139] M. B. Moretto, C. Funchal, A. Q. Santos et al., "Ebselen protects glutamate uptake inhibition caused by methyl mercury but does not by Hg2+," *Toxicology*, vol. 214, no. 1-2, pp. 57–66, 2005.

[140] M. Farina, K. C. S. Dahm, F. D. Schwalm et al., "Methylmercury increases glutamate release from brain synaptosomes and glutamate uptake by cortical slices from suckling rat pups: modulatory effect of ebselen," *Toxicological Sciences*, vol. 73, no. 1, pp. 135–140, 2003.

[141] C. Funchal, M. B. Moretto, L. Vivian, G. Zeni, J. B. T. Rocha, and R. Pessoa-Pureur, "Diphenyl ditelluride- and methylmercury-induced hyperphosphorilation of the high molecular weight neurofilament subunit is prevented by organoselenium compounds in cerebral cortex of young rats," *Toxicology*, vol. 222, no. 1-2, pp. 143–153, 2006.

[142] A. Dreiem, C. C. Gertz, and R. F. Seegal, "The effects of methylmercury on mitochondrial function and reactive oxygen species formation in rat striatal synaptosomes are age-dependent," *Toxicological Sciences*, vol. 87, no. 1, pp. 156–162, 2005.

Hindawi Publishing Corporation
Journal of Biomedicine and Biotechnology
Volume 2012, Article ID 681016, 9 pages
doi:10.1155/2012/681016

Research Article

Dietary Mercury Exposure Resulted in Behavioral Differences in Mice Contaminated with Fish-Associated Methylmercury Compared to Methylmercury Chloride Added to Diet

Jean-Paul Bourdineaud,[1] Masumi Marumoto,[2] Akira Yasutake,[2] and Masatake Fujimura[2]

[1] *Bordeaux University-CNRS, UMR EPOC 5805, Arcachon Marine Station, place du Docteur Peyneau, 33120 Arcachon, France*
[2] *Pathology Section, Department of Basic Medical Sciences, National Institute for Minamata Disease, 4058-18 Hama, Minamata, Kumamoto 867-0008, Japan*

Correspondence should be addressed to Masatake Fujimura, fujimura@nimd.go.jp

Received 16 April 2012; Revised 12 June 2012; Accepted 19 June 2012

Academic Editor: João B.T. Rocha

Methylmercury (MeHg) is a potent neurotoxin, and humans are mainly exposed to this pollutant through fish consumption. However, in classical toxicological studies, pure methylmercury chloride (MeHgCl) is injected, given to drink or incorporated within feed assuming that its effects are identical to those of MeHg naturally associated to fish. In the present study, we wanted to address the question whether a diet containing MeHg associated to fish could result in observable adverse effects in mice as compared to a diet containing the same concentration of MeHg added pure to the diet and whether beneficial nutriments from fish were able to counterbalance the deleterious effects of fish-associated mercury, if any. After two months of feeding, the fish-containing diet resulted in significant observable effects as compared to the control and MeHg-containing diets, encompassing altered behavioral performances as monitored in a Y-shaped maze and an open field, and an increased dopamine metabolic turnover in hippocampus, despite the fact that the fish-containing diet was enriched in polyunsaturated fatty acids and selenium compared to the fish-devoid diets.

1. Introduction

Methylmercury chloride (MeHgCl), the most toxic form of mercury, is a potent neurotoxin, to which human beings are mainly exposed through fish consumption. In classical toxicological studies, MeHg is most often injected intraperitoneally, instilled in the trachea, or dissolved in drinking water, which are not the natural routes for MeHg entrance into the body. In other cases MeHgCl is added to the diet. On the other hand, we have in a recent past exposed mice to diets containing fish and mimicking the Wayana Amerindians mercurial contamination or the Western populations' average fish consumption [1, 2]. A more classical approach consisting in dispersing a given quantity of methylmercury (MeHg) within diet preparations had been precluded because our working postulate is that the supramolecular form under which MeHg enters the body is of crucial importance, contrarily to the common assumption according to which the toxicological outcome of MeHg incorporated into fish tissue is identical to that from MeHgCl added to the same matrix. In this context, a higher faecal excretion and lower tissue accumulation, as well as metallothionein induction in rats following exposure to methylmercury naturally incorporated in fish compared to methylmercury chloride added to the same matrix, have been reported [3]. Moreover, the chemical form of methylmercury in fish has been identified as methylmercury-cysteine (MeHg-cysteine), probably as part of larger peptides [4]. Therefore, one can suspect a different trophic transfer rate through the intestinal barrier and a different early toxicity for ingested free and

protein-bound methylmercury. It has well been established that MeHg-cysteine was not hydrolyzed by gastric acidic juice and remained intact [4]. The acute toxicity of solubilized MeHg-cysteine has been reported to be considerably lower than MeHgCl both *in vivo* [5] and *in vitro* [6]. In addition, many compounds within fish flesh or brought by diet are likely to influence the trophic transfer rate and the toxicity outcomes of MeHgCl. For instance, selenium and vitamin E are decreasing the overall toxicity of MeHgCl [7, 8], whereas fruit or tea consumption decreases mercury accumulation within body for the same number of eaten fish meals [9, 10]. The precise quality of dietary fats also modulates the MeHg toxicity and the steroidogenesis in rats [11, 12].

Therefore, the aim of the present work was to address the questions of whether a diet containing "naturally" accumulated MeHg in fish flesh could result in differential biological outcomes as compared to MeHgCl added pure to diet. After two months of feeding with such diets, we analyzed the mercury accumulation in tissues, the mice body growth kinetics, the possible behavioral impairments, and the concentration of neurotransmitters in various brain structures.

2. Materials and Methods

2.1. Preparation of the Mice Diets. The *H. aimara* fish whose flesh was used was caught in French Guiana in the Sinnamary River, known to be contaminated by methylmercury mostly originating from the Petit-Saut hydroelectric reservoir [13]. The dry flesh of this animal contained 5 μg Hg/g. The *H. aimara* fish were thawed, minced, and lyophilized. Then the fish powder was ground in a kitchen blender. The nutrient composition and the metal content of the *H. aimara* fish powder have already been described [1]. The special diets were manufactured by Special Diets Services (Witham, Essex, United Kingdom; French commercial representation: Dietex, Saint-Gratien, France). The control diet was vegetarian (Rat and Mouse no. 1 maintenance diet, abbreviated to RM1 diet, Special Diets Services). The *H. aimara* fish-containing diet was prepared by adding 4.88% of lyophilized fish powder to the diet ingredients. The methylmercury chloride-containing diet was prepared by adding 1 L of a 1.5 mg/L solution of MeHg in a blend of ingredients allowing the preparation of 6 kg of diet. The nutrient compositions of the control RM1 and the two prepared regimens are given in Table 1 (the analyses were carried out by Special Diets Services). A comparison of the diets' compositions showed some minor differences between the control and the MeHg diets on one side, and the fish diet on the other side. Apart from the content in mercury, the control and MeHg diets are identical. The fish diet contained 3.7% more protein than the control and MeHg diets (18.1% compared to 14.4%). It also contained more of some polyunsaturated fatty acids (PUFA): 290, 70, 40, and 150 ppm of docosahexaenoic, docosapentaenoic, erucic, and eicosapentaenoic acids against <20, <20, 20, and <20 ppm for control and MeHg diets. We quantified the total mercury content of the two prepared regimens and found 253 ± 38 and 237 ± 23 ng Hg/g of food pellets for the MeHg and fish diets, respectively.

Table 1: The composition of the diets used[a].

	Control and MeHg diets	Fish diet[b]
Moisture	10	10
Fat	2.71	2.66
Protein	14.38	18.08
Fibre	4.65	4.45
Ash	6.00	5.94
Carbohydrates		
Starch	45.0	43.3
Sugar	4.05	3.85
Pectin	1.52	1.45
Hemicellulose	10.17	9.67
Cellulose	4.32	4.11
Lignin	1.68	1.60
Fatty acids		
Saturated fatty acids		
C12:0 lauric	0.02	0.02
C14:0 myristic	0.14	0.14
C16:0 palmitic	0.31	0.31
C18:0 stearic	0.04	0.04
Monounsaturated fatty acids		
C14:1 (ω5) myristoleic	0.02	0.02
C16:1 (ω7) palmitoleic	0.09	0.09
C18:1 (ω9) oleic	0.77	0.74
Polyunsaturated fatty acids		
C18:2 (ω6) linoleic	0.69	0.66
C18:3 (ω3) linolenic	0.06	0.05
C20:4 (ω6) Arachidonic	0.13	0.13
C20:5 (ω3) (EPA) Eicosapentaenoic	**<0.002**	**0.015**
C22:1 (ω9) erucic	0.002	0.004
C22:5 (ω3) (DPA) Docosapentaenoic	**<0.002**	**0.007**
C22:6 (ω3) (DHA) Docosahexaenoic	**<0.002**	**0.029**

[a] Nutrients and compounds are given as their percentages in the diets.
[b] Fish diet : mice were fed a 4.9% aimara flesh-containing diet.

The control RM1 diet contained 7.8 ± 1.9 ng Hg/g of food pellets. The content of several other metals in the control, MeHg and fish-containing regimens was assessed (Table 2). Metals were assayed by ICP-MS (Antellis, Toulouse, France). The diets metal levels were below the detection threshold for Ag (<0.02 mg/kg), As (<0.1 mg/kg), Au (<0.05 mg/kg), Bi (<0.02 mg/kg), Sb (<0.5 mg/kg), Sn (<0.5 mg/kg), Tl (<0.05 mg/kg), and V (<0.5 mg/kg). The MeHg and fish diets contained equal amounts of mercury whereas the control diet was 30-times poorer in this metal. The fish diet was richer in selenium than the control and MeHg diets (0.48 ppm against 0.30), and apart from mercury and selenium, the 3 diets were comparable.

Table 2: The metal and selenium composition of the diets used[a].

Element	Diets		
	Control diet	MeHg[b]	Fish[c]
Al	41.1	41.1	39.1
Cd	64.10^{-3}	64.10^{-3}	62.10^{-3}
Co	0.80	0.80	0.76
Cr	0.72	0.72	0.69
Cu	7.99	7.99	7.63
Hg (total)	**8.10^{-3}**	**253.10^{-3}**	**237.10^{-3}**
Ni	0.39	0.39	0.38
Pb	0.165	0.165	0.161
Se	**0.30**	**0.30**	**0.48**
Zn	41.1	41.1	40.0

[a] Metals and selenium are given in mg/kg.
[b] MeHg: mice were fed a MeHg-containing diet.
[c] Fish: mice were fed a 4.9% aimara flesh-containing diet.

2.2. Mice Treatment and Tissue Sampling. Subjects were naïve male mice of the C57Bl/6 Jico inbred strain obtained from Japan Clear (Tokyo, Japan) at the age of 3 weeks weighing 10.4 ± 0.24 g. Young animals were used because they grow quickly and because they are more sensitive to MeHg than older animals. They were first ink-labeled on the ear skin. They were socially housed in standard conditions: room temperature (23°C), 12/12 light cycles, and *ad libitum* food and water. Experiments were performed in compliance with the European Community Council directive of 24 November 1986 (8616091 EEC). Three groups of 16 mice each (8 of which dedicated for each kinetic time points at 29 and 57 days) were fed for 29 and 57 days as follows: one with the control RM1 diet and the two other groups with the MeHg and fish diets. Mice from the three groups were weighed twice a week throughout the experiment. At the end of the exposure period, mice were subjected to an open-field maze test, in order to quantify anxiety levels, and to a Y-shaped maze test, to assess cognitive ability. Thereafter, mice were anesthetized with Pentothal (50 mg/kg, i.p.), blood was immediately collected by puncturing the vena cava, and the vascular system was washed for traces of blood by perfusion with saline (0.9% NaCl). Then, tissues were dissected for mercury quantification (blood, several brain structures, liver, kidney) and neurotransmitters quantification (6 different brain structures).

2.3. Activity Test Using an Open-Field Maze. The anxiety test was assessed using the open-field apparatus after Tanaka et al. [14] with slight modifications. This behavioural test was performed using randomly chosen animals (8 mice per group) on days 28 and 56, one day before their sacrifice for tissue sampling. The apparatus consisted of a floor (50 × 50 cm) surrounded by a 50 cm high opaque wall. A CCD camera fixed above the apparatus was connected to a Macintosh computer, and the movement of the mouse was analyzed using Image OF (O'Hara & Co., Ltd., Tokyo, Japan), a modified NIH Image program (developed at the US National Institutes of Health and available on the Internet at http://rsb.info.nih.gov/nih-image/). In this software, the position of the animal was defined as the position of the gravity center of the animal's image, which was calculated every 0.5 s. The total distance traversed by the gravity center was calculated and converted into centimeters. Also, floor area was divided into 25 squares (10 × 10 cm), and the location of the animal was classified either as central (nine areas that did not have direct contact with the walls) or peripheral (remaining sixteen areas). Behavior was monitored for 5 min after placing one mouse at the center of the floor. Between each trial, floor and walls were cleaned with 70% alcohol followed by wiping with wet cotton.

2.4. Cognitive Test Using a Y-Shaped Maze. To assess spatial learning and memory, mice were tested in the Y-maze. This behavioural test was performed using randomly chosen animals (8 mice per group) on days 27 and 55, two days before their sacrifice for tissue sampling. Each mouse was placed at the end of one arm facing the centre of the maze and allowed to move freely within the maze for a period of 5 min. The arm length was 40 cm, the arm width, 10 cm, and the arm height, 35 cm. The total number of arms entered by the mouse and the order of arm entries were recorded. The total number of arms entered provides an indication of locomotor activity, and the order of arm entries provides a measure of spontaneous alternation behaviour [15]. Success in this test is indicated by a high rate of alternation in the control group, indicating that the animals can remember which arm was entered last.

2.5. Mercury Quantification. Animals were chosen randomly for mercury analysis (at day 57, 8 for blood, 6 for liver and kidney, and 3 for each brain structure). Samples were analyzed for total mercury at the National Institute for Minamata Disease (NIMD), Japan, using reliable and sensitive techniques following described procedures [16]. Tissue samples were homogenized (10%, w/v) in distilled water using a Polytron homogenizer (Kinematica GmbH, Littau, Switzerland). Blood was collected from the femoral vein into heparinised tubes. A portion of blood was haemolysed by distilled water (1 : 50). Total Hg levels in the homogenates (100 μL) and haemolysed blood (100 μL) were determined by an oxygen-combustion gold amalgamation method using an atomic absorption mercury analyzer MD-A (Nippon Instruments Co., Ltd., Osaka, Japan). To control the quality of measurements, a standard reference material from dogfish, DORM2 (National Research Council, Canada) was included in analyses. The certified value of total mercury is 4.64 ± 0.26 μg/g (mean ± SEM). Our qualification data were 4.51 ± 0.14 μg/g for total mercury (mean ± SEM).

2.6. Neurotransmitters and Associated Metabolites Quantification. The brain homogenates were prepared as follows. Brain regions including the frontal cortex, cortex, hypothalamus, striatum, cerebellum, and midbrain were dissected out from frozen brains. Dissected tissues were frozen immediately in liquid nitrogen and stored at −80°C until use. These tissues were homogenized in 0.2 M perchloric acid (Nacalai Tesque, Kyoto, Japan) containing 100 mM

EDTA and isoproterenol (ISO) (100 ng/μL) as an internal standard. Homogenates were centrifuged for 15 min at 0°C and 20,000 g. The pH of each supernatant was adjusted to 3.0 with 1 M sodium acetate and supernatants were then used to determine monoamine levels. Each sample (10 mL) was injected into an HPLC system (HTEC-500MAD) with electrochemical detection (ECD-300 EICOM, Kyoto, Japan) with an ODS column (EICOMPAC SC-5, 3.0 mm × 150 mm, EICOM). The mobile phase was sodium acetate-citric acetate buffer (pH 3.5)/methanol/sodium octane-1-sulfonate (85%/15%/0.21 g), the flow rate was maintained at 0.5 mL/min, and the temperature of the column was 25°C. Tissue levels of dopamine (DA), 5-hydroxytryptamine or serotonin (5-HT), and noradrenalin (NE) as well as their metabolites 3,4-dihydroxyphenylacetic acid (DOPAC), 3-methoxytryptamine (3-MT) and homovanillic acid (HVA) as DA metabolites, and 5-hydroxyindole-acetic acid (5-HIAA) as a 5-HT metabolite were measured. Each of the standard solutions for the 6 catecholamines (DA, NE, DOPAC, HVA, 3-MT, and ISO) and 2 indoleamines (5-HT and 5-HIAA) was prepared at a concentration of 0.1 ng/mL. Each standard (10 μL) was analyzed by HPLC, and the standard chromatographic peaks per 1 ng for each sample were obtained. The amount of each monoamine was determined with peak-area ratios using an HPLC chromatogram analysis software, eDAQ Power Chrom (eDAQ, NSW, Australia). Values were normalized for the amount of protein in each sample.

2.7. Statistical Analysis. Statistical significance was determined by a one-way ANOVA followed by a Dunnett's multiple comparison test. Data were expressed as the mean ± SEM. A difference was considered statistically significant when $P < 0.05$.

3. Results and Discussion

During the timecourse of the present experiment we observed no mortality, and no clinical or histopathological signs of lesions in mice brains whatever the diet given. The mice growth was recorded for two months. It appeared that the weight gain was faster for mice fed the MeHg and fish diets than the control diet during the first 10 days (Figure 1). At days 3, 7 and 10, the mercury-contaminated mice got body weights 7, 4, and 4% higher than the control mice, respectively. This effect is easily attributable to the beneficial effects of fish nutriments in the case of the fish-containing diet but remains hard to explain in the case of the MeHg diet.

3.1. Mercury Quantification. As expected, after 57 days of exposure the mercury concentrations in tissues of both groups of mice dietary contaminated with mercury were much higher than those from mice fed the control diet (Table 3). The mercury concentration increases ranged between 11 and 25 times those recorded in tissues from mice fed the control diet. We could notice a 20-fold increase in blood mercury for both MeHg and fish mice compared to control mice. Kidneys were the organs accumulating the highest concentrations of mercury in the organism, reaching

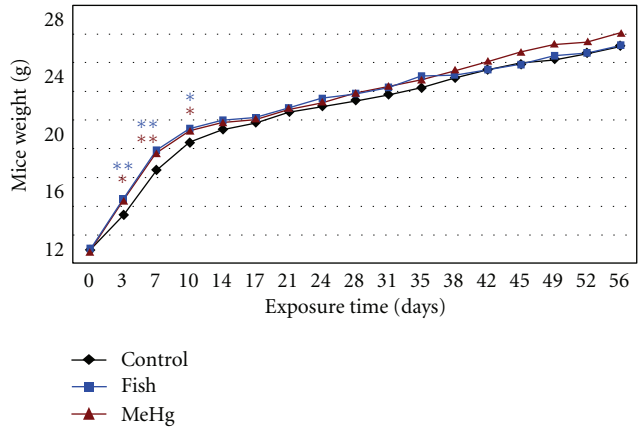


Figure 1: Influence of diet on the body weight growth. Mice were exposed to a control diet ("Control" plot with black diamonds), or a MeHg-containing diet ("MeHg" plot with red triangles), or a fish-containing diet ("Fish" plot with blue squares). Weights (in grams) were collected at the indicated kinetic time points and the mean weights plotted. Means and standard deviations were calculated from 16 control-, MeHg- and fish-fed mice from the beginning to the 35th day. From days 38 to 56, they were calculated from 8 animals per diet. The asterisks indicate a significant difference in body weight for mice fed the MeHg- and fish-containing diet compared to those fed the control diet, as determined with a one-way ANOVA followed by a Dunnett's multiple comparison method, $^{*}P < 0.05$, and $^{**}P < 0.01$.

7.3 and 6.8 mg Hg/g in mice fed the MeHg and fish diets, respectively, a multiplication factor of 17 and 16 compared to the control diet. Worth to note is the heterogeneity of brain structures in terms of mercury accumulation since, for instance frontal cortex accumulated 2.7 and 2 times more mercury than brain stem in mice fed the MeHg and fish diets, respectively. Except in the striatum, which accumulated 33% less mercury in animals fed the fish diet compared to those fed the MeHg diet, the mercury concentrations in tissues of mice fed the MeHg and the fish diets displayed no statistical significant differences, although a trend of smaller concentrations was observed in tissues of mice fed the fish diet. This lower mercury concentration in the striatum of mice fed the fish diet compared to those fed the MeHg diet cannot be explained by a lower blood mercury concentration since we measured the same blood mercury concentrations for both groups of mercury-contaminated mice. This is probably due to the fact that different mercurial chemical species are not transported into tissue cells with the same efficiency by transporters. For instance, the ubiquitous transporters LAT1 and LAT2 efficiently catalyze the uptake of MeHg-L-cysteine but not that of MeHg-D-cysteine or MeHg-glutathione [17]. Furthermore, solubilised MeHg-cysteine is thought to be, at least partly, actively transported via neutral amino acids carrier systems [18–20], while MeHgCl would be transported via other facilitated processes [20].

In order to mimic the mercury exposure in the Amazon riverside population, rats have been fed with a diet containing 20% of carnivorous fish caught in the Tapajós River and which flesh contained 1.95 μg MeHg/g, making a mercury concentration in feed of 0.39 μg MeHg/g [21].

Table 3: Total mercury concentrations in mice tissues[a].

Tissues	Control	MeHg[b]	Fish[c]
Blood ($n = 8$)	0.010 ± 0.002	0.21 ± 0.02	0.20 ± 0.01
Brain stem ($n = 3$)	0.016 ± 0.006	0.27 ± 0.07	0.20 ± 0.07
Cerebellum ($n = 3$)	0.016 ± 0.002	0.33 ± 0.06	0.26 ± 0.04
Frontal cortex ($n = 3$)	0.031 ± 0.013	0.73 ± 0.16	0.41 ± 0.13
Hippocampus ($n = 3$)	0.024 ± 0.002	0.62 ± 0.26	0.34 ± 0.08
Midbrain ($n = 3$)	0.020 ± 0.003	0.41 ± 0.20	0.23 ± 0.04
Striatum ($n = 3$)	0.018 ± 0.002	0.49 ± 0.05	***0.33 ± 0.01**
Liver ($n = 6$)	0.038 ± 0.008	0.94 ± 0.11	0.79 ± 0.12
Kidney ($n = 6$)	0.416 ± 0.091	7.3 ± 1.4	6.8 ± 0.5

[a] In μg/g for solid tissues or μg/mL for blood (mean ± SD).
[b] MeHg: mice were fed a MeHg-containing diet.
[c] Fish: mice were fed a 4.9% aimara flesh-containing diet.
All total mercury concentrations in MeHg and fish diet samples significantly higher than those in control diet samples, as determined with a one-way ANOVA followed by a Dunnett's multiple comparison method ($P < 0.01$).
The symbol * indicates a significant difference in mercury concentrations for mice fed the fish-containing diet compared to those fed the MeHg-containing diet, as determined with a one-way ANOVA followed by a Dunnett's multiple comparison method. $^*P < 0.05$. The corresponding value appears in bold characters.

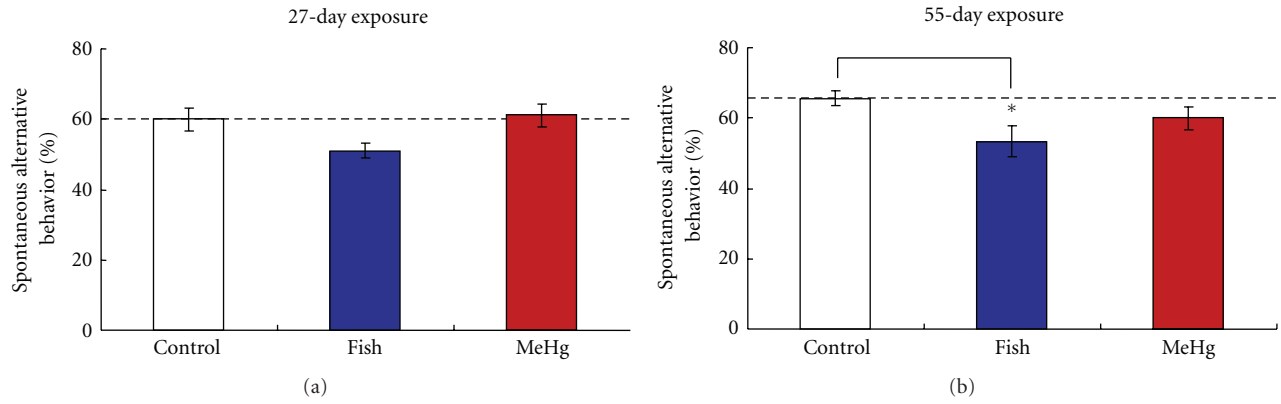


Figure 2: Mice behavior in a Y-shaped maze. Spontaneous alternations were recorded for 5 min (mean ± SEM, $n = 8$). The asterisk indicates a significant difference between the spontaneous alternations presented in the Y maze by mice fed the fish-containing diet compared to those fed the control diet, as determined with a one-way ANOVA followed by a Dunnett's multiple comparison method, $^*P < 0.05$, and $^{**}P < 0.01$.

After 84 days of contamination rats presented in their blood cells an increased lipid peroxidation and genotoxicity and an increased systolic blood pressure. Mercury concentrations were 1.31 μg/mL in blood, 0.87, 0.89, and 0.1 μg/g in liver, kidney, and brain, respectively. In the present study, we gave to mice a diet containing 0.24 μg MeHg/g, resulting after 57 days of exposure in mercury concentrations 6.5 and 1.1 times weaker in blood and liver but 8.6 and 4.1 times larger in kidney and brain, meaning that the distribution of methylmercury from blood to tissues is differing between rats and mice.

3.2. Behavioral Modification. The activity of mice was assessed in an open-field maze (Table 4). The quantity of emitted feces, along with the number of rearing events and the number of squares crossed, could not distinguish the three different groups of mice tested after 28 or 56 days of exposure. However, after 56 days of exposure the number of grooming events was significantly decreased for the fish diet mice as compared to the MeHg-fed and control mice, with this decrease reaching 45 and 40% of the values of the MeHg-fed and control mice, respectively. Additionally, after 56 days of exposure the time spent in center was significantly higher for the fish diet mice as compared to the MeHg-fed and control mice, with this increase representing 2 and 3-fold the values of the MeHg-fed and control mice, respectively. Anxious mice tend to avoid the center of the open field and to groom more frequently. Therefore, a decrease of grooming events associated to an increase of time spent in the center of the open field indicates an antianxious behaviour.

The Y-maze allowed for the testing of memory function in mice (Figure 2). Spontaneous alternation is a behavioural test dealing with spatial learning and memory. The purpose of the test for the mouse consists in remembering which

Table 4: Mice behavior in an open field maze[a].

	Control		MeHg[b]		Fish[c]	
	28th day	56th day	28th day	56th day	28th day	56th day
Squares crossed	93 ± 7	86 ± 4	86 ± 6	83 ± 5	78 ± 6	84 ± 5
Rearing	23 ± 2	26 ± 2	21 ± 2	28 ± 3	22 ± 3	24 ± 2
Grooming	1.7 ± 0.3	1.25 ± 0.16	1.5 ± 0.2	1.38 ± 0.18	1.9 ± 0.3	$^{\circ,\S}$**0.75 ± 0.16**
Fecal pellets	1.4 ± 0.8	3.8 ± 1.2	3.7 ± 1.8	1.7 ± 0.6	3.9 ± 1.1	2.3 ± 0.9
Time spent in center (s)	5.3 ± 1.0	3.6 ± 0.4	3.8 ± 0.5	4.7 ± 1.2	3.7 ± 0.6	$^{\circ\circ,\S}$**10.0 ± 1.5**

[a] Mean ± SEM, $n = 8$.
[b] MeHg: mice were fed a MeHg-containing diet.
[c] Fish: mice were fed a 4.9% aimara flesh-containing diet.
Values appearing in bold characters highlight those significantly different from the control values.
Circles indicate significant differences in behavioral parameters for mice fed the fish-containing diet compared to those fed the control diet, as determined with the Students test, $^{\circ}P < 0.05$, and $^{\circ\circ}P < 0.01$.
The symbol § indicates significant differences in behavioral parameters for mice fed the fish-containing diet compared to those fed the MeHg-containing diet, as determined with the Students test, $^{\S}P < 0.05$.

maze arm was last visited and trying to enter as many different arms as possible. The task is testing hippocampal memory and can be weakened by lesions to the hippocampus. The sequence of arm entries and total amount of arm entries are scored and a percentage is calculated. The cognition of the animal can be assessed based on the score where a lower score is considered cognitively impaired. After 55 days of exposure, mice fed the fish diet displayed a significant lower rate of spontaneous alternations in the maze compared to MeHg-fed and control mice, strongly suggesting that these animals had difficulties remembering which arm was entered last. After 27 days of exposure, mice fed the fish-containing diet already presented a downward trend concerning the rate of spontaneous alternations but this was statistically nonsignificant.

4. Neurotransmitters and Associated Metabolites Quantification in Various Brain Structures

The levels of noradrenalin (NE), dopamine (DA), and 5-hydroxytryptamine or serotonin (5-HT) were quantified in various brain structures after two months of feeding with mercury-containing diets. In addition to these three neurotransmitters, the metabolite resulting from the degradation of serotonin, 5-hydroxyindole-acetic acid (5-HIAA) and those from the degradation pathway of DA, 3,4-dihydroxyphenylacetic acid (DOPAC), and homovanillic acid (HVA) were also quantified (Table 5).

No differences in levels of neurotransmitters or their metabolites could be observed after one month of exposure in the various brain structures collected. Only after 2 months of exposure could we see differences. The levels of serotonin appeared to be 2 times decreased in brainstem of mice fed the MeHg diet as compared to those fed the control diet. However, the level of serotonin in the same brain structure from mice fed the fish diet was not significantly different from that of the MeHg-containing diet, and therefore this decrease cannot account for the differences observed in mice behavior. And the same holds true with the 5-HIAA level which was 67% higher in the striatum of mice fed the fish diet compared to control mice, since the value from the MeHg-fed mice was not significantly different. In cerebellum of mice fed the fish diet, the DOPAC level was 2-times higher than that of the control mice. For mice fed the MeHg diet, the DOPAC level in this brain structure was as high as for mice fed the fish diet, but due to a great variability of values the mean could not be considered significantly different from that of control mice, and therefore the values from both the MeHg and fish diets cannot be taken as different. The dopamine metabolite DOPAC levels were significantly increased in hippocampus and striatum of mice fed the MeHg diet as compared to those fed the control diet, with increase factors reaching 15- and 2.6-fold the values of the control mice, respectively. Accordingly, the dopamine metabolite HVA level was 2.6 times increased in striatum of mice fed the MeHg diet as compared to those fed the control diet. It has already been described the striatal increase in dopamine and its acidic metabolite DOPAC after exposure of rats to MeHgCl [22]. However in this latter study the dose of injected MeHgCl was very high (0.1–2 mg/kg/day) compared to the dose used through diet in the present study (50 μg/kg/day), suggesting that striatum is more sensitive to methylmercury associated to fish than added pure in the diet. In hippocampus of mice fed the fish diet, the DOPAC level was significantly intermediate between those of mice fed the control and MeHg diets, since it reached 5 and 0.33 times those of control and MeHg-fed mice, respectively. Additionally, the metabolic turnover was calculated as the ratio of the sum of DOPAC and HVA levels to dopamine to estimate the activity of this metabolic pathway. We calculated a dopamine metabolic turnover of 0.36, 0.26, and 0.52 for mice fed the control, MeHg-containing, and fish-containing diets, respectively. This indicates that the rate of consumption of dopamine is greater in hippocampus of mice fed the fish-containing diet compared to the other two diets. Previous reports demonstrated that hippocampal dopamine receptors might be necessary for executive function including working memory [23]. The greater dopamine consumption in hippocampus might show the abnormality of hippocampal dopamine receptors. This may explain the decreased

Table 5: Neurotransmitter levels and associated decay products in brain structures of mice fed MeHg- and fish-containing diets.

Brain structures and diets	Days	Neurotransmitters and decay products (pg/mg, mean ± SEM, $n = 3$)					
		NE	DA	DOPAC	HVA	5-HT	5-HIAA
Frontal cortex							
Control	28	10.8 ± 3.7	2.1 ± 0.8	1.4 ± 0.6	3.5 ± 1.4	4.3 ± 1.8	8.2 ± 3.2
	56	11.1 ± 3.2	1.8 ± 0.4	0.25 ± 0.02	1.4 ± 0.2	4.4 ± 2.6	3.6 ± 0.8
MeHg[a]	28	9.4 ± 1.5	2.0 ± 0.6	1.1 ± 0.5	1.7 ± 1.0	2.2 ± 0.6	4.4 ± 0.6
	56	5.8 ± 0.7	1.7 ± 0.3	0.36 ± 0.08	0.9 ± 0.6	0.3 ± 0.3	2.6± 0.5
Fish[b]	28	16.9 ± 4.9	2.2 ± 0.7	0.9 ± 0.2	3.8 ± 1.5	5.8 ± 2.2	6.9 ± 0.7
	56	6.8 ± 3.9	2.0 ± 0.2	0.7 ± 0.2	1.1 ± 0.6	2.1 ± 1.2	5.7 ± 2.9
Brainstem							
Control	28	15.7 ± 2.8	7.2 ± 1.8	1.1 ± 0.5	2.0 ± 1.0	4.4 ± 0.8	21 ± 7
	56	12.3 ± 1.4	3.4 ± 0.4	0.56 ± 0.11	0.8 ± 0.1	4.7 ± 0.1	10 ± 1
MeHg	28	13.2 ± 0.2	3.6 ± 0.3	0.73 ± 0.04	1.04 ± 0.08	4.1 ± 0.3	11 ± 1
	56	8.4 ± 4.0	2.3 ± 0.7	0.68 ± 0.19	0.73 ± 0.05	*__2.3 ± 0.6__	8.9 ± 0.7
Fish	28	14.3 ± 1.7	6.3 ± 1.5	1.2 ± 0.6	1.8 ± 0.8	4.8 ± 0.6	15 ± 3
	56	10.2 ± 2.2	3.2 ± 0.8	0.63 ± 0.15	0.8 ± 0.1	3.1 ± 1.0	10 ± 2
Hippocampus							
Control	28	18.5 ± 4.6	9.4 ± 3.6	6.6 ± 5.8	5.4 ± 3.6	7.1 ± 2.1	26 ± 7
	56	7.9 ± 1.8	2.4 ± 0.7	0.17 ± 0.03	0.7 ± 0.1	5.0 ± 1.2	7.6 ± 1.9
MeHg	28	13.8 ± 2.4	4.6 ± 0.2	2.0 ± 0.8	3.2 ± 1.6	5.4 ± 1.3	12.3 ± 0.4
	56	14.4 ± 10.6	12.8 ± 0.8	*__2.55 ± 0.06__	0.8 ± 0.2	12.4 ± 9.7	49 ± 3
Fish	28	10.5 ± 2.4	4.2 ± 0.4	1.0 ± 0.7	1.8 ± 0.7	6.1 ± 1.9	10.1 ± 1.3
	56	12.5 ± 3.3	4.3 ± 1.2	°,§__0.85 ± 0.18__	1.4 ± 0.2	7.5 ± 2.8	13 ± 3
Striatum							
Control	28	6.0 ± 1.3	120 ± 29	68 ± 15	56 ± 18	11.6 ± 4.8	19 ± 7
	56	1.3 ± 0.7	79 ± 9	31 ± 5	22.0 ± 1.4	5.7 ± 0.1	5.2 ± 0.3
MeHg	28	4.6 ± 1.5	53 ± 17	60 ± 21	32 ± 10	6.5 ± 1.9	10 ± 2
	56	2.8 ± 0.6	65 ± 10	*__82 ± 12__	*__43 ± 5__	7.5 ± 3.3	13.4 ± 5.1
Fish	28	5.2 ± 1.6	49 ± 11	23 ± 8	15 ± 4	5.0 ± 1.1	6 ± 1
	56	4.9 ± 1.6	61 ± 10	54 ± 14	32 ± 7	5.0 ± 1.0	°__8.7 ± 1.0__
Cerebellum							
Control	28	4.7 ± 1.4	1.2 ± 0.4	0.33 ± 0.11	1.0 ± 0.5	0.4 ± 0.1	5.1 ± 2.4
	56	9.0 ± 2.8	1.0 ± 0.3	0.36 ± 0.03	0.81 ± 0.09	2.5 ± 1.0	3.4 ± 0.7
MeHg	28	6.2 ± 0.9	0.96 ± 0.08	0.29 ± 0.16	0.47 ± 0.05	0.38 ± 0.07	2.6 ± 0.1
	56	6.3 ± 1.4	0.5 ± 0.2	0.59 ± 0.21	0.5 ± 0.1	1.6 ± 1.0	3.3 ± 1.1
Fish	28	5.1 ± 0.7	0.5 ± 0.2	0.18 ± 0.05	0.44 ± 0.05	0.23 ± 0.06	2.5 ± 0.2
	56	3.4 ± 1.7	0.6 ± 0.3	°°__0.61 ± 0.03__	0.3 ± 0.1	0.16 ± 0.09	1.7 ± 0.9
Midbrain							
Control	28	13.8 ± 2.8	6.8 ± 1.8	2.0 ± 0.8	3.3 ± 1.3	3.7 ± 0.8	22 ± 5
	56	17.0 ± 2.9	9.0 ± 1.4	2.1 ± 0.2	3.5 ± 0.5	9.5 ± 2.2	23 ± 4
MeHg	28	14.1 ± 2.5	8.6 ± 1.0	2.2 ± 0.4	3.7 ± 0.8	5.6 ± 1.2	19 ± 3
	56	11.8 ± 7.3	4.0 ± 1.1	2.5 ± 0.4	3.1 ± 0.4	7.8± 5.6	14.5 ± 0.9

Table 5: Continued.

Brain structures and diets	Days	Neurotransmitters and decay products (pg/mg, mean ± SEM, $n = 3$)					
		NE	DA	DOPAC	HVA	5-HT	5-HIAA
Fish	28	13.1 ± 1.4	7.1 ± 1.2	1.7 ± 0.6	3.2 ± 1.2	5.7 ± 1.0	17± 4
	56	10.0 ± 1.3	5.8 ± 1.1	1.8 ± 0.1	3.29 ± 0.05	2.8 ± 1.3	16 ± 1

[a] MeHg: mice were fed a MeHg-containing diet.
[b] Fish: mice were fed a 4.9% aimara flesh-containing diet.
NE: noradrenalin, DA: dopamine, DOPAC: 3,4-dihydroxyphenylacetic acid, HVA: homovanillic acid, 5-HT: 5-hydroxytryptamine or serotonin, 5-HIAA: 5-hydroxyindole-acetic acid.
Values appearing in bold characters highlight those significantly different from the control values.
Asterisks indicate significant differences in the levels of neurotransmitters or decay products in the brain structures of mice fed the MeHg-containing diet compared to those fed the control diet, as determined with the Students test, $^{*}P < 0.05$.
Circles indicate significant differences in the levels of neurotransmitters or decay products in the brain structures of mice fed the fish-containing diet compared to those fed the control diet, as determined with the Students test, $^{\circ}P < 0.05$, and $^{\circ\circ}P < 0.01$.
The symbol § indicates significant differences in the levels of neurotransmitters or decay products in the brain structures of mice fed the fish-containing diet compared to those fed the MeHg-containing diet, as determined with the Students test, $^{\S}P < 0.05$.

cognitive performance of the mice fed the fish diet after 2 months of exposure.

5. Conclusion

The two mercury-containing diets are differing by the fact that mercury was brought by the addition of either pure methylmercury chloride or by mercurial species associated to fish. Therefore, any differential effects observed between MeHg-containing and fish-containing diets should be attributed to different chemical species of mercury present in one diet and absent from the other and vice-versa along with the possible intervening role of fish PUFA and selenium. If the beneficial role of fish nutriments such as PUFA and selenium was to counteract MeHg effects, the pattern of effects displayed after exposure to the fish-containing diet should appear less severe than that observed with the MeHg-containing diet. But in the present study, the mice fed the fish-containing diet displayed worse behavioral performances than those fed the control and the MeHg-containing diets, although the brain structures of both mercury-contaminated groups of mice contained comparable levels of mercury and even less in the striatum of those fed the fish diet. Therefore, the different chemical species of mercury within fish flesh are likely to explain the deficit in cognitive performance in the Y maze and the decreased locomotory activity in the open-field maze.

Acknowledgments

The authors thank Ayumi Onitsuka for their technical help and the Agence Nationale de la Recherche for its financial support (Grant no. 05 SEST 034 01).

References

[1] J. P. Bourdineaud, N. Bellance, G. Bénard et al., "Feeding mice with diets containing mercury-contaminated fish flesh from French Guiana: a model for the mercurial intoxication of the Wayana Amerindians," *Environmental Health*, vol. 7, article 53, 2008.

[2] J. P. Bourdineaud, M. Fujimura, M. Laclau, M. Sawada, and A. Yasutake, "Deleterious effects in mice of fish-associated methylmercury contained in a diet mimicking the Western populations' average fish consumption," *Environment International*, vol. 37, no. 2, pp. 303–313, 2011.

[3] M. H. G. Berntssen, K. Hylland, A. K. Lundebye, and K. Julshamn, "Higher faecal excretion and lower tissue accumulation of mercury in Wistar rats from contaminated fish than from methylmercury chloride added to fish," *Food and Chemical Toxicology*, vol. 42, no. 8, pp. 1359–1366, 2004.

[4] G. N. George, S. P. Singh, R. C. Prince, and I. J. Pickering, "Chemical forms of mercury and selenium in fish following digestion with simulated gastric fluid," *Chemical Research in Toxicology*, vol. 21, no. 11, pp. 2106–2110, 2008.

[5] H. H. Harris, I. J. Pickering, and G. N. George, "The chemical form of mercury in fish," *Science*, vol. 301, no. 5637, p. 1203, 2003.

[6] Y. Oyama, Y. Yamazaki, Y. Okada, K. Takahama, M. Satoh, and H. Hayashi, "Toxicity of methylmercury conjugated with L-cysteine on rat thymocytes and human leukemia K562 cells in comparison with that of methylmercury chloride," *Environmental Toxicology and Pharmacology*, vol. 9, no. 1-2, pp. 49–55, 2000.

[7] H. E. Ganther, C. Goudie, M. L. Sunde, M. J. Kopecky, and P. Wagner, "Selenium: relation to decreased toxicity of methylmercury added to diets containing tuna," *Science*, vol. 175, no. 4026, pp. 1122–1124, 1972.

[8] H. E. Ganther, "Modification of methylmercury toxicity and metabolism by selenium and vitamin E: possible mechanisms," *Environmental Health Perspectives*, vol. 25, pp. 71–76, 1978.

[9] C. J. Passos, D. Mergler, E. Gaspar et al., "Eating tropical fruit reduces mercury exposure from fish consumption in the Brazilian Amazon," *Environmental Research*, vol. 93, no. 2, pp. 123–130, 2003.

[10] R. Canuel, S. B. de Grosbois, M. Lucotte, L. Atikessé, C. Larose, and I. Rheault, "New evidence on the effects of tea on mercury metabolism in humans," *Archives of Environmental and Occupational Health*, vol. 61, no. 5, pp. 232–238, 2006.

[11] X. Jin, E. Lok, G. Bondy et al., "Modulating effects of dietary fats on methylmercury toxicity and distribution in rats," *Toxicology*, vol. 230, no. 1, pp. 22–44, 2007.

[12] M. J. McVey, G. M. Cooke, I. H. A. Curran et al., "An investigation of the effects of methylmercury in rats fed different dietary fats and proteins: testicular steroidogenic enzymes and

serum testosterone levels," *Food and Chemical Toxicology*, vol. 46, no. 1, pp. 270–279, 2008.

[13] A. Boudou, R. Maury-Brachet, M. Coquery, G. Durrieu, and D. Cossa, "Synergic effect of gold mining and damming on mercury contamination in fish," *Environmental Science and Technology*, vol. 39, no. 8, pp. 2448–2454, 2005.

[14] M. Tanaka, R. Ohtani-Kaneko, M. Yokosuka, and C. Watanabe, "Low-dose perinatal diethylstilbestrol exposure affected behaviors and hypothalamic estrogen receptor-α-positive cells in the mouse," *Neurotoxicology and Teratology*, vol. 26, no. 2, pp. 261–269, 2004.

[15] J. Podhorna and R. E. Brown, "Strain differences in activity and emotionality do not account for differences in learning and memory performance between C57BL/6 and DBA/2 mice," *Genes, Brain and Behavior*, vol. 1, no. 2, pp. 96–110, 2002.

[16] A. Yasutake, M. Nagano, and A. Nakano, "Simple method for methylmercury estimation in biological samples using atomic absorption spectroscopy," *Journal of Health Science*, vol. 51, no. 2, pp. 220–223, 2005.

[17] T. A. Simmons-Willis, A. S. Koh, T. W. Clarkson, and N. Ballatori, "Transport of a neurotoxicant by molecular mimicry: the methylmercury-L-cysteine complex is a substrate for human L-type large neutral amino acid transporter (LAT) 1 and LAT2," *Biochemical Journal*, vol. 367, no. 1, pp. 239–246, 2002.

[18] L. E. Kerper, N. Ballatori, and T. W. Clarkson, "Methylmercury transport across the blood-brain barrier by an amino acid carrier," *American Journal of Physiology*, vol. 262, no. 5, pp. R761–R765, 1992.

[19] N. Ballatori, "Transport of toxic metals by molecular mimicry," *Environmental Health Perspectives*, vol. 110, no. 5, pp. 689–694, 2002.

[20] J. J. Leaner and R. P. Mason, "Methylmercury accumulation and fluxes across the intestine of channel catfish, *Ictalurus punctatus*," *Comparative Biochemistry and Physiology C*, vol. 132, no. 2, pp. 247–259, 2002.

[21] D. Grotto, J. Valentini, J. M. Serpeloni et al., "Evaluation of toxic effects of a diet containing fish contaminated with methylmercury in rats mimicking the exposure in the Amazon riverside population," *Environmental Research*, vol. 111, no. 8, pp. 1074–1082, 2011.

[22] L. R. F. Faro, R. Durán, J. L. M. do Nascimento, M. Alfonso, and C. W. Picanço-Diniz, "Effects of methyl mercury on the in vivo release of dopamine and its acidic metabolites DOPAC and HVA from striatum of rats," *Ecotoxicology and Environmental Safety*, vol. 38, no. 2, pp. 95–98, 1997.

[23] H. Takahashi, M. Kato, H. Takano et al., "Differential contributions of prefrontal and hippocampal dopamine D 1 and D2 receptors in human cognitive functions," *The Journal of Neuroscience*, vol. 28, no. 46, pp. 12032–12038, 2008.

Hindawi Publishing Corporation
Journal of Biomedicine and Biotechnology
Volume 2012, Article ID 256965, 7 pages
doi:10.1155/2012/256965

Research Article

Mercury Disposition in Suckling Rats: Comparative Assessment Following Parenteral Exposure to Thiomersal and Mercuric Chloride

Maja Blanuša, Tatjana Orct, Maja Vihnanek Lazarus, Ankica Sekovanić, and Martina Piasek

Analytical Toxicology and Mineral Metabolism Unit, Institute for Medical Research and Occupational Health, Ksaverska cesta 2, P.O. Box 291, HR-10001 Zagreb, Croatia

Correspondence should be addressed to Tatjana Orct, torct@imi.hr

Received 5 April 2012; Accepted 25 May 2012

Academic Editor: João B. T. Rocha

Due to the facts that thiomersal-containing vaccine is still in use in many developing countries, and all forms of mercury have recognised neurotoxic, nephrotoxic, and other toxic effects, studies on disposition of ethylmercury and other mercury forms are still justified, especially at young age. Our investigation aimed at comparing mercury distribution and rate of excretion in the early period of life following exposure to either thiomersal (TM) or mercuric chloride ($HgCl_2$) in suckling rats. Three experimental groups were studied: control, TM, and $HgCl_2$, with 12 to18 pups in each. Both forms of mercury were administered subcutaneously in equimolar quantities (0.81 μmol/kg b.w.) three times during the suckling period (on the days of birth 7, 9, and 11) to mimic the vaccination regimen in infants. After the last administration of TM or $HgCl_2$, total mercury retention and excretion was assessed during following six days. In TM-exposed group mercury retention was higher in the brain, enteral excretion was similar, and urinary excretion was much lower compared to $HgCl_2$-exposed sucklings. More research is still needed to elucidate all aspects of toxicokinetics and most harmful neurotoxic potential of various forms of mercury, especially in the earliest period of life.

1. Introduction

Mercury is a pervasive environmental contaminant with proven toxic properties in mammals. Major risks recognized due to mercury exposure are dietary methylmercury exposure from fish and seafood, elemental mercury vapour from amalgam in tooth "silver fillings," and thiomersal-contained ethylmercury in vaccines [1–3]. Thiomersal (thimerosal, merthiolate) has been banned in the United States and Canada since 1999 and in the European Union since 2001 from vaccines recommended for children below seven years [4–6].

The molecule of thiomersal is sodium ethylmercury-thiosalycilate that dissociates to ethylmercury and thiosalicylate [7]. Ethylmercury is acting as a preservative against bacterial and fungal contamination of the vaccines that are repeatedly given to infants (Diphtheria-Tetanus-acellular-Pertussis vaccine, 3 to 7 times) up to 6 months of age. A potential threat of neurodevelopmental toxic effect of mercury lies in the fact that the exposure occurs in the most vulnerable period of life, when the brain is developing and growing [8]. Organic forms of mercury are more easily absorbed when ingested and are less readily eliminated from the body than its inorganic forms [1].

By now considerable amount of evidence has been collected to prove that doses of thiomersal in human vaccines do not pose harm, except for the risk of local hypersensitivity reactions [9–19]. In a recent overview Dórea [20] integrated experimental neurotoxicity studies of low-dose thiomersal in vaccines and concluded that doses relevant to thiomersal-containing vaccines exposure possess the potential to affect human neurodevelopment. A recently published experimental study in thiomersal-exposed infant rats reopens the debate on thiomersal-induced neurotoxic threat showing perturbations in the balance between excitatory and inhibitory amino acids in the brain, shifting it towards excessive neuroexcitation that may lead to neurodevelopmental disorders [21].

Recent large European cohort studies, such as the one conducted in Spain, show that increased perinatal mercury exposure is mostly related to maternal fish consumption, which means methylmercury intake [3]. However, the fact remains that during the first six months of life some infants might be exposed to cumulative levels of mercury that could exceed United States Environmental Protection Agency reference doses (5.8 μg/L of methylmercury or 6.4 μg/L of total mercury). The latter has been taken into account when setting up the recommendation for reduction of total mercury exposure and elimination of mercury use whenever possible [2].

Due to the present widespread use of thiomersal-containing vaccine in many developing countries and the fact that mercury is well recognised toxic metal, studies on disposition of ethylmercury and other mercury forms in mammalian organism including humans are still justified since numerous controversies still exist. Due to bioethical obstacles associated with such studies in infants, more studies on experimental animals are needed to quantify distribution and rate of mercury excretion after thiomersal exposure. We conducted our investigation to obtain a more detailed insight into distribution and rate of excretion in rodents in early period of life. The scope of our investigation was to assess organ retention and excretion of mercury following exposure to thiomersal compared to inorganic mercury (mercuric chloride) in suckling rats. We utilized the data and experience gained through foregoing experimental work on inorganic mercury that was conducted in our Unit by research team of Kostial et al. [22–25]. Past research confirmed that under same exposure conditions higher mercury retention is found in the gut and the brain of young compared to adult experimental animals. In our present investigation, we administered both forms of mercury subcutaneously, three times during suckling period, to mimic the type of administration (parenteral, intramuscular application) and usual vaccination regimen in infants. After the last thiomersal administration, mercury retention, and excretion was followed up during six consecutive days. Special attention was paid to the comparison of excretion rate (measured as total mercury) in thiomersal versus inorganic mercury-exposed study groups.

2. Material and Methods

2.1. Experimental Animals. Experimental rats (Wistar strain reared in the Laboratory Animal Unit of the Institute for Medical Research and Occupational Health in Zagreb, Croatia) were supplied with feed for small laboratory animals (Muccedola, Milano, Italy) and tap water *ad libitum*. Animal facility was kept under constant indoor conditions (20–22°C, constant humidity of 40%, and 12 h light/dark cycles). Animal cages were provided by sterilised pine shaving beddings. Two weeks after mating (in ratio male:female 1 : 3 over a week), pregnant rats were placed into small individual polycarbonate cages (20.7 × 26.5 × 14.0 cm, Ehret, Germany) where they gave birth and reared the litters. Six mother rats with the litters that delivered on the same day were designated for the experiment. The litters were normalized to eight male pups per litter one day after birth. Pups' body weights were recorded every morning throughout the experiment. All procedures with animals were carried out in accordance with national Law on the Protection of Animal Welfare. The experimental protocol was approved by the Institute's Bioethical Committee and was conducted within the framework of the research project granted by the Croatian Ministry of Science, Education and Sports. The implementation of the protocol was officially permitted by the Veterinary Administration of the Croatian Ministry of Agriculture, Forestry and Water Management.

2.2. Experimental Design. The pups were assigned into three experimental groups: Control, Thiomersal, and $HgCl_2$ with two to three pups per group within each litter, with total number of pups per group 12, 18, and 18. The experiment started at pups' age of seven days by subcutaneous injection of deionised water, thiomersal (ethylmercurythiosalicilic acid sodium salt, SERVA Electrophoresis, Germany), or mercuric chloride ($HgCl_2$ *p.a.*, Kemika, Croatia), depending on the assignment to the experimental group. The dose of administered mercury in both forms was equimolar, that is, 0.81 μmol/kg b.w. and was given in the volume of 0.05 mL. Compounds were dissolved in deionised water and freshly prepared each time before injection. The dose of mercury used in the experiment was calculated to be 10% of LD_{50} dose based on a previous finding for mercuric chloride in suckling rats provided by our Unit. Subcutaneous administration of both mercury forms was repeated three times; first time on the day of birth 7 (as described above), second time on day 9, and third time on the day of birth 11. The morning after the last parenteral exposure of either mercury form, from day of birth 12 through 17, during six consecutive days, we euthanized all pups from one litter of each experimental group after intraperitoneal administration of the combination of anaesthetics Narketan plus Xylapan (Vetoquinol AG, Switzerland) in doses 0.8 plus 0.6 mL/kg b.w. We then sampled the blood, selected organs, urine, and gut with its content for mercury analysis. Blood was collected from the heart in heparinised syringes. Urine was collected from the urinary bladder with a syringe immediately after opening the abdominal cavity. The brain, kidneys, liver, and entire small and large intestine were dissected after exsanguination from the abdominal aorta.

2.3. Analytical Procedure. Wet (fresh) weights of samples were recorded, and the samples were frozen at −20°C before analysis. Blood and urine samples were analysed directly without digestion. Frozen organ samples were digested, and total mercury analysed as described earlier [26, 27]. The results of mercury mass fraction in organs were expressed as micrograms or nanograms per gram of wet tissue weight (μg or ng/g w.w.) and concentrations of mercury in blood and urine as micrograms per litter (μg/L).

2.4. Statistical Analysis. The hypothesis of normal distribution of data was tested by Shapiro-Wilk's W test. The results are presented as arithmetic means and standard deviations

or median with a range of minimum and maximum values. Differences between parameters in the rats given two different forms of mercury were analysed at each sampling point by Student's t-test. Correlations (Pearson's correlation coefficient) between two forms of mercury in different tissues obtained during the entire six-day collection period were calculated. We used Statistica Programme (StatSoft, Inc., version 9.0) for the statistical analysis. The level of $P < 0.05$ was considered significant.

3. Results

During the period of exposure to mercury, between day of birth 7 and 12, body weight gain was 2.2 ± 0.4 g a day. No differences between experimental groups were found in either body weight gain or organ weights at the end of experiment. Three doses of 0.81 μmol/kg of either mercury form caused no signs of general toxicity. The timing of injection of two forms of mercury was a compromise of imitation of infant vaccination regimen and age when pups are suckling, and not yet reaching for solid feed on the cage. Subcutaneous injection was chosen as a mode of parenteral mercury administration instead of intramuscular injection in growing tiny muscle mass in suckling rats. Total mercury fraction in all analysed tissues of the control pups ($N = 12$; 2 pups in each litter) was more than 1000 times lower than values found in the exposed groups. Therefore, we pooled the values of control pups and presented them separately in Table 1, and only the values of two exposed groups were compared by statistical evaluation.

The concentrations in whole blood and urine and mass fractions of mercury in the selected organs of two mercury-exposed groups are presented graphically to show the differences and daily course during six-day collection of samples (Figure 1). In all sampling points, concentrations and mass fractions of total mercury in urine and kidney in $HgCl_2$-exposed group were significantly higher than in TM-exposed group. In the liver and in both small and large intestine, total mercury values were lower, although not always statistically significant. However, total mercury in TM-exposed group was significantly higher both in blood and in brain than in $HgCl_2$-exposed group. During six-day period of sampling, only whole blood showed a decline with time in both mercury-exposed groups (Figure 1).

In the urine, the excretion decreased within six days only in the inorganic mercury exposed group. To find out the similarity of mercury behaviour during six-day sampling period in two experimental groups, correlation between organic and inorganic mercury given to animals was tested in each analyzed organ, whole blood and urine. Statistically significant correlations were found only in whole blood and large intestine (Table 2).

4. Discussion

We investigated disposition of two different forms of mercury other than methylmercuy during the critical period of brain maturation process, which occurs in rats during early postnatal period up to age of 3-4 weeks after birth [8]. Our results show that mercury levels decreased in blood and urine in a time-dependent manner while mercury mass fractions in all selected organs remained relatively constant during six days following the parenteral exposure. The later finding indicates slow mercury elimination from internal organs. Other authors described similar findings obtained under different experimental conditions following administration of different forms of mercury to neonatal mice or infant monkeys. In mice, after receiving a single intramuscular injection of methylmercury or thiomersal (ethylmercury), mercury levels decreased after seven days in the blood and were unchanged in the brain [28]. Infant monkeys were measured three times after exposure to either methylmercury or thiomersal. The calculated washout ($T_{1/2}$) of total mercury in the brain was significantly longer than the $T_{1/2}$ for total mercury in the blood, indicating slow mercury elimination from the brain [29].

In our study, higher mercury retention in internal organs other than brain when given in inorganic form, and higher quantities of excreted mercury in urine and in small and large intestine content when given in organic form of mercury, shows higher excretion rate of inorganic form of mercury. On the other hand, higher concentration of mercury given in organic form in whole blood and the brain points to higher toxic potential of organic mercury at this early age. Although the latter results were expected, they also point to much higher absorption rate of organic mercury and easier transport into brain mass [30]. Significant differences between the two mercury-exposed groups in the blood and the brain at all collection time points (Figure 1) confirmed our preliminary observation [31, 32] when mercury was measured in only one sampling point and not determined in small and large intestine.

Concentrations of mercury in small and large intestine given as thiomersal, in spite of being lower than those given as inorganic mercury, show significant enteral mercury excretion. Such data in rodents have not been revealed in the literature so far, especially not at this early age. The latter is in line with finding of increased rate of mercury excretion in infants' stool after parenteral administration of thiomersal during intramuscular vaccination, which lead to an assumption that ethylmercury might be excreted through gastrointestinal system [7]. Our finding of high mercury mass fraction in the small and large intestine of pups

Table 1: Total mercury in whole blood, urine, liver, kidneys, small intestine, large intestine, and brain of the control pups.

	Median	Range (min.–max. value)
Whole blood (μg/L)	0.32	0.24–0.51
Urine (μg/L)	0.19	0.1–0.74
Liver (ng/g)	3.56	2.97–4.51
Kidneys (ng/g)	10.1	6.04–12.37
Small intestine (ng/g)	7.58	5.77–9.74
Large intestine (ng/g)	14.2	10.26–15.43
Brain (ng/g)	2.24	1.82–2.63

Total number of animals in the control group was 12, that is, 2 pups in each litter of the six litters in total.

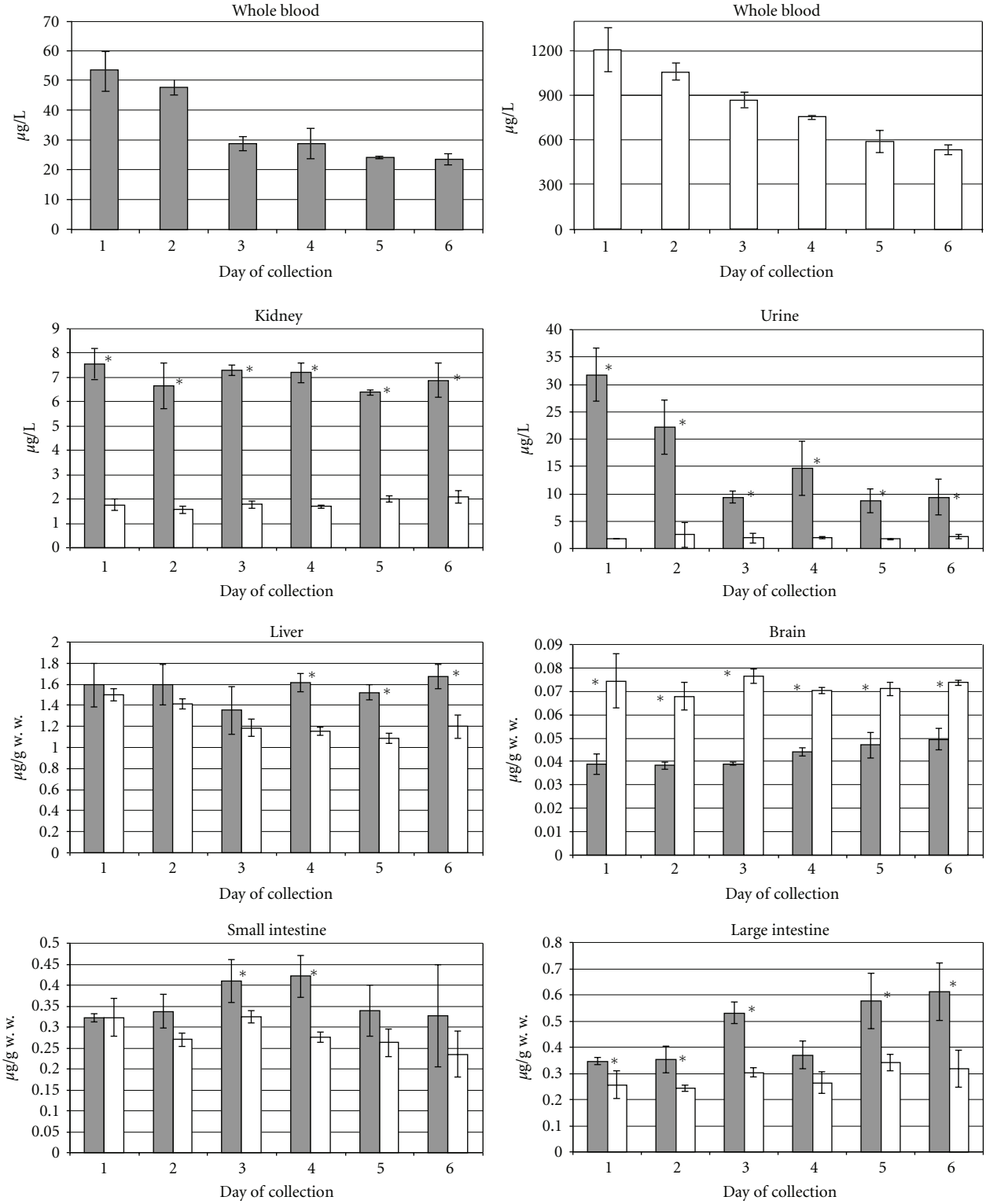


Figure 1: Concentrations in whole blood and urine and mass fractions of mercury in organs (kidneys, liver, brain, small intestine, and large intestine) of suckling rats exposed to mercuric chloride ($HgCl_2$; grey bars) or thiomersal (ethylmercury; white bars). Data are presented within six days after administration of either form of mercury as means ± SD; *statistically significant difference between exposed groups (at $P < 0.05$).

Table 2: Descriptive characteristics and Pearson's correlation coefficient between two experimental groups of suckling rats exposed to $HgCl_2$ (mercuric chloride) or thiomersal (ethylmercury). Total mercury was measured in whole blood, urine, and tissues (liver, kidneys, small intestine, large intestine, and brain). Values are presented as μg/L in whole blood and urine, and as μg/g w.w. in the liver, kidneys, small intestine, large intestine, and brain.

Study group	Samples	Mean	Std. dev.	N	P value	Pearson's r
$HgCl_2$	Whole blood	33.2	11.0	18	0.000	0.899
Thiomersal		831	257	18		
$HgCl_2$	Urine	15.9	9.10	18	0.742	0.089
Thiomersal		2.06	0.95	18		
$HgCl_2$	Liver	1546	172	18	0.77	0.079
Thiomersal		1251	158	18		
$HgCl_2$	Kidneys	6958	589.6	18	0.746	−0.088
Thiomersal		1818	253.5	18		
$HgCl_2$	Small intestine	369.9	70.76	18	0.51	0.178
Thiomersal		276.9	42.43	18		
$HgCl_2$	Large intestine	471.7	134.4	18	0.026	0.554
Thiomersal		284.5	48.72	18		
$HgCl_2$	Brain	42.7	5.69	18	0.444	0.206
Thiomersal		72.0	5.48	18		

N = Number of rats.

proves that endogenous faecal excretion given in the form of ethylmercury or thiomersal is an important route of excretion and it is probably more important than urinary excretion. This observation was supported by the findings of about three times lower mercury in the kidney and ten times lower mercury in urine of thiomersal-exposed compared to inorganic mercury-exposed pups.

The parallel downward disappearance of mercury from blood is shown by very high correlation between these two mercury forms given to animals (Table 2). Other tissues were either constantly high (kidneys, liver, brain) or even with tendency of increasing (small and large intestine) during the six-day sampling period. Correlations between mercury retention in two experimental groups in other tissues, apart from blood, were mostly not significant except in large intestine where a weak significant correlation was found (Table 2). Such different disposition may be due to easier transport of organic mercury through cell membranes and to partial transformation of organic mercury form during metabolic pathways into inorganic mercury form. It was reported that a high percentage of total mercury in the brain was in the form of inorganic mercury for the thiomersal-exposed infant monkeys [29]. The latter also means that after entering into brain, a substantial part of ethylmercury is transformed into inorganic form. Rodrigues and coworkers [30] recently found by speciation analysis that 48 hours after oral thiomersal administration to adult rats the predominant form of mercury in blood was inorganic. In the brain and in other organs, inorganic mercury was predominant as well. There are, however, no speciation data in the literature about the fate of ethylmercury in the brain and other organs given to very young and undeveloped mammals.

In conclusion, although analytical methods that we used did not allow discerning between different mercury species, our experimental design showed that parenterally administered mercury in the form of thiomersal during the suckling period underwent different distribution, retention, and elimination compared to inorganic mercury given under same experimental conditions. In the case of thiomersal exposure, mercury retention is evidently higher in the brain, its urinary excretion is much lower, and enteral excretion is similar to that of inorganic mercury. Our results contribute to the evidence on mercury disposition in the early period of life, comparing in a simple original experimental design the distribution and retention in the brain and other tissues, and elimination of two types of mercury: thiomersal and mercuric chloride mercury. Both mercury forms are present in real life, including the most vulnerable period of growth and development. Our findings are in line with the overall conclusion reached so far in the research initiatives in this area that more work is still needed to elucidate especially neurodevelopmental toxic potential of various forms of mercury and their fate in body in the earliest period of human life.

Acknowledgments

The experimental results presented in this paper are based on a long-standing research work of Professor Krista Kostial and several generations of her coworkers in the area of mercury toxicology. This work has provided a considerable contribution to the evidence that all forms of mercury are more toxic when administered to infant than adult mammals by showing that under same exposure conditions higher mercury retention is found in the gut and the brain of young compared to adult experimental animals. Our new investigation presented in this paper was supported by the Ministry of Science Education and Sports of the Republic of

Croatia within approved National Research Project (Grant no. 022-0222148-2135). The technical assistance of Mrs. Đurđa Breški and Mrs. Marija Ciganović is gratefully acknowledged.

References

[1] T. W. Clarkson, "The three modern faces of mercury," *Environmental Health Perspectives*, vol. 110, no. 1, pp. 11–23, 2002.

[2] World Health Organisation, *Exposure to Mercury: A Major Public Health Concern*, WHO Document Production Services, Geneva, Switzerland, 2007.

[3] R. Ramon, M. Murcia, X. Aguinagalde et al., "Prenatal mercury exposure in a multicenter cohort study in Spain," *Environment International*, vol. 37, no. 3, pp. 597–604, 2011.

[4] L. Magos, "Review on the toxicity of ethylmercury including its presence as a preservative in biological and pharmaceutical products," *Journal of Applied Toxicology*, vol. 21, no. 1, pp. 1–5, 2001.

[5] L. Magos, "Neurotoxic character of thimerosal and the allometric extrapolation of adult clearance half-time to infants," *Journal of Applied Toxicology*, vol. 23, no. 4, pp. 263–269, 2003.

[6] S. K. Parker, B. Schwartz, J. Todd, and L. K. Pickering, "Thimerosal-containing vaccines and autistic spectrum disorder: a critical review of published original data," *Pediatrics*, vol. 114, no. 3, pp. 793–804, 2004.

[7] M. E. Pichichero, A. Gentile, N. Giglio et al., "Mercury levels in premature and low birth weight newborn infants after receipt of thimerosal-containing vaccines," *Journal of Pediatrics*, vol. 155, no. 4, pp. 495–499, 2009.

[8] D. Rice and S. Barone Jr., "Critical periods of vulnerability for the developing nervous system: evidence from humans and animal models," *Environmental Health Perspectives*, vol. 108, supplement 3, pp. 511–533, 2000.

[9] L. K. Ball, R. Ball, and R. D. Pratt, "An assessment of thimerosal use in childhood vaccines," *Pediatrics*, vol. 107, no. 5, pp. 1147–1154, 2001.

[10] N. Andrews, E. Miller, A. Grant, J. Stowe, V. Osborne, and B. Taylor, "Thimerosal exposure in infants and developmental disorders: a retrospective cohort study in the United Kingdom does not support a causal association," *Pediatrics*, vol. 114, no. 3, pp. 584–591, 2004.

[11] C. J. Clements, "The evidence for the safety of thiomersal in newborn and infant vaccines," *Vaccine*, vol. 22, no. 15-16, pp. 1854–1861, 2004.

[12] J. D. Clemens and L. Jodar, "Translational research to assist policy decisions about introducing new vaccines in developing countries," *Journal of Health, Population and Nutrition*, vol. 22, no. 3, pp. 223–231, 2004.

[13] J. Heron, J. Golding, and ALSPAC Study Team, "Thimerosal exposure in infants and developmental disorders: a prospective cohort study in the United Kingdom does not support a causal association," *Pediatrics*, vol. 114, no. 3, pp. 577–583, 2004.

[14] P. Grandjean and P. Landrigan, "Developmental neurotoxicity of industrial chemicals," *The Lancet*, vol. 368, no. 9553, pp. 2167–2178, 2006.

[15] J. G. Dórea, "Making sense of epidemiological studies of young children exposed to thimerosal in vaccines," *Clinica Chimica Acta*, vol. 411, no. 21-22, pp. 1580–1586, 2010.

[16] CIDRAP (Center for Infectious Disease Research and Policy), http://www.cidrap.umn.edu/cidrap/content/other/news/feb-0210scan.html, 2010.

[17] The Editors of The Lancet, "Retraction-Ileal-lymphoid-nodular hyperplasia, non-specific colitis, and pervasive developmental disorder in children," [Retraction of A. J. Wakefield, S. H. Murch, A. Anthony etal., *The Lancet*, vol. 351, no. 9103, pp. 637–641, 1998], *The Lancet*, vol. 3475, no. 9713, p. 445, 2010.

[18] H. L. Evans, "Cognitive complications of mercury exposure," in *Cognitive and Behavioral Abnormalities of Pediatric Diseases*, R. D. Nass and Y. French, Eds., pp. 617–619, Oxford University Press, New York, NY, USA, 2010.

[19] S. T. Schultz, "Does thimerosal or other mercury exposure increase the risk for autism? A review of current literature," *Acta Neurobiologiae Experimentalis*, vol. 70, no. 2, pp. 187–195, 2010.

[20] J. G. Dórea, "Integrating experimental (in vitro and in vivo) neurotoxicity studies of low-dose thimerosal relevant to vaccines," *Neurochemical Research*, vol. 36, no. 6, pp. 927–938, 2011.

[21] M. Duszczyk-Budhathoki, M. Olczak, M. Lehner, and M. D. Majewska, "Administration of thimerosal to infant rats increases overflow of glutamate and aspartate in the prefrontal cortex: protective role of dehydroepiandrosterone sulfate," *Neurochemical Research*, vol. 37, no. 2, pp. 436–447, 2012.

[22] K. Kostial, D. Kello, and S. Jugo, "Influence of age on metal metabolism and toxicity," *Environmental Health Perspectives*, vol. 25, pp. 81–86, 1978.

[23] K. Kostial, I. Rabar, M. Blanuša, and M. Landeka, "Effect of age on heavy metal absorption," *Proceedings of the Nutrition Society*, vol. 38, no. 2, pp. 251–256, 1979.

[24] K. Kostial, I. Šimonović, I. Rabar, M. Blanuša, and M. Landeka, "Age and intestinal retention of mercury and cadmium in rats," *Environmental Research*, vol. 31, no. 1, pp. 111–115, 1983.

[25] K. Kostial, M. Blanuša, T. Maljković et al., "Age and sex influence the metabolism and toxicity of metals," in *Trace Elements in Man and Animals-7*, B. Momčilović, Ed., pp. 1–6, Institute for Medical Research and Occupational Health, Zagreb, Croatia, 1991.

[26] L. Prester, D. Jureša, and M. Blanuša, "Decomposition of fish samples for determination of mercury," *Arhiv za Higijenu Rada i Toksikologiju*, vol. 49, no. 4, pp. 343–348, 1998.

[27] H. M. Boylan, R. C. Richter, H. M. Skip Kingston, and A. Ricotta, "Rapid mercury analysis for the field: method development and application to natural gas utility sites," *Water, Air, and Soil Pollution*, vol. 127, no. 1–4, pp. 255–270, 2001.

[28] G. J. Harry, M. W. Harris, and L. T. Burka, "Mercury concentrations in brain and kidney following ethylmercury, methylmercury and Thimerosal administration to neonatal mice," *Toxicology Letters*, vol. 154, no. 3, pp. 183–189, 2004.

[29] T. M. Burbacher, D. D. Shen, N. Liberato, K. S. Grant, E. Cernichiari, and T. Clarkson, "Comparison of blood and brain mercury levels in infant monkeys exposed to methylmercury or vaccines containing thimerosal," *Environmental Health Perspectives*, vol. 113, no. 8, pp. 1015–1021, 2005.

[30] J. L. Rodrigues, J. M. Serpeloni, B. L. Batista, S. S. Souza, and F. Barbosa, "Identification and distribution of mercury species in rat tissues following administration of thimerosal or methylmercury," *Archives of Toxicology*, vol. 84, no. 11, pp. 891–896, 2010.

[31] T. Orct, M. Blanuša, M. Lazarus, V. M. Varnai, and K. Kostial, "Comparison of organic and inorganic mercury distribution in suckling rat," *Journal of Applied Toxicology*, vol. 26, no. 6, pp. 536–539, 2006.

[32] T. Orct, M. Blanuša, M. Lazarus, V. M. Varnai, and K. Kostial, "Effect of thiomersal and mercuric chloride on mercury distribution in suckling rats," *Toxicology Letters*, vol. S164, no. 6, pp. S191–S192, 2006.

Hindawi Publishing Corporation
Journal of Biomedicine and Biotechnology
Volume 2012, Article ID 635075, 7 pages
doi:10.1155/2012/635075

Research Article

Methyl Mercury Exposure at Niigata, Japan: Results of Neurological Examinations of 103 Adults

Kimio Maruyama,[1] Takashi Yorifuji,[2] Toshihide Tsuda,[2] Tomoko Sekikawa,[3] Hiroto Nakadaira,[4] and Hisashi Saito[5]

[1] *Department of Social Welfare and Psychology, Faculty of Nursing, Social Welfare and Psychology, Niigata Seiryo University, 1-5939 Suido-cho, Chuo-ku, Niigata 951-8121, Japan*
[2] *Department of Human Ecology, Okayama University Graduate School of Environmental and Life Science, 3-1-1 Tsushima-naka, Kita-ku, Okayama 700-8530, Japan*
[3] *Department of Internal Medicine, Nuttari Clinic, 6-4-12 Nuttarihigasi, Chuo-ku, Niigata 950-0075, Japan*
[4] *Department of Nursing, Faculty of Nursing, Social Welfare and Psychology, Niigata Seiryo University, 1-5939 Suido-cho, Chuo-ku, Niigata 951-8121, Japan*
[5] *Department of Internal Medicine, Kido Hospital, 4-13-3 Takeo, Higashi-ku, Niigata 950-0862, Japan*

Correspondence should be addressed to Kimio Maruyama, k-maruyama@n-seiryo.ac.jp

Received 6 April 2012; Revised 2 June 2012; Accepted 8 June 2012

Academic Editor: Jose G. Dorea

Background. Large-scale poisonings caused by methyl mercury (MeHg) have occurred in Japan (Minamata in the 1950s and Niigata in the 1960s) and Iraq (in the 1970s). The current WHO neurological risk standard for adult exposure (hair level: 50 μg/g) was based partly on evidence from Niigata which did not consider any cases who were diagnosed later and/or exposed to low level of MeHg (hair mercury level less than 50 μg/g). *Methods*. Early in the Niigata epidemic in June 1965 there were two extensive surveys. From these two surveys, we examined 103 adults with hair mercury measurement who consulted two medical institutions. We compared the prevalence and the distribution of neurological signs related to MeHg poisoning between exposure categories. *Result*. We found 48 subjects with neurological signs related to MeHg poisoning who had hair mercury concentration less than 50 μg/g. Among the neurological signs, sensory disturbance of the bilateral distal extremities was observed more frequently, followed by disequilibrium, hearing impairment, and ataxia, in groups with hair MeHg concentration both below 50 μg/g and over 50 μg/g. *Conclusion*. The present study suggests the possibility that exposure to MeHg at levels below the current WHO limits could cause neurologic signs, in particular, sensory disturbance.

1. Introduction

Large-scale poisonings caused by methyl mercury (MeHg) have occurred in Japan (Minamata in the 1950s and Niigata in the 1960s) and Iraq (in the 1970s) [1–3]. These poisonings provided substantial data regarding the neurologic signs caused by MeHg: sensory disturbance, ataxia, constriction of the visual field, speech disturbance, and hearing impairment [1–7]. In Minamata, the etiologic agent (MeHg) was not acknowledged until three years after official identification of the exposed cases (in 1956); thus, there were few attempts to evaluate the association between MeHg exposure and neurologic signs (i.e., dose-response relationships). In contrast, in the settings of Niigata and Iraq, MeHg was recognized as an etiologic agent in the early phase of the poisonings [1, 5, 7], which provided the occasions to examine the dose-response relationships. The findings from these two poisonings therefore provided significant insights for World Health Organization (WHO) conclusion on mercury and health [1, 8].

Indeed, WHO published two reports of Environmental Health Criteria in 1976 and 1990 based on the findings from the Japanese and Iraqi outbreaks [1, 8–10]. Then, WHO concluded that "Certain groups with high fish consumption

may attain a high blood methyl mercury level (about 200 μg/liter, corresponding to 50 μg/g of hair) associated with a low (5%) risk of neurological damage to adults" [9]. The reports also mentioned that "No adverse effects have been detected with long-term daily methyl mercury intakes of 3–7 μg/kg body weight (hair mercury concentrations of approximately 50–125 μg/g)" [9]. In the process of making the conclusion, WHO reports cited publications from Niigata which mentioned that the mercury content in hair samples from 30 initial patients, officially certified as having MeHg poisoning, ranged from 52 μg/g to 570 μg/g [3, 11, 12]. However, the publications did not consider any cases who were diagnosed later and/or exposed low level of MeHg (hair mercury level less than 50 μg/g), possibly distorting the conclusion of WHO.

Early on in the Niigata poisoning in 1965, there were two extensive surveys of people living along the Agano River and the adjacent areas, which included assessment of hair mercury concentration. In the present study, we targeted 103 adults from the surveys and examined whether neurological signs related to MeHg exposure were observed below hair mercury concentration of 50 μg/g as well as the distributions of the neurological signs among the participants. Because MeHg exposure through fish consumption has attracted intense public health interest [13], the findings from Niigata would be valuable.

2. Material and Methods

2.1. Study Areas and Subjects. In Niigata Japan, the first case of MeHg poisoning was found in January, the second in April, and the third in May, of 1965. Seven patients were confirmed before June and all lived around the lower stream of the Agano River. Their exposure was due to consumption of fish and shellfish contaminated by MeHg discharged from a chemical factory (Showa Denko Kanose Factory) located on the upper reaches of the Agano River [3, 5, 6, 11, 12, 14]. The total mercury content of fish samples collected in June 1965 from the Agano River was elevated (barbel, 21.0~23.6 ppm; snakehead mullet, 12.3 ppm; chub, 4.6~8.38 ppm) and one eel captured near Tsugawa town (a town along the Agano River) had 41.0 ppm [7].

In June 1965, two surveys were carried out for all officially registered inhabitants of five administrative districts located within 15 km from the mouth of the river where the seven patients lived. The administrative districts were composed of the parts of 2 cities, 2 towns, and one village, consisting of 65 settlements. The first survey was conducted in 6 settlements where the first 7 patients lived between June 14, 1965 and June 18, 1965 by medical doctors, interns, and students from the Niigata University Medical Department. The second survey was performed between June 21, 1965 and June 24, 1965 by the Niigata Prefecture Sanitation Department in the remaining 59 settlements of the districts. The second survey included public health nurses from the prefectural health centers as well [5, 7, 15].

The sampling process of the study subjects from these two surveys is shown in Figure 1. In the first survey, 2,813 inhabitants were interviewed using a standardized questionnaire to determine the extent of their MeHg exposure. The survey focused on symptoms related to MeHg exposure and river fish consumption. Subjects with symptoms suggestive of MeHg exposure were subsequently examined neurologically. The second survey was done in the same fashion on 19,888 inhabitants of the lower reaches of the Agano River basin.

From these interviews, 1,386 persons were identified as being exposed because they had symptoms or findings suggestive of MeHg exposure; because other family members had symptoms related to MeHg poisoning; or because they consumed river fish in large quantities. Subsequently, their total hair mercury concentration was measured [5, 7]. After that, in July 1965, Niigata Prefecture restricted the taking of fish and shellfish on the lower reaches of the Agano River.

Among 1,386 persons, 109 subjects who had symptoms related to MeHg poisoning as chief complaints voluntarily consulted at either (or both) of two medical institutions (Nuttari clinic and Kido hospital) for medical care for the first time during the period of 1965 to 1986. The two institutions are medical institutions which have followed MeHg poisoning cases in Niigata just after the outbreak in 1965, cooperating with Niigata University. Most of the patients still consult at either (or both) of the two medical institutions for medical care. In the present study, we included 103 adults ($\geqq$20 years old) and considered the neurological manifestations observed at their first consultation.

2.2. Measurement of MeHg in Hair. The subjects had their hair collected before the restriction of fish and shellfish (in July 1965) and were believed to be consuming the contaminated fish and shellfish when mercury was measured. The mercury content of hair samples was measured using 3 different techniques: the Dithizone method, Neutron Activation Analysis, and Atomic Absorption Spectroscopy at the Niigata University [5, 7]. We report the values without any interpolation.

2.3. Measurement of Outcomes (Diagnosis of Neurological Signs from MeHg Exposure). The 103 subjects were evaluated using a standard neurological examination by at least two well-trained physicians including two of the authors in the present study (T. Sekikawa and H. Saito) between 1965 and 1986. In particular, one of the authors (H. Saito) has been treating the MeHg poisoning cases since the outbreak in 1965 at the two medical institutions up to the present and was involved in all of the neurological examinations. Thus, neurological examination was standardized over the study period and between the examiners. Data were collected on neurological signs related to MeHg poisoning, namely, sensory disturbance of the distal extremities, perioral area, and whole body; ataxia; visual constriction; hearing impairment; speech disturbance; disequilibrium. However, not all subjects underwent all neurological tests: data on whole body sensory disturbance, visual constriction, hearing impairment, speech disturbance, and disequilibrium were missing for 7, 8, 9, 7, 1 study subjects, respectively. In the previous study in

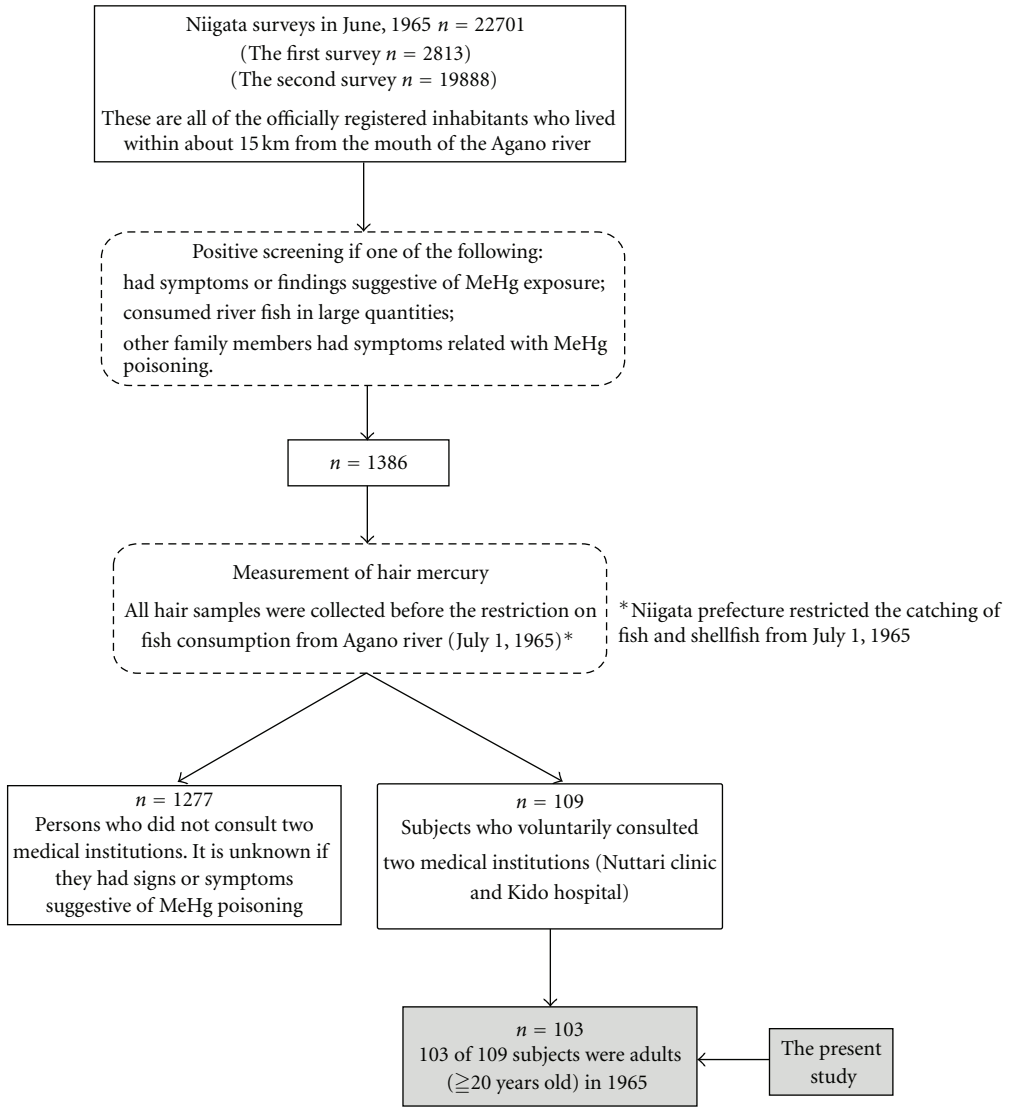


Figure 1: Sampling process of the study subjects.

Minamata, it was noted that sensory disturbance of the bilateral distal extremities could be used as the gold standard for the diagnosis of Minamata Disease (methyl mercury poisoning-related neurological disorders) in the exposed areas [16, 17].

Tactile sensation was estimated using a small paint brush and pain sensation was tested using a roulette wheel. Visual fields were examined by confrontation. Hearing impairment was examined by tuning fork of 512 Hz and a ticking watch. Speech disturbances were examined by phonation of labial and lingual sounds. Ataxia was determined by 3 tests; Adiadochokinesis, Finger-Nose, and Heel-Knee. Disequilibrium was tested by 3 tests; Romberg test, Mann test, and standing on one foot. Mann test is used to investigate the loss of motor coordination. The procedure is as follows: the subject stands with both legs on a straight line putting one toenail behind the heel of the other foot and looks straight ahead with eyes open and then closes both eyes. The subject repeats the procedure reversing the positions of his feet. Mann test is judged positive when the subject sways. For ataxia and disequilibrium, each test was scored as positive (+), equivocal (±), or negative (−). If one test in the group was positive that category was classified as positive. If there were no positives in the three tests and there was one and over (±) in the category it was scored as equivocal. When all 3 tests were negative, the category was classified as negative. When the categories were classified as positive or equivocal, ataxia and disequilibrium are scored positive in this study. The subjects were all examined between June 1965 and July 1986. In the present study, we examined neurological symptoms and signs at the first consultation for the two medical institutions (i.e., the closest findings to the hair mercury measurement of June, 1965).

3. Analysis

First, we described the demographic characteristics of the subjects by exposure category (0–≤20 μg/g, 20–50 μg/g,

50–100 μg/g, and >100 μg/g). We then compared the prevalence proportions of neurological signs between the exposure categories. We estimated the adjusted prevalence odds ratios of exposure using a logistic regression model, employing the lowest measured category as reference. We adjusted for age and sex. We also conducted statistical tests for trend. In a sensitivity analysis, we restricted the subjects whose hair mercury concentration was measured by the Dithizone method, which the previous publications from Niigata adopted, and repeated the same analysis. All confidence intervals were calculated at the 95% level. PASW software version 18.0 was used for all analyses.

4. Results

The distribution of the 103 subjects among 1,386 persons (who were measured mercury concentration) separated by the exposure category is shown in Table 1. The persons who had lower level of mercury concentration in hair tended not to be consulted at the medical institutions. The characteristics of the study subjects separated by the exposure category are also shown in Table 2. The subjects in the highest exposure category (>100 μg/g) tended to be younger in 1965, men, and examined earlier after their signs appeared. About seven-tenths of the samples (69.9%) were measured using the Dithizone method, while about one-fourth (25.2%) by Neutron Activation Analysis, and only 5 samples (4.9%) by Atomic Absorption Spectroscopy.

Figure 2 and Table 3 show the frequency of neurological signs related to MeHg poisoning separated by the hair mercury content group. Distal sensory disturbance of the bilateral extremities was most frequently detected (95.1%), followed by disequilibrium (86.3%), hearing impairment (75.5%), and ataxia (70.9%). Half of the subjects manifested sensory disturbance of the perioral area (57.3%) and visual constriction (44.2%). All of the subjects who had a perioral sensory disturbance had a sensory disturbance of the bilateral extremities. Even the subjects who had hair mercury levels less than 50 μg/g had a variety of neurological signs related to MeHg poisoning.

When we estimated the adjusted prevalence odds ratios (Table 3), the associations between exposure and neurological signs were not apparent because neurological sings were detected even in the low exposure categories. Statistical dose-response relationships were not apparent except perioral sensory disturbance ($P = 0.04$).

Even when we restricted the subjects whose hair mercury concentration was measured by the Dithizone method, the 28 subjects (Table 2) with hair mercury concentration less than 50 μg/g had the neurological signs related to MeHg poisoning.

5. Discussion

In the present study, we examined whether neurological signs related to MeHg exposure were observed below hair mercury concentration of 50 μg/g as well as the distributions of the neurological signs in Niigata, Japan. We then found that there were the 48 subjects with hair mercury concentration less than 50 μg/g had the neurological signs related to MeHg poisoning, that is, some of the subjects with the typical neurological signs had hair mercury levels less than 50 μg/g. Moreover, among the neurological signs, sensory disturbance of the bilateral distal extremities, disequilibrium, hearing impairment, and ataxia were observed more frequently.

Although the previous studies conducted in the same exposed area (Niigata) suggested that the hair mercury content from the initial patients ranged from 52 μg/g to 570 μg/g [11, 12, 14], the present study provided an additional finding that the subjects who had similar neurological signs were detected even among the 48 subjects below hair mercury concentration of 50 μg/g. Although we do not have information about the neurological signs of 48 subjects at survey time (in June 1965), they had a variety of neurological signs related to MeHg poisoning when we examined. This is probably because our study included the subjects who manifested the signs later and who had less severe signs in the low exposure level compared to the previous studies.

Given the sampling process of the present study (only 7.4% of the hair-mercury-measured subjects) (Figure 1 and Table 1), there may be a question about inferring that the persons who have hair mercury concentration less than 50 μg/g really manifest the typical neurological signs induced by MeHg exposure. However, the notion that persons with the low level of exposure manifest such neurological signs could be supported by the following evidence: first, the prevalence of such typical neurological signs among unexposed population is rare (e.g., prevalence of sensory disturbance of the bilateral distal extremities ranged from 0 to 1.1% [18–22]). Second, as mentioned, it was noted that sensory disturbance of the bilateral distal extremities could be used as the gold standard for the diagnosis of Minamata Disease (methyl mercury poisoning-related neurological disorders) in the exposed areas [16, 17]. This evidence was supported by the findings that sensory disturbance had the lowest threshold [1]; milder cases only had sensory disturbance [1, 8]; sensory disturbance was the most persistent neurologic sign [18]; the prevalence of sensory disturbance of the bilateral distal extremities was low (as mentioned). Therefore, it could be considered that the subjects in the present study who had hair mercury concentration less than 50 μg/g manifested the typical neurological signs because of MeHg exposure.

Moreover, even if we assumed that persons who did not consult at the medical institutions did not have any typical neurological signs, the prevalence of the neurological signs would be still higher than that of general population For example, 23, 23, 20, and 32 subjects had sensory disturbance of the bilateral distal extremities in each exposure category (0–<20, 20–50, 50–100, and >100 μg/g) (Table 3); thus, the prevalence of the neurological sign was 2.5, 7.5, 22.0, and 52.5% in each category assuming that persons who did not consult at the medical institutions did not have any typical neurological signs (Table 1). The prevalence, in particular in the categories of hair mercury levels less than 50 μg/g, is still higher than that of general population mentioned above (0 to 1.1% [18–22]). The present study therefore supports that long-term exposure to MeHg at levels below the current

Table 1: Distribution of the 103 subjects among 1,386 persons (whose hair mercury concentrations were measured) separated by the exposure category.

	0–≤20 μg/g (n = 926)	20–50 μg/g (n = 308)	50–100 μg/g (n = 91)	>100 μg/g (n = 61)	Total (n = 1386)
Persons who did not consult two medical institutions	901	283	67	26	1277
Subjects in the present study	24 + 1*(2.7%)	24 + 1(8.1%)	21 + 3(26.4%)	34 + 1(57.4%)	103 + 6(7.9%)

* Number after the plus sign shows the number of persons younger than 20 years old. Consequently, 103 subjects were included in the present study.
Source: Ministry of Health and Welfare.

Table 2: Characteristics of subjects by hair mercury content.

	All (n = 103)	0–≤20 μg/g (n = 24)	20–50μg/g (n = 24)	50–100μg/g (n = 21)	>100 μg/g (n = 34)
Mean age in years (1965) ± SD	44.5 ± 12.9	45.3 ± 12.9	44.8 ± 12.4	49.5 ± 13.1	40.6 ± 12.4
Sex					
Men	61 (59.2%)	11 (45.8%)	12 (50.0%)	12 (57.1%)	26 (76.5%)
Women	42 (40.8%)	13 (54.2%)	12 (50.0%)	9 (42.9%)	8 (23.5%)
Date symptoms began	1964–1973	1964–1973	1964–1970	1964–1972	1964–1971
Mean age in years (±SD) that symptoms began	45.5 ± 12.8	46.0 ± 12.8	46.5 ± 13.5	51.4 ± 13.0	40.4 ± 10.7
Mean age in years of assessment of neurologic signs ± SD	50.7 ± 13.0	53.9 ± 12.4	52.4 ± 12.5	56.3 ± 11.7	43.6 ± 11.7
Mean hair mercury content ± SD (μg/g)	107.6 ± 123.1	13.2 ± 4.5	32.6 ± 8.5	72.6 ± 13.9	248.8 ± 121.4
Hair mercury content (μg/g) (min-max)	4.2–570.0	4.2–19.5	20.5–46.6	56.0–100.0	104.0–570.0
Analysis method of hair mercury (1965)					
Dithizone	72 (69.9%)	10 (41.7%)	18 (75.0%)	13 (61.9%)	31 (91.2%)
Neutron activation	26 (25.2%)	13 (54.2%)	6 (25.0%)	5 (23.8%)	2 (5.9%)
Atomic absorption	5 (4.9%)	1 (4.1%)	0 (0%)	3 (14.3%)	1 (2.9%)

SD: standard deviation.

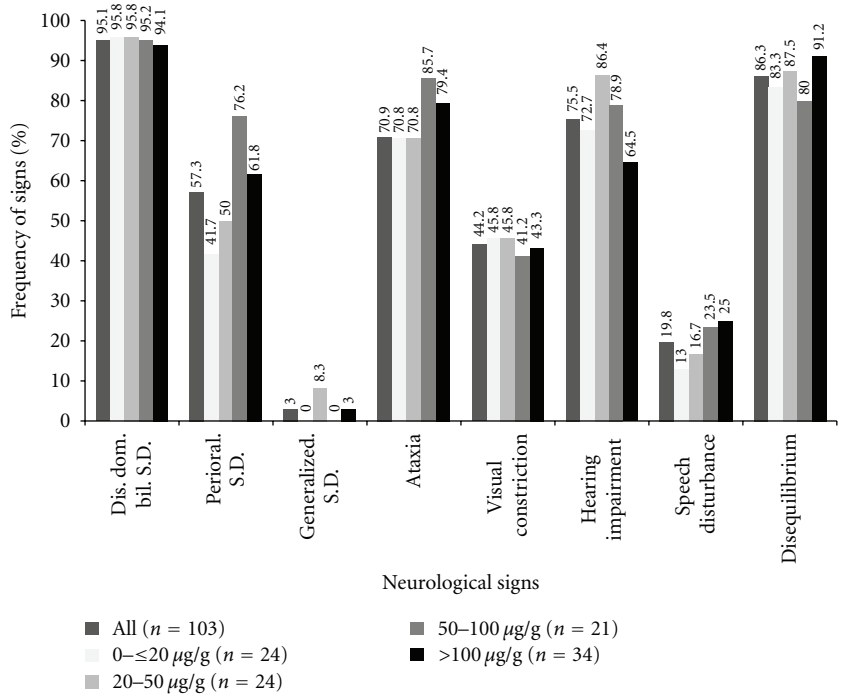


Figure 2: Hair mercury content and frequency of neurological signs. Dis.dom.bil. S.D.: distal dominant bilateral Sensory Disturbance S.D.: sensory disturbance.

Table 3: Adjusted PORs* and their 95% C.I.s between mercury content and neurological signs.

	0–≤20 μg/g ($n = 24$)	20–50μg/g ($n = 24$)	50–100μg/g ($n = 21$)	>100 μg/g ($n = 34$)	P value for trend
Dis.dom.bil. S.D. n (%)	23 (95.8)	23 (95.8)	20 (95.2)	32 (94.1)	
PORs and 95% CIs	1	1.0 (0.1, 18.1)	0.6 (0, 11.7)	0.7 (0, 10.4)	0.74
Perioral S.D. n (%)	10 (41.7)	12 (50)	16 (76.2)	21 (61.8)	
PORs and 95% CIs	1	1.4 (0.5, 4.5)	4.3 (1.2, 16)	2.7 (0.9, 8.3)	0.04
Whole body S.D. n (%)	0 (0)	2 (8.3)	0 (0)	1 (3)	
PORs and 95% CIs	1	NE	NE	NE	0.98
Ataxia n (%)	17 (70.8)	17 (70.8)	18 (85.7)	27 (79.4)	
PORs and 95% CIs	1	1.0 (0.3, 3.8)	2.2 (0.5, 10.5)	2.3 (0.6, 8.9)	0.14
Visual constriction n (%)	11 (45.8)	11 (45.8)	7 (41.2)	13 (43.3)	
PORs and 95% CIs	1	1.0 (0.3, 3.5)	0.5 (0.1, 2)	1.1 (0.3, 3.6)	0.89
Hearing impairment n (%)	16 (72.7)	19 (86.4)	15 (78.9)	20 (64.5)	
PORs and 95% CIs	1	2.6 (0.5, 13.2)	1.1 (0.2, 5.1)	0.6 (0.1, 2.2)	0.25
Speech disturbance n (%)	3 (13)	4 (16.7)	4 (23.5)	8 (25)	
PORs and 95% CIs	1	1.2 (0.2, 6.9)	1.4 (0.2, 8.4)	1.8 (0.4, 8.8)	0.45
Disequilibrium n (%)	20 (83.3)	21 (87.5)	16 (80)	31 (91.2)	
PORs and 95% CIs	1	1.4 (0.3, 7.1)	0.7 (0.1, 3.4)	1.9 (0.3, 10)	0.66

* Prevalence odds ratios were estimated using 0–20 ppm as a reference and were adjusted for age and sex.
Abbreviations: C.I.: confidence interval; Dis.dom.bil.: distal dominant bilateral; NE: not estimatable; POR: prevalence odds ratio.

WHO limits could cause neurologic signs, in particular, sensory disturbance, which is consistent with the view of the National Research Council (US) [23] and the previous studies conducted in Brazil [24] and Minamata [10].

The finding that the dose-response relationship was observed for perioral sensory disturbance (Table 3) was consistent with the previous study in Minamata [10]. The reason why the dose-response relationships were not observed for other neurological sings could be explained by the sampling process. It is likely that the subjects who did not have the neurological signs in the lower level of the mercury exposure did not consult the doctors, probably diminishing the dose-response relationships.

There is a concern that the hair mercury level measured in June 1965 may not reflect the peak exposure of the subjects. However, the hair mercury concentration of the subjects was measured at the relatively early phase of the outbreak. In addition, all of the hair samples were collected before the restriction of fishing; thus, the subjects were considered to be consuming the contaminated fish (at that time) and their hair mercury values were thought to be close to the peak exposure value. Thus, such a concern would not invalidate our result.

In the present study, a sensory disturbance of the bilateral distal extremities was most frequently detected (95.1%), followed by disequilibrium (86.3%), hearing impairment (75.5%), and ataxia (70.9%). Half of the subjects had sensory disturbance of the perioral area (57.3%) and visual constriction (44.2%). This tendency that the sensory disturbance was the most prevalent sign is consistent with the previous studies in Niigata [25] and Minamata [4] which examined the prevalence of the neurological signs among the exposed subjects.

The possible limitation of the present study is that there were three different methods for hair mercury measurement. Hoshino et al. [26] measured total mercury content in hair samples from 3 Niigata Minamata disease patients and their 17 family members by using two techniques (the Neutron Activation Analysis and the Dithizone Method) and showed that the values from the two methods were similar. Moreover, Tsubaki [14] measured same samples by using the Dithizone Method and Atomic Absorption Analysis, then they showed that the values were the same between the two methods. Therefore, the different methods for hair mercury measurement would not explain the present results. Moreover, restricting the subjects whose hair mercury concentration was measured by the Dithizone Method did not alter the results in the sensitivity analysis.

As mentioned, another limitation is the sampling process of the present study. We could not obtain the information about health status of the 1277 persons who did not consult the doctors. This may affect the dose-response relationships and the obtained prevalence of the neurological signs in each exposure category. However, this limitation does not affect the present finding that there were subjects with the neurological signs related to MeHg poisoning in the exposure category of hair mercury levels less than 50 μg/g.

6. Conclusions

The present study, conducted in Niigata Japan, showed that the subjects with the neurological signs related to MeHg poisoning had hair mercury levels less than 50 μg/g and suggested the possibility that long-term exposure to MeHg at levels below the current WHO limits could cause neurologic signs, in particular, sensory disturbance. Among the neurological signs, sensory disturbances of bilateral extremities were the most common. These findings may provide additional evidence for MeHg toxicity from contaminated seafood intake.

Ethical Approval

This study was approved by the ethics committee of Niigata Seiryo University.

Conflict of Interests

The authors declared that they have no conflict of interests.

Acknowledgments

The authors would like to appreciate the contributions of the late professor Kenichi Shirakawa. The authors are truly grateful to Professor Gray J. Myers from the University of Rochester, Rochester, New York for his advice and editorial assistance. The study was conducted without any specific funding. This research was carried out at Kido Hospital.

References

[1] F. Bakir, S. F. Damluji, L. Amin-Zaki et al., "Methyl mercury Poisoning in Iraq," *Science*, vol. 181, pp. 230–241, 1973.

[2] M. Harada, "Minamata disease: methylmercury poisoning in Japan caused by environmental pollution," *Critical Reviews in Toxicology*, vol. 25, no. 1, pp. 1–24, 1995.

[3] T. Tsubaki, "Clinical epidemiology about niigata minamata disease," in *Minamata Disease, Rresearches during 20 Years and the Problems at Present*, S. Arima, Ed., pp. 291–300, Seirinsha, Tokyo, Japan, 1979.

[4] H. Tokuomi, "Clinical investigation on minamata disease. Minamata disease in human adult," in *Minamata Disease*, Study Group of Minamata Disease, Ed., pp. 48–81, Kumamoto University, Kumamoto, Japan, 1968.

[5] Ministry of Health and Welfare, "Research on diagnosis of the mercury poisoning," in *Special Research Report on Organic Mercury poisoning in Niigata*, pp. 3–24, Ministry of Health and Welfare, Tokyo, Japan, 1967.

[6] T. Tsubaki, "Organic mercurial poisoning along the River Agano," *Internal Medicine*, vol. 21, no. 5, pp. 871–875, 1968 (Japanese).

[7] Ministry of Health and Welfare, "Epidemiological research on the mercury poisoning," in *Special Research Report on Organic Mercury Poisoning in Niigata*, p. 58, Ministry of Health and Welfare, Tokyo, Japan, 1967.

[8] World Hearth Organization, *Environmental Health Criteria 1: Mercury*, World Health Organization, gneva, Switzerland, 1976.

[9] World Hearth Organization, *Environmental Health Criteria: 101. Methyl Mercury*, World Health Organization, Geneva, Switzerland, 1990.

[10] T. Yorifuji, T. Tsuda, S. Takao, E. Suzuki, and M. Harada, "Total mercury content in hair and neurologic signs: historic data from minamata," *Epidemiology*, vol. 20, no. 2, pp. 188–193, 2009.

[11] T. Tsubaki, "The follow up of Niigata Minamata Disease," *Science*, vol. 42, pp. 526–531, 1972 (Japanese).

[12] T. Tsubaki, "Methyl mercury poisoning along the Agano River, researches by Niigata University," *Clinical Neurology*, vol. 8, pp. 511–520, 1968.

[13] T. W. Clarkson, L. Magos, and G. J. Myers, "The toxicology of mercury—current exposures and clinical manifestations," *New England Journal of Medicine*, vol. 349, no. 18, pp. 1731–1737, 2003.

[14] T. Tsubaki, K. Hirota, K. Shirakawa, K. Kondo, and T. Sato, "Clinical, epidemiological and toxicological studies on methyl mercury poisoning," in *Proceedings of the 1st International Congress on Toxicology*, G. L. Plaa and W. A. M. Duncan, Eds., pp. 339–357, Academic Press, New York, NY, USA.

[15] K. Kondo, "Incidence of Minamata disease in communities along the Agano river, Niigata, Japan—patterns of the exposure and official diagnosis of patients," *Nihon Eiseigaku Zasshi*, vol. 51, no. 2, pp. 599–611, 1996 (Japanese).

[16] The Committee on Reseach Human Right in The Japanese Society of Psychiatry and Neurology, "An opinion on the criteria on acquired Minamata disease," *Psychiatria et Neurologia Japonica*, vol. 100, pp. 765–790, 1998 (Japanese).

[17] T. Yorifuji, T. Tsuda, S. Inoue, S. Soshi Takao, M. Harada, and I. Kawachi, "Critical Appraisal of the 1977 Diagnostic Criteria for Minamata Disease," *Archives of Environmental and Occupational Health*. In press.

[18] T. Ninomiya, H. Ohmori, K. Hashimoto, K. Tsuruta, and S. Ekino, "Expansion of methylmercury poisoning outside of Minamata: an epidemiological study on chronic methylmercury poisoning outside of Minamata," *Environmental Research*, vol. 70, no. 1, pp. 47–50, 1995.

[19] T. Fujino, "A clinical and epidemiological study regarding effects of methylmercury exposure in island—second report," *Kumamoto Igakukai Zassi*, vol. 51, pp. 90–147, 1977 (Japanese).

[20] T. Kumamoto, *Reserch Regarding the Effect of Aging on Neurological Disorders*, Japan Public Health Association, Tokyo, Japan, 1993.

[21] M. Nou, *Effects of Aging on Clinical Features of Minamata Disease Patients*, Japan Public Health Association, 1993.

[22] H. Tokuomi, T. Okajima, S. Kawasaki, R. Inoue, and Y. Kinoshita, "Problems of Minamata disease from the perspective of elder's health checkup," *Environmental Health Report*, vol. 37, pp. 149–156, 1976 (Japanese).

[23] National Research Council, *Toxicological Effect of Methylmercury*, National Academy Press, Washington, DC, USA, 2000.

[24] E. M. Yokoo, J. G. Valente, L. Grattan, S. L. Schmidt, I. Platt, and E. K. Silbergeld, "Low level methylmercury exposure affects neuropsychological function in adults," *Environmental Health*, vol. 2, article 1, pp. 1–11, 2003.

[25] T. Tsubaki, K. Hirota, and K. Shirakawa, "Clinical symptoms and hair mercury content on niigata minamata disease," *Environmental Health Report*, vol. 37, pp. 118–122, 1976 (Japanese).

[26] O. Hoshino, K. Tanzawa, M. Terao, and T. Ukita, "Quantitative determination of mercury in hair by activation analysis," *Eiseikagaku*, vol. 12, pp. 94–99, 1966 (Japanese).

Hindawi Publishing Corporation
Journal of Biomedicine and Biotechnology
Volume 2012, Article ID 589569, 8 pages
doi:10.1155/2012/589569

Review Article

Oral Lichenoid Contact Lesions to Mercury and Dental Amalgam—A Review

Helen McParland[1] and Saman Warnakulasuriya[2]

[1] *Oral Medicine Unit, Guy's and St Thomas' NHS Foundation Trust, London SE1 9RT, UK*
[2] *Oral Medicine Unit, Department of Clinical & Diagnostic Sciences, King's College London Dental Institute and WHO Collaborating Centre for Oral Cancer, Bessemer Road, London SE5 9RS, UK*

Correspondence should be addressed to Saman Warnakulasuriya, s.warne@kcl.ac.uk

Received 3 February 2012; Accepted 19 March 2012

Academic Editor: Jose G. Dorea

Human oral mucosa is subjected to many noxious stimuli. One of these substances, in those who have restorations, is dental amalgam which contains mercury. This paper focuses on the local toxic effects of amalgam and mercury from dental restorations. Components of amalgam may, in rare instances, cause local side effects or allergic reactions referred to as oral lichenoid lesions (OLLs). OLLs to amalgams are recognised as hypersensitivity reactions to low-level mercury exposure. The use of patch testing to identify those susceptible from OLL is explored, and recommendations for removing amalgam fillings, when indicated are outlined. We conclude that evidence does not show that exposure to mercury from amalgam restorations poses a serious health risk in humans, except for an exceedingly small number of hypersensitivity reactions that are discussed.

1. Introduction

Human oral mucosa is often subjected to many noxious stimuli, either hot or cold, acidic or alkaline substances, spiced or not so spicy foods. Among substance misusers, the oral mucosa is also in constant contact with tobacco, alcohol, or other substances taken through the mouth. In people with restored teeth, one material that is present in significant amounts is dental amalgam. Mercury is the primary ingredient incorporated during making an amalgam filling. This paper focuses on the local toxic effects of amalgam and mercury from dental restorations with particular reference to oral lichenoid reactions (lesions) as a result of low-level mercury exposure.

2. Dental Amalgam

Dental amalgam is an alloy composed of a mixture of approximately equal parts of liquid mercury and a powder consisting of silver (~22–32%), tin (~14%), copper (~8%), and other trace metals, including zinc [1]. Elemental mercury has been used in clinical dentistry since 1830s when it began to be used in fillings. Dental amalgam now has been used for well over one hundred and eighty years and remains the most commonly placed filling material in the world. Around the 1970s, approximately 22 million amalgam restorations were placed each year in NHS treatment in England and Wales [2], though the numbers have declined recently. On average in a British adult about 7 amalgam restorations can be found. Amalgam use though is declining; the main reason is that dental caries rates among school children and young adults are dropping. Improved alternative filling materials are also now available. A recent US survey, however, showed that 48% of US dentists were using dental amalgam [3]. Few countries like Sweden, Denmark, and Germany have restrictions on dental amalgam use, and Norway has completely banned amalgam. In the rest of the globe amalgam remains popular because it is strong, durable, and relatively inexpensive. It is considered a safe material but despite this concerns over its use remain because of the continuous low-level release of mercury, a substance which is known to be toxic [4–8]. Summaries of some selected research reports concerning amalgam fillings can be found at http://www.yourhealthbase.com/amalgams.html.

Mercury vapour is released during insertion, condensation, and carving of amalgam. The amount of mercury in the restoration can be reduced by 6–10% by good condensation. During later dental interventions such as polishing a restoration may also result in release of further mercury. However, for newer amalgams polishing to improve the surface is no longer required [9], but in older adults with corroded amalgams this may still be necessary. A similar rise in mercury vapour occurs during removal of amalgams but that can be minimised by the use of adequate water coolants and high speed suction [10].

During the functional life of a restoration, chewing during eating can release mercury as a vapour or as a salt dissolved in saliva [11, 12], and studies have shown that the amount released directly correlates to the amount of amalgams present and their total surface area [12]. The daily absorbed dose of mercury from amalgam for the average individual is low, 1.2 μg by inhaled mercury and 1.5 μg by ingested mercury [13]. This represents less than of the daily amount derived from the food and nondental sources and falls below the threshold allowable or safe intake of mercury which has recently been reduced to 0.1 microgram/day per kilogram of body weight [14], but note is made that this EPA recommendation is mainly based on oral exposure to methyl mercury [15]. There are concerns, but no current scientific evidence, that mercury emitted from amalgam fillings may cause or worsen degenerative diseases such as amyotrophic lateral sclerosis (ALS), Alzheimer's disease, multiple sclerosis, and Parkinson's disease. Studies carried out regarding elemental mercury and cancer in humans are inconclusive due to lack of valid exposure data and confounding factors. In summary, the current scientific evidence does not show that exposure to mercury from amalgam restorations poses a serious health risk in humans, except for an exceedingly small number of hypersensitivity reactions [16] that are discussed below.

3. Reactions of Oral Mucosa to Mercury and Amalgam

Holmstrup [17] describes 3 distinct reactions to amalgam fillings in susceptible patients: type IV sensitivity, toxic reactions, and a much rarer phenomenon, acute or generalized sensitivity, the management of which differs considerably. These will be discussed separately below.

4. Delayed or Type IV Hypersensitivity Reactions

The most common reaction to amalgam is the development of oral lichenoid reactions/lesions (OLRs/OLLs) involving mucosae in direct contact with amalgam restorations (Figure 1).

An OLL generally represents a type IV hypersensitivity reaction [18, 19]. Type IV hypersensitivity is often called a delayed type of hypersensitivity as the reaction takes a long period to develop and, in this case, could be months to years. Unlike the other types, it is not antibody mediated

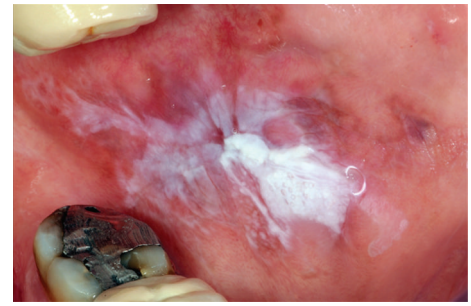


Figure 1: An oral lichenoid lesion or a toxic reaction on buccal mucosa found close to a large amalgam restoration. A positive patch test result to dental materials may confirm this to be an OLL.

but rather is a type of cell-mediated response. Mercury salts that accumulate in healthy and damaged oral mucosa [20] will cause this hypersensitivity reaction in only a susceptible minority of the population with resulting reticular white patches, papules, plaques, erosions, or ulceration, similar to that found in oral lichen planus (OLP)—hence the terminology lichenoid. These lesions can be asymptomatic or sore especially with hot or spicy food. Nonspecific toxic reactions, not as a result of hypersensitivity, can also manifest as OLL.

Hypersensitivity to dental amalgam is rare and according to Holmstrup is due to corrosion products of amalgam restorations, and it seems to be related to mercury in almost all cases, with only a few cases implicating silver, copper, or tin [21, 22].

OLLs of the oral mucosa caused by dental amalgam represent contact allergy. Contact allergies, although a common entity in skin, are relatively rare in oral mucosa [23]. Prolonged intimate contact of the oral mucosa with amalgam fillings over a long period, often many years, appears to be necessary, and in one study the mean age of patients with OLL was 54.6 years [24]. In order for a contact allergic reaction to be established, mercury salts and other metal ions which are leached from amalgam have to penetrate the epithelial lining and bind with host keratinocyte surface proteins. In susceptible individuals this results in a cell-mediated response directed at basal keratinocytes [25–28]. Previous sensitisation of the individual must have occurred. In the case of mercury, dental amalgam may provide the first exposure but it may be also through other sources of mercury including disinfectants, cosmetics, dyes, foods, and vacuum preservatives [29]. It is possible that this is genetically determined by the HLA type but little evidence exists at present.

The pathophysiology of type 1 V hypersensitivity is complex. CD8+ cytotoxic T cells and CD4+ helper T cells recognize the antigen (metal elements in this case) in a complex with either type 1 or 2 major histocompatibility complex. The antigen-presenting cells normally are macrophages that secrete interleukins which stimulate the proliferation of further CD4+ T cells. These activated cells further induce the release of other type 1 cytokines, thus mediating the immune response.

5. Toxic Reactions

Little is known about toxic reactions to irritants such as dental amalgam or its constituents but it is thought they can develop if an irritant substance is in direct contact with the mucosa over several years. Clinically they resemble OLLs (see Figure 1) which are caused by hypersensitivity reactions and can only be differentiated by exclusion based on a negative patch test [30]. Toxic reactions may be more common in amalgams with a higher zinc content [31].

6. Clinical Features and Differential Diagnosis

The lesions of OLL resemble those of oral lichen planus (OLP), and it is therefore necessary to exclude likely OLLs when making a diagnosis of OLP. While some authors do not differentiate the two [32, 33], we believe, along with others [24, 34–37], that the two conditions are distinct. OLP is a more widespread condition involving many anatomical sites within the oral cavity (or elsewhere including skin and genitalia) and distinct from OLL. Both OLP and OLL can be considered potentially malignant [38, 39]. It is important for subsequent management to be able to accurately diagnose each condition.

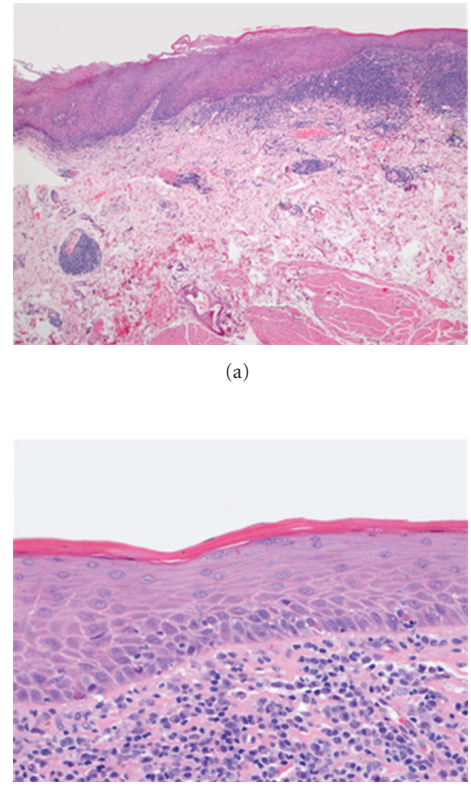
Typically the clinical presentation in both conditions can be reticular white patches, papules, or plaques with or without erosions or ulcerated areas. The clinical diagnosis is further complicated because similar oral lesions can occur as a result of drug-related lichenoid reactions or as graft versus host disease (GVHD), discoid lupus erythematosus (DLE), and systemic lupus erythematosus (SLE). These conditions too have a similar clinical appearance. Diagnosis is facilitated by detailed history, clinical findings, and immunohistological findings. It is beyond this paper to discuss these other conditions.

OLLs caused by hypersensitivity to amalgam or its constituents typically have a clear anatomical relationship to the dental amalgam fillings [24], so they are usually unilateral and not symmetrical. They are most commonly seen on the buccal mucosae and tongue where the covering lining mucosa comes in contact with restorations. The gingivae, palate, or floor of mouth, being sites further away from restorations, are rarely affected, and patients almost never have associated cutaneous symptoms. These clinical features help to distinguish OLL from OLP and other conditions, but it can still be difficult for the clinician to make a clear distinction, if amalgam restorations are widespread in the mouth.

7. Histopathology

It is common practice to biopsy red and white lesions of the mouth to aid diagnosis [40] and to exclude dysplasia [41]. Classical histological features of an OLL are shown in Figure 2.

In 1973 Pinkus [42] coined the term "lichenoid tissue reaction" to describe the histological pattern featuring damage to keratinocytes, now referred to as apoptosis, infiltrate of inflammatory cells in the connective tissue which may extend into the epithelium and keratosis or hyperkeratosis.



(a)

(b)

Figure 2: A biopsy taken from a lichenoid lesion. Photomicrographs show oral mucosa covered by stratified squamous epithelium which is atrophic and orthokeratinized. (a) The superficial lamina propria contains a dense and a well-demarcated lymphoplasmacytic infiltrate. A perivascular lymphoplasmacytic infiltrate also present in the deeper lamina propria. (b) There is thickening of basement membrane region, loss of well-defined basal cell layer, and frequent keratinocyte apoptosis. Photomicrographs; courtesy of Prof Edward Odell.

He described this histopathological pattern common to several diseases referred to above rather than a clinical entity. Since then, further histological features have been identified by Schiodt to distinguish, for example, oral DLE from OLP [43], namely, keratin plugging, atrophy of the rete processes, a deep inflammatory infiltrate, oedema in the lamina propria, and a thick PAS deposit in the basement membrane zone. It has also been suggested that a mixed cell subepithelial infiltrate and a deeper diffuse distribution in lamina propria can help to distinguish a lichenoid lesion [44].

Distinguishing between OLP and OLL remains a challenge. A study carried out by Thornhill et al. [24] confirmed the difficulty of making the distinction between OLL and OLP on purely histological grounds. Overall the pathologists were able to distinguish between the two conditions only a third of the time. Most pathologists report it is either OLP or OLL.

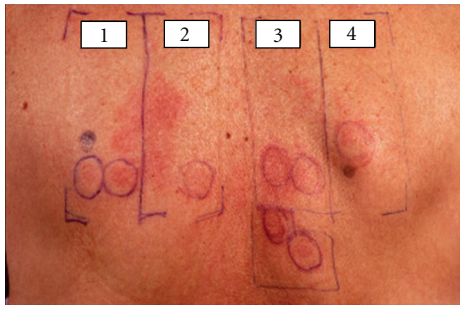


Figure 3: Positive skin reactions in the case illustrated include Row 1: cobalt chloride and balsam of peru, Row 2: nickel sulphate, Row 3: menthol, methyl methacrylate, palladium chloride, amalgam, and Row 4: ammoniated mercury

8. Patch Testing

Patch testing may be useful to identify those patients with suspected hypersensitivity reactions to amalgam or mercury [24, 45, 46]. However, studies investigating their usefulness have shown conflicting results [24]. It is likely that these earlier studies may have failed to clinically distinguish OLP from OLL when ordering patch testing. The test should be carried out in a specialist dermatology or oral medicine centre and is achieved by using commercially available kits which are typically placed on the skin of the back or fore arm in wells and held in place for 48 hours with hypoallergenic adhesive tape. The standard tests take into account that mercury from amalgam restorations may be in the form of metal, organic substances, or organic salts. Few patients react to all three forms. There is no worldwide consensus regarding the allergens used but generally it is accepted that 5% amalgam and 1% ammoniated mercury are suitable for screening [45]. The tests are usually carried out with an European series and includes other dental materials to screen for additional allergens.

The test results are generally read at 48 and 72 hours but evidence has shown that late readings at 10–14 days can capture previously missed positive reactions [34, 47, 48].

A skin reaction with erythema (Figure 3) and effusion with possible papulovesicles (eczema reaction) is considered a positive reaction [46].

Skin testing is preferable to mucosal testing due to a higher sensitivity and specificity and due to the simpler procedure [17]. Furthermore, allergen concentration on mucosa needs to be 5–12 times higher than that to develop skin reactions [49], and toxic reactions may occur at these high concentrations [17].

The routine use of patch testing for all patients with lichen planus like lesions should be avoided as the test itself is time consuming and may provoke sensitisation in the patient [50]. Holmstrup [21] lists the basis of patients requiring allergy testing as

(1) the presence of oral mucosal lesions as lichen planus or mucositis resistant to treatment,

(2) clear anatomical relationship between oral mucosal lesions and the suspected restorative material,

(3) lack of symmetry of affected sites.

The anatomical relationship appears to be the most powerful predictor of an OLL, and studies have shown that 70% of patients who had strong physical relationship of their mucosal lesions to amalgam tested positive to amalgam or ammoniated mercury, contrasting with 3.9% with weakly associated lesions. Patch tests, however, will not be 100% reliable as false positives will arise. 3.2% of the general population appear to be sensitised to dental amalgam or mercury. False negatives will arise for the minority of toxic reactions noted to mercury.

9. Resolution of OLL following Removal of Amalgam Fillings

Although a positive patch test may facilitate diagnosis of OLL caused by a hypersensitivity reaction, this can only be proved if resolution occurs after the offending amalgam has been removed. The resolution or partial resolution of lichenoid lesions following removal of amalgam restorations is illustrated in Figure 4. In one study lesions with direct contact with amalgam responded better when the restoration was removed than those exceeding the contact zone [51]. Some benefit was noted in 97% of such patients regardless of the patch test result but complete healing was seen more often in patients who had a positive patch test [51]. Some authors found a good response to replacement of amalgams in patients with the patch test reactions to mercury salts [20, 34, 36] while others did not [52–54].

Amalgam removal had strongest effect on tongue lesions [51]. In another study relief of symptoms as early as 2-3 days after amalgam removal was found but this could take up to 5 weeks [55] or longer [56].

10. Recommendations for Replacement following Detection of an OLL

(1) OLL should be suspected clinically when

(i) lesions of the buccal mucosa or tongue are unilateral or not symmetrical,

(ii) lesions are in intimate contact with amalgam fillings,

(iii) lesions fail to heal following treatment.

They are unlikely in combination with

(i) cutaneous lesions,

(ii) desquamative gingivitis.

(2) Patch testing may help with diagnosis of OLL and is useful to check for sensitivities to other dental materials if amalgam fillings are to be replaced. We recommend that

(i) patch testing should be carried out in a specialist centre,

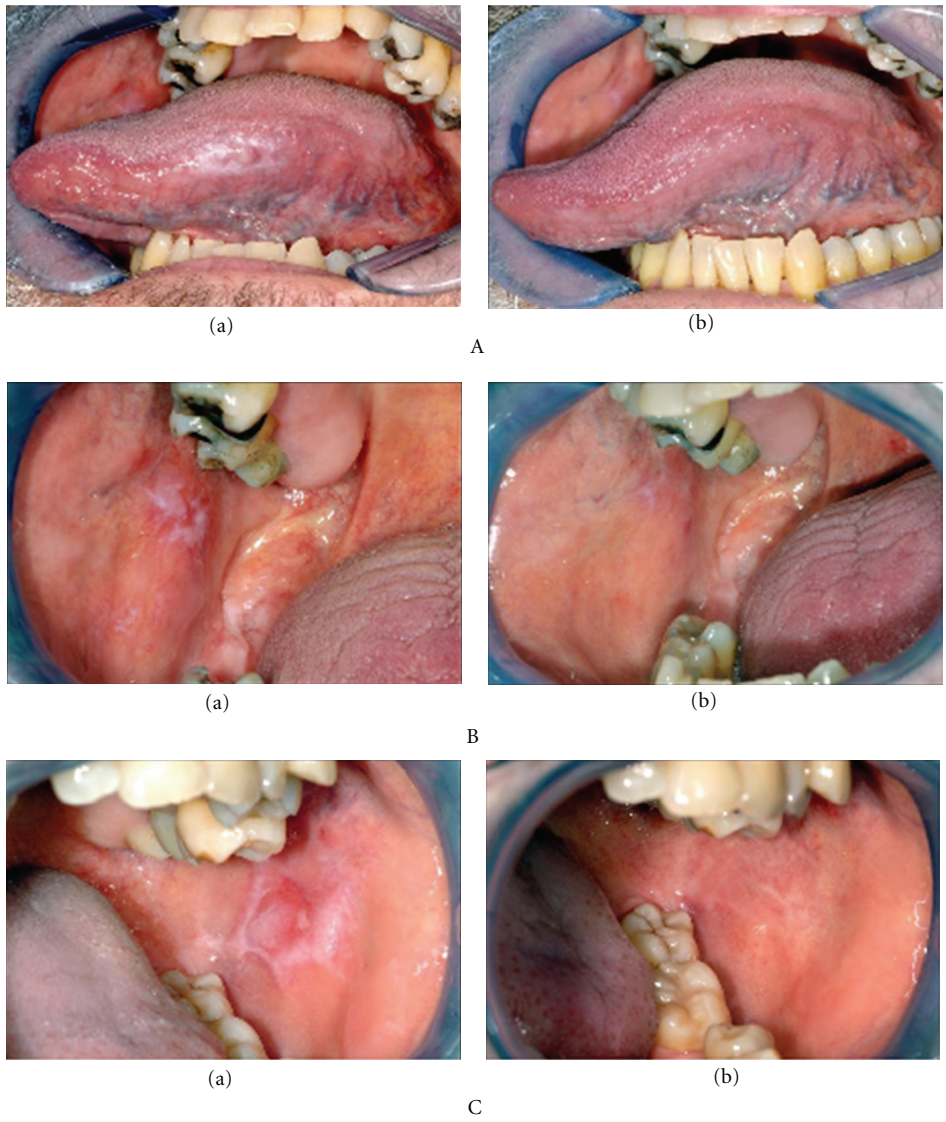


Figure 4: Illustrations showing resolution or partial resolution of lichenoid lesions following removal of amalgam restorations. All were in close contact with an amalgam filling. Panel A at the time of detection and Panel B after removal of the offending amalgam restoration.

(ii) products should be placed on the skin rather than mucosa,

(iii) 5% amalgam, 1% ammoniated mercury are suitable allergens,

(iv) test results are read late, 10–14 days in addition to usual 48 hours.

(3) Following a positive patch test, only amalgams in close contact to OLL should be removed. Placing a rubber dam during replacement is recommended.

(4) If the patch test is negative but there is a strong clinical suspicion of an OLL due to amalgam, either the crown can be covered to avoid amalgam contacting the mucosa or a single test amalgam can be removed to check healing before other amalgams are replaced.

11. Acute or Generalized Sensitivity Reactions

Reports on acute or generalized sensitivity reaction to amalgam or its constituents are rare. One report stated that this could occur in susceptible individuals after inhalation or absorption of mercury vapour, for example, during or directly after placement of an amalgam filling. Symptoms include the development of a cutaneous, erythematous, urticarial rash affecting the face and limbs, usually on the flexural aspect [17]. These reactions are on the same side of the body as the dental intervention. Acute mucosal reactions intraorally are much more rare but may present as vesicles which rupture to form erosions [57]. Symptoms usually appear within hours [57–62] of amalgam placement or removal and typically are self-limiting and resolve over the course of a few days [61].

The nature of these reactions is not fully understood but it is thought they may represent type 1 hypersensitivity reactions [63], and some authors recommend the use of antihistamine therapy for relief of symptoms [60, 64].

Following an acute reaction, unlike with OLL, existing amalgams can remain in place and need only be removed if symptoms persist [44] but alternatives to amalgam should be chosen for future restorations. If amalgam removal is necessitated, the use of rubber dam and high speed suction and copious water coolant is recommended and antihistamine therapy given prior to treatment [56, 60].

For acute reactions, patch testing for amalgam is not recommended as it is not useful in type I sensitivity.

12. Conclusions

Health policy for amalgams can be based on scientific reports from many advisory and regulatory bodies. Based on the guidance given by The Centre for Disease Control (May 2010) [16], at present, there is scant evidence that the health of the vast majority of people with amalgam is compromised nor that removing amalgam fillings has a beneficial effect on health. There is insufficient evidence of a link between dental mercury and health problems [65], except in rare instances of an allergic reaction. The WHO highlights the importance of reporting systems on adverse side effects of dental materials during dental care [66]. This paper highlights the diagnosis and clinical findings of contact allergic reactions to mercury and amalgam. This update should enable dentists to undertake appropriate measures when patients have a suspected or proven contact allergy on their oral mucosa to mercury or other metallic constituents of amalgam.

Acknowledgments

The authors thank Pam Chart and Barbara Burke for secretarial assistance.

References

[1] S. J. Jensen, "Maximum contents of mercury in dental silver amalgams," *Scandinavian Journal of Dental Research*, vol. 93, no. 1, pp. 84–88, 1985.

[2] Office of Health Economics, *Compedium of Health Statistics*, Office of Health Economics, London, UK, 7th edition, 1989.

[3] http://thewealthydentist.com/surveyresults/16_mercuryamalgam_results.htm.

[4] M. Hanson and J. Pleva, "The dental amalgam issue. A review," *Experientia*, vol. 47, no. 1, pp. 9–22, 1991.

[5] ADA Council on Scientific Affairs, "Dental amalgam update on safety concerns," *The Journal of the American Dental Association*, vol. 129, pp. 494–503, 1998.

[6] S. B. Corbin and W. G. Kohn, "The benefits and risks of dental amalgam: current findings reviewed," *The Journal of the American Dental Association*, vol. 125, no. 4, pp. 381–388, 1994.

[7] B. M. Eley, "The future of dental amalgam: a review of the literature Part 6: possible harmful effects of mercury from dental amalgam," *British Dental Journal*, vol. 182, no. 12, pp. 455–459, 1997.

[8] M. Levy, "Dental amalgam: toxicological evaluation and health risk assessment," *Journal of the Canadian Dental Association*, vol. 61, no. 8, pp. 667–671, 1995.

[9] K. O. Frykholm, "On mercury from dental amalgam;its toxic and allergenic effects and some comments on occupational hygiene," *Acta Odontologica Scandinavica*, vol. 15, supplement 22, pp. 1–108, 1957.

[10] J. M. Richards and P. J. Warren, "Mercury vapour released during the removal of old amalgam restorations," *British Dental Journal*, vol. 159, no. 7, pp. 231–232, 1985.

[11] M. J. Vimy and F. L. Lorscheider, "Serial measurements of intra-oral air mercury: estimation of daily dose from dental amalgam," *Journal of Dental Research*, vol. 64, no. 8, pp. 1072–1075, 1985.

[12] A. Berglund, "Estimation by a 24-hour study of the daily dose of intra-oral mercury vapor inhaled after release from dental amalgam," *Journal of Dental Research*, vol. 69, no. 10, pp. 1646–1651, 1990.

[13] J. R. Mackert Jr., "Factors affecting estimation of dental amalgam mercury exposure from measurements of mercury vapor levels in intra-oral and expired air," *Journal of Dental Research*, vol. 66, no. 12, pp. 1775–1780, 1987.

[14] T. W. Clarkson, L. Magos, and G. J. Myers, "The toxicology of mercury—current exposures and clinical manifestations," *New England Journal of Medicine*, vol. 349, no. 18, pp. 1731–1737, 2003.

[15] Environmental Protection Agency, *Reference Dose for Chronic Oral Exposure to Methylmercury*, Integrated Information System, Greenbelt, Md, USA, 2001.

[16] http://www.cdc.gov/oralhealth/publications/factsheets/amalgam.htm.

[17] P. Holmstrup, "Reactions of the oral mucosa related to silver amalgam: a review," *Journal of Oral Pathology and Medicine*, vol. 20, no. 1, pp. 1–7, 1991.

[18] R. A. Cawson and E. W. Odell, Eds., *Cawson's Essentials of Oral Medicine and Pathology*, Churchill Livingston, London, UK, 2008.

[19] M. Jolly, A. J. Moule, R. W. Bryant, and S. Freeman, "Amalgam-related chronic ulceration of oral mucosa," *British Dental Journal*, vol. 160, no. 12, pp. 434–437, 1986.

[20] J. Bolewska, P. Holmstrup, B. Moller-Madsen, B. Kenrad, and G. Danscher, "Amalgam associated mercury accumulations in normal oral mucosa, oralmucosal lesions of lichen planus and contact lesions associated with amalgam," *Journal of Oral Pathology and Medicine*, vol. 19, no. 1, pp. 39–42, 1990.

[21] P. Holmstrup, "Oral mucosa and skin reactions related to amalgam," *Advances in Dental Research*, vol. 6, pp. 120–124, 1992.

[22] J. Banoczy, B. Roed Petersen, J. J. Pindborg, and J. Inovay, "Clinical and histologic studies on electrogalvanically induced oral white lesions," *Oral Surgery Oral Medicine and Oral Pathology*, vol. 48, no. 4, pp. 319–323, 1979.

[23] K. Merritt, *International Workshop: Biocompatibility, Toxicity and Hypersensitivity to Alloy Systems Used in Dentistry*, 1986.

[24] M. H. Thornhill, M. N. Pemberton, R. K. Simmons, and E. D. Theaker, "Amalgam-contact hypersensitivity lesions and oral lichen planus," *Oral Surgery, Oral Medicine, Oral Pathology, Oral Radiology, and Endodontics*, vol. 95, no. 3, pp. 291–299, 2003.

[25] M. H. Thornhill, "Immune mechanisms in oral lichen planus," *Acta Odontologica Scandinavica*, vol. 59, no. 3, pp. 174–177, 2001.

[26] L. R. Eversole, "Immunopathogenesis of oral lichen planus and recurrent aphthous stomatitis," *Seminars in Cutaneous Medicine and Surgery*, vol. 16, no. 4, pp. 284–294, 1997.

[27] S. R. Porter, A. Kirby, I. Olsen, and W. Barnett, "Immunological aspects of dermal and oral lichen planus; a review," *Oral Surgery, Oral Medicine, Oral Pathology, Oral Radiology, and Endodontology*, vol. 83, pp. 358–366, 1997.

[28] P. B. Sugerman, N. W. Savage, L. J. Walsh et al., "The pathogenesis of oral lichen planus," *Critical Reviews in Oral Biology and Medicine*, vol. 13, no. 4, pp. 350–365, 2002.

[29] S. Enestrom and P. Hultman, "Does amalgam affect the immune system? A controversial issue," *International Archives of Allergy and Immunology*, vol. 106, no. 3, pp. 180–203, 1995.

[30] M. Jontell and P. Holmstrup, "Red and white lesions of the oral mucosa," in *Burket's Oral Medicine*, M. S. Greenberg, M. Glick, and J. A. Ship, Eds., pp. 77–78, BC Decker, Hamilton, Canada, 2008.

[31] M. Kaga, N. S. Seale, T. Hanawa, J. L. Ferracane, and T. Okabe, "Cytotoxicity of amalgams," *Journal of Dental Research*, vol. 67, no. 9, pp. 1221–1224, 1988.

[32] I. M. C. Lundstrom, "Allergy and corrosion of dental materials in patients with oral lichen planus," *International Journal of Oral Surgery*, vol. 13, no. 1, pp. 16–24, 1984.

[33] J. A. Yiannias, R. A. El-Azhary, J. H. Hand, S. Y. Pakzad, and R. S. Rogers, "Relevant contact sensitivities in patients with the diagnosis of oral lichen planus," *Journal of the American Academy of Dermatology*, vol. 42, no. 2, pp. 177–182, 2000.

[34] J. Laine, K. Kalimo, and R. P. Happonen, "Contact allergy to dental restorative materials in patients with oral lichenoid lesions," *Contact Dermatitis*, vol. 36, no. 3, pp. 141–146, 1997.

[35] P. O. Ostman, G. Anneroth, and A. Skoglund, "Oral lichen planus lesions in contact with amalgam fillings: a clinical, histologic, and immunohistochemical study," *Scandinavian Journal of Dental Research*, vol. 102, no. 3, pp. 172–179, 1994.

[36] M. Pecegueiro, M. F. Sachse, J. Amaro, P. Farinha, and I. Fonseca, "Oral lichen planus versus oral lichenoid eruption as a manifestation of contact allergy," *Contact Dermatitis*, vol. 40, no. 6, pp. 333–334, 1999.

[37] J. Bratel, M. Hakeberg, and M. Jontell, "Effect of replacement of dental amalgam on oral lichenoid reactions," *Journal of Dentistry*, vol. 24, no. 1-2, pp. 41–45, 1996.

[38] M. D. Mignogna, L. Lo Muzio, L. Lo Russo, S. Fedele, E. Ruoppo, and E. Bucci, "Clinical guidelines in early detection of oral squamous cell carcinoma arising in oral lichen planus: a 5-year experience," *Oral Oncology*, vol. 37, no. 3, pp. 262–267, 2001.

[39] E. H. van der Meij, H. Mast, and I. van der Waal, "The possible premalignant character of oral lichen planus and oral lichenoid lesions: a prospective five-year follow-up study of 192 patients," *Oral Oncology*, vol. 43, no. 8, pp. 742–748, 2007.

[40] R. J. Melrose, "Failure to diagnose pathology: an avoidable complication in oral and maxillofacial surgery," *Oral and Maxillofacial Surgery Clinics of North America*, vol. 23, no. 3, pp. 465–473, 2011.

[41] S. Warnakulasuriya, J. Reibel, J. Bouquot, and E. Dabelsteen, "Oral epithelial dysplasia classification systems: predictive value, utility, weaknesses and scope for improvement," *Journal of Oral Pathology and Medicine*, vol. 37, no. 3, pp. 127–133, 2008.

[42] H. Pinkus, "Lichenoid tissue reactions; a speculative review of the clinical spectrum of epidermal cell damage with special reference to erythema dyschromium pestans," *Archives of Dermatology*, vol. 107, pp. 840–843, 1973.

[43] M. Schiodt, "Oral discoid lupus erythematosus. III. A histopathologic study of sixty-six patients," *Oral Surgery Oral Medicine and Oral Pathology*, vol. 57, no. 3, pp. 281–293, 1984.

[44] L. W. Solomom, T. N. Helm, C. Stevens, M. E. Neidas, and V. Kumar, "Clincal and immunopathiological findings in oral lichen planus pemphigoides," *Oral Surgery, Oral Medicine, Oral Pathology, Oral Radiology & Endodontics*, vol. 103, pp. 808–813, 2007.

[45] S. Fregert, *Manual of Contract Dermatitis*, Copenhagen, Munksgaard, Danmark, 2nd edition, 1981.

[46] D. Ditrichova, S. Kapralova, and M. Tichy, "Oral lichenoid lesions and allergy to dental materials," *Biomedical Papers of the Medical Faculty of the University Palacký, Olomouc Czechoslovakia*, vol. 15, no. 2, pp. 333–339, 2007.

[47] P. Koch and F. A. Bahmer, "Oral lesions and symptoms related to metals used in dental restorations: a clinical, allergological, and histologic study," *Journal of the American Academy of Dermatology*, vol. 41, no. 3, pp. 422–430, 1999.

[48] H. Mobacken, K. Hersle, K. Sloberg, and H. Thilander, "Oral lichen planus: hypersensitivity to dental restoration material," *Contact Dermatitis*, vol. 10, no. 1, pp. 11–15, 1984.

[49] G. Lüders, "Exogen induzierte erkrarkungen der mundschleimhaut," *Zeitschrift für Hautkrankheiten*, vol. 62, pp. 603–612, 1987.

[50] W. G. Shafer, M. K. Hine, and B. M. Levy, *A Textbook of Oral Pathology*, Saunders, Philadelphia, Pa, USA, 4th edition, 1983.

[51] A. Dunsche, I. Kastel, H. Tenheyden, I.N.G. Springer, E. Christopers, and J. Brash, "Oral lichenoid reactions associated with amalgam; improvement after amalgam removal," *British Journal of Dermatology*, vol. 148, pp. 70–76, 2003.

[52] P. O. Ostman, G. Anneroth, and A. Skoglund, "Oral lichen planus lesions in contact with amalgam fillings: a clinical, histologic, and immunohistochemical study," *Scandinavian Journal of Dental Research*, vol. 102, no. 3, pp. 172–179, 1994.

[53] A. Skoglund, "Value of epicutaneous patch testing in patients with oral, mucosal lesions of lichenoid character," *Scandinavian Journal of Dental Research*, vol. 102, no. 4, pp. 216–222, 1994.

[54] K. Kalimo, "Oral lichenoid lesions caused by allergy to mercury in amalgam fillings," *Contact Dermatitis*, vol. 35, no. 1, p. 69, 1996.

[55] E. R. Smart, R. I. Macleod, and C. M. Lawrence, "Resolution of lichen planus following removal of amalgam restorations in patients with proven allergy to mercury salts: a pilot study," *British Dental Journal*, vol. 178, no. 3, pp. 108–112, 1995.

[56] S. H. Ibbotson, E. L. Speight, R. I. Macleod, E. R. Smart, and C. M. Lawrence, "The relevance and effect of amalgam replacement in subjects with oral lichenoid reactions," *British Journal of Dermatology*, vol. 134, no. 3, pp. 420–423, 1996.

[57] L. R. Eversole, "Allergic stomatitides," *Journal of Oral Medicine*, vol. 34, no. 4, pp. 93–102, 1979.

[58] W. A. Wiltshire, M. R. Ferreira, and A. J. Ligthelm, "Allergies to dental materials," *Quintessence International*, vol. 27, no. 8, pp. 513–520, 1996.

[59] J. Thomson and J. A. Russell, "Dermatitis due to mercury following amalgam dental restorations," *British Journal of Dermatology*, vol. 82, no. 3, pp. 292–297, 1970.

[60] F. A. Wright, "Allergic reaction to mercury after dental treatment," *New Zealand Dental Journal*, vol. 67, no. 310, pp. 251–252, 1971.

[61] H. Nakayama, F. Niki, M. Shono, and S. Hada, "Mercury exanthem," *Contact Dermatitis*, vol. 9, no. 5, pp. 411–417, 1983.

[62] A. J. Duxbury, R. D. Ead, S. McMurrough, and D. C. Watts, "Allergy to mercury in dental amalgam," *British Dental Journal*, vol. 152, no. 2, pp. 47–48, 1982.

[63] B. McGivern, M. Pemberton, E. D. Theaker, J. A. Buchanan, and M. H. Thornhill, "Delayed and immediate hypersensitivity reactions associated the use of amalgam," *British Dental Journal*, vol. 188, no. 2, pp. 73–76, 2000.

[64] R. R. White and R. L. Brandt, "Development of mercury hypersensitivity among dental students," *The Journal of the American Dental Association*, vol. 92, no. 6, pp. 1204–1207, 1976.

[65] J. Eyeson, I. House, Y. H. Yang, and K. A. A. S. Warnakulasuriya, "Relationship between mercury levels in blood and urine and complaints of chronic mercury toxicity from amalgam restorations," *British Dental Journal*, vol. 208, no. 4, p. E7, 2010.

[66] World Health Organization, "Future use of materials for dental restoration: report of the meeting convened at WHO HQ," Geneva, Switzerland, prepared by Dr. Poul Erik Petersen, 2009.

Hindawi Publishing Corporation
Journal of Biomedicine and Biotechnology
Volume 2012, Article ID 254582, 12 pages
doi:10.1155/2012/254582

Research Article

Exposure to Low Dose of Cinnabar (a Naturally Occurring Mercuric Sulfide (HgS)) Caused Neurotoxicological Effects in Offspring Mice

Chun-Fa Huang,[1,2] Chuan-Jen Hsu,[3] Shing-Hwa Liu,[2] and Shoei-Yn Lin-Shiau[4]

[1] *School of Chinese Medicine, College of Chinese Medicine, China Medical University, No. 91 Hsueh-Shih Road, Taichung 404, Taiwan*
[2] *Institute of Toxicology, College of Medicine, National Taiwan University, No. 1, Section 1, Jen-Ai Road, Taipei 100, Taiwan*
[3] *Department of Otolaryngology, College of Medicine, National Taiwan University, No. 1, Section 1, Jen-Ai Road, Taipei 100, Taiwan*
[4] *Institute of Pharmacology, College of Medicine, National Taiwan University, No. 1, Section 1, Jen-Ai Road, Taipei 100, Taiwan*

Correspondence should be addressed to Shoei-Yn Lin-Shiau, syshiau@ntu.edu.tw

Received 6 April 2012; Accepted 24 May 2012

Academic Editor: Marcelo Farina

Cinnabar, a naturally occurring mercuric sulfide (HgS), has long been used in Chinese mineral medicine for more than 2000 years. Although mercury is well-known for its toxicity, whether cinnabar induces neurotoxicity, especially in infants and children, is unknown. The purpose of this study was to explore the neurotoxic effects of low-dose of cinnabar (10 mg/kg/day) on developing mice. The results revealed neurobehavioral defects in F1-C-Cin group, which were associated with Hg accumulation, increased NO_x levels in whole blood, and Na^+/K^+-ATPase activities in brain tissues. F1- and F2-Cin-V groups were found to increase brain Hg contents and prominent neurobehavioral defects compared with F1-C-V group, suggesting that the fetal brain was more susceptible to irreversible effects for cinnabar-induced damage. Moreover, F1- and F2-Cin-Cin groups had severely neurobehavioral dysfunctions, closely correlated with the further alteration of NO_x levels and Na^+/K^+-ATPase activities than F1- and F2-C-Cin groups. Effects in F2-Cin-Cin group were more significant than those in F1-Cin-Cin group. In conclusion, this study demonstrates that exposure to low-dose of cinnabar during the perinatal and developmental stages results in irreversible and severe injuries of the neurotoxicity in offspring, and NO_x and Na^+/K^+-ATPase activities may exist potential and useful biomarkers for neurotoxicity-induced by low-doses of mercuric compounds.

1. Introduction

Mercury is a toxic metal that is widespread in the environment and harmful to human and mammalian. The different chemical forms (organic and inorganic) of mercurial compounds exhibit different degrees of toxicity with effects including hearing loss, vision disturbance, motor deficits, and retarded or abnormal walking ability [1, 2]. Cinnabar (an inorganic mercurial compound) contains more than 95% mercury sulfide (HgS) and has been used for many thousands of years in traditional Chinese medicine (TCM) and in Asian and Middle Eastern countries as a sedative and hypnotic [3–6]. Although cinnabar is supposed to have many therapeutic properties and is generally disregarded to result in significant mercury absorption from the gastrointestinal (G-I) tract following oral administration, its high mercury content highlights the need for studies on its possible toxic effects. Various reports have reported that cinnabar can be absorbed following oral administration at high doses and accumulated in the brain and other tissues, causing mercury intoxication [5, 7, 8]. Many studies have reported that the total amount of mercury accumulated in tissues from methylmercuric chloride (MeHgCl) was about 5000-fold higher than that from HgS [9], but that exposure to a high-dose of cinnabar or HgS (1.0 g/kg/day, for 7 or 14 consecutive days) was able to cause neurotoxicity, including dysfunction of the vestibule-ocular reflex (VOR) system, an abnormal of auditory brainstem response, learning memory deficits,

impairment of spontaneous locomotor activity, and suppression of compound muscle action potentials (CMAPs), as has been reported for MeHg [10–12]. Recently, Huang et al. [13] also reported that long-term exposure to low dose of cinnabar (10 mg/kg/day, for more than 77 consecutive days) induced neurotoxicological effects, which were associated with significant Hg accumulation in the brain. Moreover, cinnabar-containing TCMs, such as Ba Paul San, which is used as a sedative and for the management of external infections in infants and children, have been reported to contain excess amounts of cinnabar that cause occasional intoxication in the Chinese population [3, 9, 14]. Therefore, expectant infants and children exposed to cinnabar may cause toxic effects because of overdosage and long-term administration. Nonetheless, available data on the toxicological effects and action mechanisms of cinnabar in children at the perinatal stage exposed to low doses are still insufficient.

Auditory brainstem response (ABR) test, a method to provide a clear and reliable indicator of hearing function in the central auditory system, is a useful indicator of neurotoxicity in body burdens of toxic metals [15, 16]. It has been demonstrated the significant correlation between the abnormal ABR test (the hearing loss and the latency of wave III and V delayed) and Hg accumulation during prenatal, postnatal, development, adult stage exposed to mercury or existed in patients with mercury-contaminated area [2, 17–20]. In experimental animals, the irreversible loss of hearing after exposure to chemicals (such as MeHg or cisplatin) has been reported to be associated with toxicity to the central auditory system, including auditory loss or damage to the cochlea [16, 21, 22]. However, possible ototoxic effects of low dose of cinnabar exposure remain unclear, especially during the perinatal or developmental periods.

On the other hand, it has been shown that chronic mercury intoxication is characterized by inhibition of neuronal Na^+/K^+-ATPase, which is a key enzyme for neurological function [23, 24]. Na^+/K^+-ATPase of the neuronal membranes, which is responsible for the active transport of sodium and potassium ions in the nervous system, plays a critical role in the maintenance of cellular ionic homeostasis and in the physiological function of the inner ear as well as the nervous system [25, 26]. The inactivation of Na^+/K^+-ATPase leads to partial membrane depolarization, which allows excessive Ca^{2+} entry into neurons with resultant toxic events similar to excitotoxicity and has been implicated in pathological and physiological abnormalities and neurodegenerative diseases [26–28]. Recently, the accumulated evidence has revealed that mercurial compounds specifically bind to this enzyme and cause cell or organ dysfunction [10, 21, 22, 29, 30].

Based on these findings, we attempted through this study to clarify the toxicological effects of low dose of cinnabar in offspring that were exposed after weaning, only during the perinatal and weaning stages, or across all these stages. Subsequently, we verified the working hypothesis that long-term exposure to low dose of cinnabar induced neurobehavioral abnormalities and central auditory system dysfunction through interference with Na^+/K^+-ATPase activities. We, therefore, examined a low dosage of cinnabar (10 mg/kg/day) and a longer duration (7 weeks) of oral application in mice and then monitored various neurobehavioral effects (including spontaneous locomotor activities, pentobarbital-induced sleeping time, and motor equilibrium performance) and hearing function (by ABR test, a useful parameter for indicating mercuric compounds-induced oto-toxicity), followed by analyzing changes in the Hg content and Na^+/K^+-ATPase activities of the brain tissues and NO_x levels of whole blood at the end of the treatment.

2. Materials and Methods

2.1. Animal Preparation. Randomly bred, male and female ICR mice were obtained from the Animal Center of the College of Medical, National Taiwan University (Taipei, Taiwan). The protocol was approved by the Institutional Animal Care and Use Committee (IACUC), and the care and use of laboratory animals were conducted in accordance with the guidelines of the Animal Research Committee of College of Medicine, National Taiwan University. Mice were housed seven per cage under standard laboratory conditions at a constant temperature (23 ± 2°C), 50 ± 20% relative humidity, given a solid diet and tap water ad libitum and 12 hrs for light-dark cycles. Mice were acclimatized to the laboratory conditions prior to the experiments, and all experiments were carried out between 8:00 AM and 05:00 PM. The adult male and female ICR mice (4∼5 weeks old, 22–25 g) (breeders of F0 and F1 generations) were randomly assigned to four initial dosing groups and then were orally gavaged distilled water or 10 mg/kg/day cinnabar for 4 consecutive weeks before mating, and then two females were placed per cage with one male breeder for mating. Gestational day 0 (GD 0) was confirmed by the presence of a vaginal plug in the morning. At that time, the female mice with vaginal plugs (dams) were placed into individual cage form GD0 to postnatal day (PND) 21 (lactation period) and then maintained exposure to cinnabar. Thus, the exclusive route of offspring exposure to mercury was through maternal milk [31]. At postnatal day (PND) 0, the offspring newborn mice (pups) were recorded the number in the litter and randomly selected from different litters (three or four per litter) and sacrificed after deep anesthesia by an intraperitoneal injection of pentobarbital (80 mg/kg), and whole blood samples of the mothers were collected to eppendorf from an eyehole vessel after light anesthesia by an intraperitoneal injection of pentobarbital (50 mg/kg). These samples were analyzed Hg contents. At PND 21, offspring (pups) within the original dose group assignment were randomly separated into two groups (seven per cage, total numbers = 12–15/group) and then orally gavaged with distilled water or 10 mg/kg/day cinnabar for 7 consecutive weeks, respectively. Figure 1 illustrates the time course of administration of cinnabar (10 mg/kg/day) to offspring and distilled water exposure during maternal gestation and weaning or following weaning. After the end of experiment, all experimental animals were sacrificed by decapitation under pentobarbital anesthesia (80 mg/kg, i.p.) after administration with the vehicle control or cinnabar fed. Various tissues were quickly removed to liquid nitrogen and

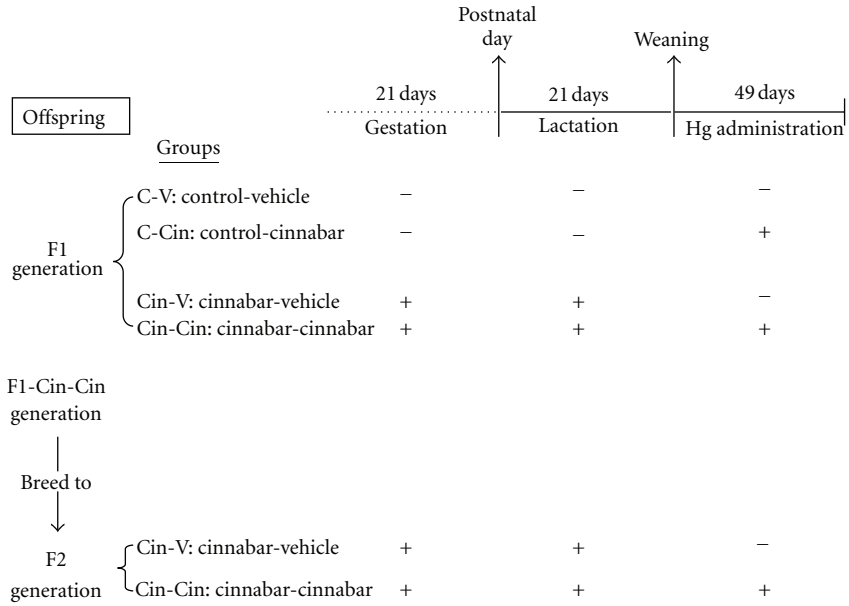


Figure 1: Schedule of oral administration of cinnabar (10 mg/kg/day) to offsprings during maternal gestation, weaning, or after weaning.

stored at −80°C until use. These tissues were analysis of Na^+/K^+-ATPase activities (cerebral cortex, cerebellar cortex and brainstem), nitric oxide levels of whole blood, and mercury content (whole blood, cerebral cortex, cerebellar cortex, and brainstem).

2.2. Determination of Mercury Contents. To determine the Hg concentrations, various tissues (300 mg of whole blood, cerebral cortex, cerebellar cortex and brainstem or offspring newborn mice) or cinnabar sample (100 mg) were placed in a 15 mL polyethylene tube, and 0.4 mL of a 3 : 1 mixture of hydrochloric acid (35%) and nitric acid (70%) was added. The tubes were capped and allowed to stand overnight at 50 degree oven. After cooling, suitable dilution buffer (0.3% nitric acid and 0.1% Triton X-100 in distilled water) was added to the digested material, and the total mercury content was determined by Inductively Coupled Plasma Mass Spectrometry (ICP-MS). The detection limit for mercury was ∼0.1 ppb (μg/L). The cinnabar sample used in this study was composed: 809.3 mg/g of mercury, 1.03 mg/g of sodium, 0.12 mg/g of magnesium, 0.06 mg/g of aluminum, 4.37 mg/g of potassium, 0.73 mg/g of calcium, 1.05 mg/g of barium, 1.05 mg/g of Chromium, 1.62 mg/g of iron, 0.05 mg/g of zinc, 0.04 mg/g of selenium, 0.01 mg/g of lead, 0.01 mg/g of copper, and 0.02 mg/g nickel.

2.3. Spontaneous Locomotor Activity. The mice were orally administered cinnabar by gavage or distilled water once every day for 7 consecutive weeks, and the effects on the spontaneous locomotor activity were measured at end of 7 weeks treatment. In the spontaneous locomotor activity tests, the experiments were performed during the day (9:00–18:00). When the drugs were administered by consecutive oral route, the mice were individually placed in an open field. A large colorless rectangular box with a metallic grid floor was used (70 cm wide, 90 cm long and 60 cm high). The photobeam activity monitors (Tru Scan coulbourn instruments) were used as well as real-time for detecting track-type plots. Overall pulses were recorded in an electromechanical counter as a gross measure of activity. Typical application of X-Y activity recording (floor plane activity) sensory ring drops over the cage and rests on ring support. Movement was detected by 16 × 16 infrared photobeam detectors and transducers set 1.5 cm above the floor of the apparatus and measured by a PC. Finally, the number of squares crossed and the plots of tracking were counted during a period of 30 min for all experiments, and quantification of data was by TruScan 99 software.

2.4. Prolongation of Pentobarbital-Induced Sleeping Time. In order to investigate the neurotoxic effect of cinnabar-induced sleep disorder in offspring mice, prolongation of pentobarbital-induced sleeping time was performed [13, 32]. Briefly, the prolongation of sleeping time was induced by an intraperitoneal injection of pentobarbital (50 mg/kg) and recorded the sleeping time from anesthesia to awakening at end of 7 weeks in the mice with or without cinnabar administration.

2.5. Motor Equilibrium Performance. The motor equilibrium performance on a rotating rod is a more complex motor skill task, which requires both fine motor coordination and precise postural control and is an useful marker for monitoring mercurial compounds-induced neurotoxicity [21, 33, 34]. The effect of cinnabar on motor coordination in the separate groups of the mice was tested using an accelerating rotating rod treadmill (Ugo Basile; Stoelting Co., Chicago, IL). The rotating rod was set in motion at a constant speed (60 rpm),

and the mice were placed into individual sections of rotating rod. Each time an animal fell, it was noted whether the fall had occurred when it sat still or when it walked. The effect of drug administration on motor equilibrium performance was measured once every week.

2.6. Recording of Auditory Brainstem Responses (ABRs). The mice were administered with distilled water or cinnabar once every day for 7 consecutive weeks. ABRs were monitored after administration with cinnabar at end of 7-week treatment. The ABR recording was based on that described by Huang et al. [21, 34]. Briefly, experimental mice were deep anesthetized with an intraperitoneal injection of pentobarbital (50 mg/kg body weight), keeping the body temperature by an electric blanket and recording the brainstem evoked response in a sound attenuated room. Subcutaneous needle electrodes with active electrodes placed in the vertex and ipsilateral retro-auricular region and a ground electrode on the neck of the animal recorded the click-evoked ABR by an auditory evoked potential system (Nicolet, Spirit, Madison, WI, USA). Mice were presented with a stimulus intensity series, which was initiated at 110 dB sound pressure level (SPL) and reached a minimum of −5 dB SPL. The intensity of the stimulus was varied in a 5 dB stepwise decrement. Click stimuli were calibrated with a calibrated B & K precision sound level meter (duration 100 μs, stimulation rate 57.7/s, and frequency from 0 to 150 Hz). ABR threshold was defined as the lowest intensity capable of eliciting replicable and detectable waveforms. The absolute wave and interwave latencies of ABR waveforms were also recorded at a 105 dB SPL signal intensity. ABR was evoked by clicks in this study because the click-elicited ABR is a simplified and effective electrophysiological test to examine the hearing loss induced by mercurial compounds, and its hearing thresholds would correlate with the enzyme activities of the brainstem of the experimental mice.

2.7. Measurement of Nitric Oxide (NO_x) Detection. The quantitative nitric oxide (NO_x) assay was based on that described by Huang et al. [21, 34] and Young et al. [12]. Briefly, the whole blood samples were collected to eppendorf from an eyehole vessel of the experimental animals after light anesthesia by an intraperitoneal injection of pentobarbital (50 mg/kg). To avoid total protein denatured incompletely, we added 95% ethanol into the eppendorf at 4°C overnight (12–16 hrs). Next day, all samples were centrifuged at 4°C for 20 min at 12000 ×g. The supernatants of these samples were collected and assayed by the NO/ozone chemiluminescence (NO Analyzer 280A SIEVERS) for quantitative NO_x levels, which measured the oxidation products (NO_2- and NO_3-) of NO using a reaction vessel containing a reducing system (0.1 M vanadium chloride, Aldrich Co., Germany). Detection of NO_x is then completed by its reaction with ozone, which leads to the emission of red light ($NO + O_3 \rightarrow NO_2^* + O_2$; $NO_2^* \rightarrow NO_2 + h\nu$). Standard curves were made prior to concentration (1, 5, 10, 15, and 20 μM NO), which were prepared using freshly prepared solutions of $NaNO_2$ in distilled water.

2.8. Na^+/K^+-ATPase Activity of Brain Tissue. The brain tissues (cerebral cortex, cerebellar cortex and brainstem) of the vehicle control and cinnabar treated mice were acquired and analyzed for Na^+/K^+-ATPase activity after 7 weeks subsequent cinnabar fed. Membrane Na^+/K^+-ATPase activities were assayed as described previously (Huang et al. [21, 34]). The method allowed for quantification of two distinct Na^+/K^+-ATPase and Mg^{2+}-ATPase activities in the same sample. The enzymatic activities were measured in triplicate in covered 96-well microliter plates at 37 ± 0.5°C on a shaker. Thirty microliters of assay buffer (118 mM NaCl, 1.67 mM KCl, 1.2 mM $MgCl_2$, 12.3 mM $NaHCO_3$, 11 mM glucose, 0.5 mM EGTA, PH: 7.4) containing 2 μg of membrane protein was added to each well. The Na^+/K^+-ATPase activity was determined by subtracting the ouabain (1.25 mM) insensitive Mg^{2+}-ATPase activity from the overall $Na^+/K^+/Mg^{2+}$-ATPase and the assay was started with the addition of 10 μl of ATP (final concentration 5 mM) making the final reaction volume of 100 μl. The reaction was terminated after preincubation at 37 ± 0.5°C by the addition of 200 μl of malachite green (MG) plus ammonium molybdate (AM) (3 : 1). The inorganic phosphate (Pi) released from the substrate ATP was colorimetrically assayed by a microplate ELISA reader (Dynatech MR7000, Ashford, Middesex, UK) at 630 nm. The absorbance values obtained were converted to activity values by linear regression using a standard curve of sodium monobasic phosphate that included in the assay procedure. The specific ATPase activities were expressed as Pi μmole (micromoles inorganic phosphate) released per mg protein per hr. Values reported represent mean ± SE of at least three separate experiments.

2.9. Statistical Analysis. The results in the text are given as mean ± standard errors (SE). The significance of difference was evaluated by the Student's *t*-test. When more than one group was compared with one control, significance was evaluated according to one-way analysis of variance (ANOVA) was used for analysis, and the Duncans's post hoc test was applied to identify group differences. The *P* value less than 0.05 was considered to be significant.

3. Results

3.1. Changes in Litter Number and Levels of Mercury in Maternal Whole Blood and Offspring at Postnatal Day (PND) 1 following Cinnabar Exposure. As shown in Figure 2, the mean of the number of offspring per litter whose dam exposed to low dose of cinnabar (10 mg/kg) was significantly decreased (12.2 ± 0.6 and 10.1 ± 0.6 in F1 and F2 generation, resp.) compared with F1-control group (14.4±0.4). The body weight of the offspring in groups exposed to low dose of cinnabar (10 mg/kg) (F1 and F2 generations) at PND 1 was also significantly lower (1.44 ± 0.02 and 1.45 ± 0.02 g, resp.) than in age-matched control (1.63 ± 0.02 g). Moreover, the Hg content of the cinnabar-exposed maternal (dams) whole blood was 8.1 ± 1.2 and 6.3 ± 0.4 ppb for F0 and F1 dams, respectively, which was significantly higher than that of the control (2.0 ± 0.3 ppb) (Table 1). Likewise, the Hg content

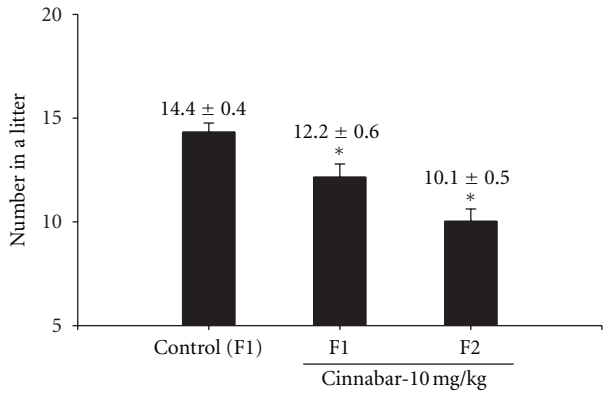


Figure 2: Decrease of litter number induced by cinnabar. Cinnabar (10 mg/kg/day) was treated with the dams by oral gavage during gestation period. Numbers of newborn pups were recorded at postnatal day 1 (PND 1) as described in the section of Materials and Methods. All data are presented as mean ± S.E. (n = 12–15/group). $^*P < 0.05$ as compared with F1 control vehicle (F1-C-V) group.

was markedly higher in the cinnabar-exposed pups at PND 1 than in age-matched control (Table 1).

3.2. Mercury Accumulation in the Brain Tissues of Offspring Mice following Exposure to Cinnabar. To investigate whether the mercury within cinnabar could be absorbed by the G-I tract, pass through the blood-brain-barrier (BBB), and accumulate in the brain, we detected the Hg content of the cerebral cortex, cerebellar cortex, and brainstem by ICP-MS. As shown in Table 2, the Hg contents in the cerebral cortex, cerebellar cortex, and brainstem were slightly increased in F1- and F2-Cin-V groups ($^*P < 0.05$ as compared with F1-C-V group (age-matched control)) and markedly increased in F1-C-Cin group, F1- and F2-Cin-Cin groups, but only the brainstem of F1-C-Cin group significantly accumulated Hg than F1- and F2-Cin-Cin groups. Moreover, the Hg levels of liver and kidney, especially in the kidney, were also significantly accumulated in F1- and F2-Cin-V groups ($^*P < 0.05$ as compared with F1-C-V group), and there were gradually and significantly increased Hg levels in F1- and F2-Cin-Cin groups more than those in F1-C-Cin group ($^{\#}P < 0.05$ as compared with F1-C-Cin group).

3.3. Changes in Body Weight, Neurobehavioral Abnormalities, and Auditory Dysfunction Induced by Low Dose of Cinnabar in Offspring Mice

3.3.1. Altered Body Weight. As shown in Figure 3, the growth of mice in both F1- and F2-Cin-V groups (as assessed by the gain in body weight) was significantly decreased compared with F1-C-V group (decreased by 12.9 ± 1.6% in F1-Cin-V; 8.3 ± 2.2% in F2-Cin-V, resp.). However, further decrease in the continuing exposure to cinnabar for 7 consecutive weeks (decreased by 12.8 ± 2.2% in F1-Cin-Cin group; 10.9 ± 1.4% in F2-Cin-Cin group) was not revealed.

3.3.2. Abnormal Spontaneous Locomotor Activities, Motor Equilibrium Performance, and Prolonged Pentobarbital-Induced Sleeping Time. To investigate neurotoxicity induced by exposure to low dose of cinnabar in offspring, we examined spontaneous locomotor activities (a useful method for detecting the central function of neurotransmission or motor dysfunction on mercury-induced neurotoxicity [21] in cinnabar-treated offspring. As shown in Figures 4(a) and 4(b), F1-C-Cin group was revealed to be hypoactive in terms of its quantitative ambulatory distances and stereotype-1 episodes following exposure to cinnabar ($^*P < 0.05$ as compared with F1-C-V group). However, those parameters were significantly increased (hyperactive) in F1-, F2-Cin-V and F1-, F2-Cin-Cin groups ($^*P < 0.05$ as compared with F1-C-V group; $^{\#}P < 0.05$ as compared with F1-C-Cin group), and only F1-Cin-Cin group showed more severely effects than F1-Cin-V group ($^{\&}P < 0.05$). In the parameter of jump (Figure 4(c)), a marked decrease was observed in F1-C-Cin group ($^*P < 0.05$ as compared with F1-C-V group). F1- and F2-Cin-V groups were found to show slight declines in this parameter, which were higher as in F1- and F2-Cin-Cin groups ($^{\&}P < 0.05$ as compared with F1- or F2-Cin-V group, resp.). Meanwhile, the motor equilibrium performance test of F1-C-Cin and F1- and F2-Cin-Cin groups showed remarkably decreased retention times on the rotating rod ($^*P < 0.05$ as compared with F1-C-V group), with the decrease being especially severe in F2-Cin-Cin group ($^{\&}P < 0.05$ as compared with F2-Cin-V; $^{\#}P < 0.05$ as compared with F1-C-Cin group) (Figure 4(d)).

In addition, the administration of cinnabar for 7 consecutive weeks after weaning (F1-C-Cin group) caused a definite prolongation of pentobarbital-induced sleeping time, which was 33.4 ± 2.4 min compared with 24.4 ± 1.0 min in F1-C-V group ($^*P < 0.05$, Figure 5). However, no significant increase in the pentobarbital-induced sleeping time was observed after exposure to cinnabar during the perinatal and weaning stages (F1- and F2-Cin-C groups). A remarkable prolongation of sleeping time by pentobarbital-induced was, however, revealed in F1- and F2-Cin-Cin groups by continuing exposure to cinnabar for further 7 consecutive weeks ($^*P < 0.05$ as compared with F1-C-V group; $^{\&}P < 0.05$ as compared with F1- or F2-Cin-V group, respectively), and this was even more significant than that in F1-C-Cin group ($^{\#}P < 0.05$).

3.3.3. Auditory Dysfunction. To further understand whether the low dose of cinnabar (10 mg/kg) induced ototoxicity in offspring, the hearing thresholds were determined using the ABR test. As shown in Figure 6, the means of hearing threshold was significantly elevated in F1-C-Cin group (11.67 ± 2.99 dB SPL in F1-C-Cin group as compared with 5.00 ± 1.60 dB SPL in F1-C-V group; $^*P < 0.05$). Both F1- and F2-Cin-V groups also had hearing thresholds (17.25 ± 2.52 and 30.00 ± 3.27 dB SPL respectively, as compared with the F1-C-V group; $^*P < 0.05$), and this effect was found to be more severe following a further 7 consecutive weeks of exposure (23.80 ± 2.02 and 50.63 ± 6.44 dB SPL in the F1-Cin-Cin and F2-Cin-Cin groups, respectively; $^*P < 0.05$ as compared with the F1-C-V group; $^{\&}P < 0.05$ as compared

Table 1: Hg contents of dam's blood and offspring (whole body at PND 1) mice after administration with low dose of cinnabar.

Dam's (whole blood)		Offspring (whole body)	Generation
F0 (Distilled water)	2.0 ± 0.3	1.8 ± 0.1	F1-control
F0 (Cinnabar-10mg/kg/day)	8.1 ± 1.2*	6.3 ± 0.5*	F1-Cinnabar
F1 (Cinnabar-10mg/kg/day)*	6.3 ± 0.4	5.2 ± 0.2*	F2-Cinnabar

At postnatal day 1 (PND 1), the offspring mice (pups) were randomly selected as representatives of their respective litters (four or five per litter). Hg content was expressed as ng/g of wet-weight and presented as mean ± SE.
*$P < 0.05$ as compared with control group.

Table 2: Hg contents of whole blood and brain tissues of offspring mice treated with cinnabar.

Group	Cerebral cortex	Cerebellar cortex	Brainstem	Liver	Kidney
F1-C-V	3.15 ± 0.17	4.35 ± 0.13	7.26 ± 0.31	10.06 ± 1.40	29.40 ± 1.15
F1-C-Cin	17.56 ± 2.10*	22.75 ± 1.66*	50.06 ± 4.66*	30.55 ± 4.96*	99.53 ± 6.04*
F1-Cin-V	8.53 ± 0.08*	8.59 ± 0.48*	13.29 ± 0.20*	14.36 ± 0.98*	43.59 ± 2.95*
F1-Cin-Cin	19.02 ± 2.27*,&	34.62 ± 0.51*,&,#	28.88 ± 2.68*,&,#	52.44 ± 4.58*,&,#	182.63 ± 39.40*,&,#
F2-Cin-V	5.73 ± 1.25*	10.36 ± 3.72*	8.23 ± 1.02	19.12 ± 3.46*	74.14 ± 10.97*
F2-Cin-Cin	13.01 ± 0.97*,&	26.26 ± 2.63*,&	27.56 ± 3.84*,&,#	68.80 ± 5.70*,&,#	369.68 ± 14.61*,&,#

Data of Hg content in various tissues were expressed as ng/g of wet-weight and presented as mean ± SE (n = 12–15/group).
*$P < 0.05$ as compared with F1-C-V group; &$P < 0.05$ as compared with F1- or F2-Cin-V group; #$P < 0.05$ as compared with F1-C-Cin group, respectively.

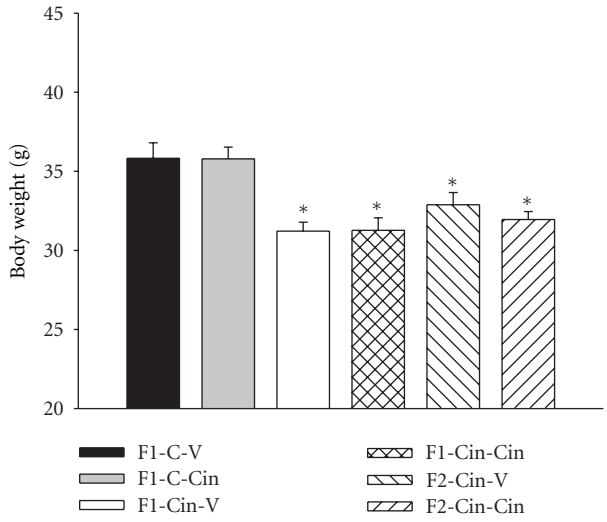


Figure 3: Effects of cinnabar on body weight gain of offspring mice. After weaning, the offspring mice were randomly selected as representatives of their respective litters (n = 12–15/group), and body weights were determined in various experimental groups after 49 consecutively treated days as described in Figure 1. Data are presented as mean ± SE. *$P < 0.05$ as compared with F1-C-V group.

with F1- or F2-Cin-V group, respectively; #$P < 0.05$ as compared with the F1-C-Cin group). Moreover, the extent of the elevation of the hearing thresholds was greater in F2-Cin-Cin group than in F1-Cin-Cin group ($P < 0.05$; Figure 6(a)).

The absolute wave and interwave latencies of ABR were also observed to be significantly delayed by low dose of cinnabar exposed (Figures 6(b) and 6(c)). The mean values of the absolute latency of wave I and III were unaffected as compared with the age-matched control group (F1-C-V group), but those of wave V were increased in all exposed groups (*$P < 0.05$ as compared with F1-C-V group; &$P < 0.05$ as compared with F1- or F2-Cin-V group, respectively), especially in F2-Cin-Cin group (#$P < 0.05$ as compared with F1-C-Cin group). In addition, the interwave latencies of I–V and III–V, but not that of I–III, were significantly increased in all exposed groups. In particularly, the III–V interwave of F2-Cin-Cin group was more markedly increased than that in F1-Cin-Cin group ($P < 0.05$; Figure 6(c)).

3.4. Exposure of Offspring Mice to Low Dose of Cinnabar Caused the Alteration of Nitric Oxide (NO_x) Levels in the Whole Blood and Na^+/K^+-ATPase Activities in the Brain Tissues. To examine whether changes in nitric oxide levels and Na^+/K^+-ATPase activities were involved in low dose of cinnabar-induced neurotoxicity in offspring mice, we first analyzed the nitric oxide (NO_x: nitrate plus nitrite) levels of whole blood by the NO/ozone chemiluminescence. As shown in Figure 7, NO_x levels in the whole blood were significantly increased in F1- and F2-Cin-V groups (28.8±1.5 and 27.4 ± 1.6 μM, respectively; *$P < 0.05$) as compared with F1-C-V group (19.9 ± 0.8 μM) and even more markedly increased in F1-C-Cin and F1-Cin-Cin groups (22.4 ± 0.9 μM and 37.5 ± 0.6 μM, resp.; *$P < 0.05$), particularly in F1-Cin-Cin group (&$P < 0.05$ as compared with F1-Cin-V group; #$P < 0.05$ as compared with F1-C-Cin group). Conversely, there was a decrease the NO_x level in F2-Cin-Cin group, which was more intensively acted as compared with F1-Cin-Cin group (16.4 ± 1.3 mM versus 19.9 ± 0.8 mM; $P < 0.05$; Figure 7).

Next, we detected Na^+/K^+-ATPase enzyme activities of the brain regions in offspring mice exposed to low dose of cinnabar for 7 consecutive weeks. As shown in Figure 8, Na^+/K^+-ATPase activities in F1-C-Cin group were increased

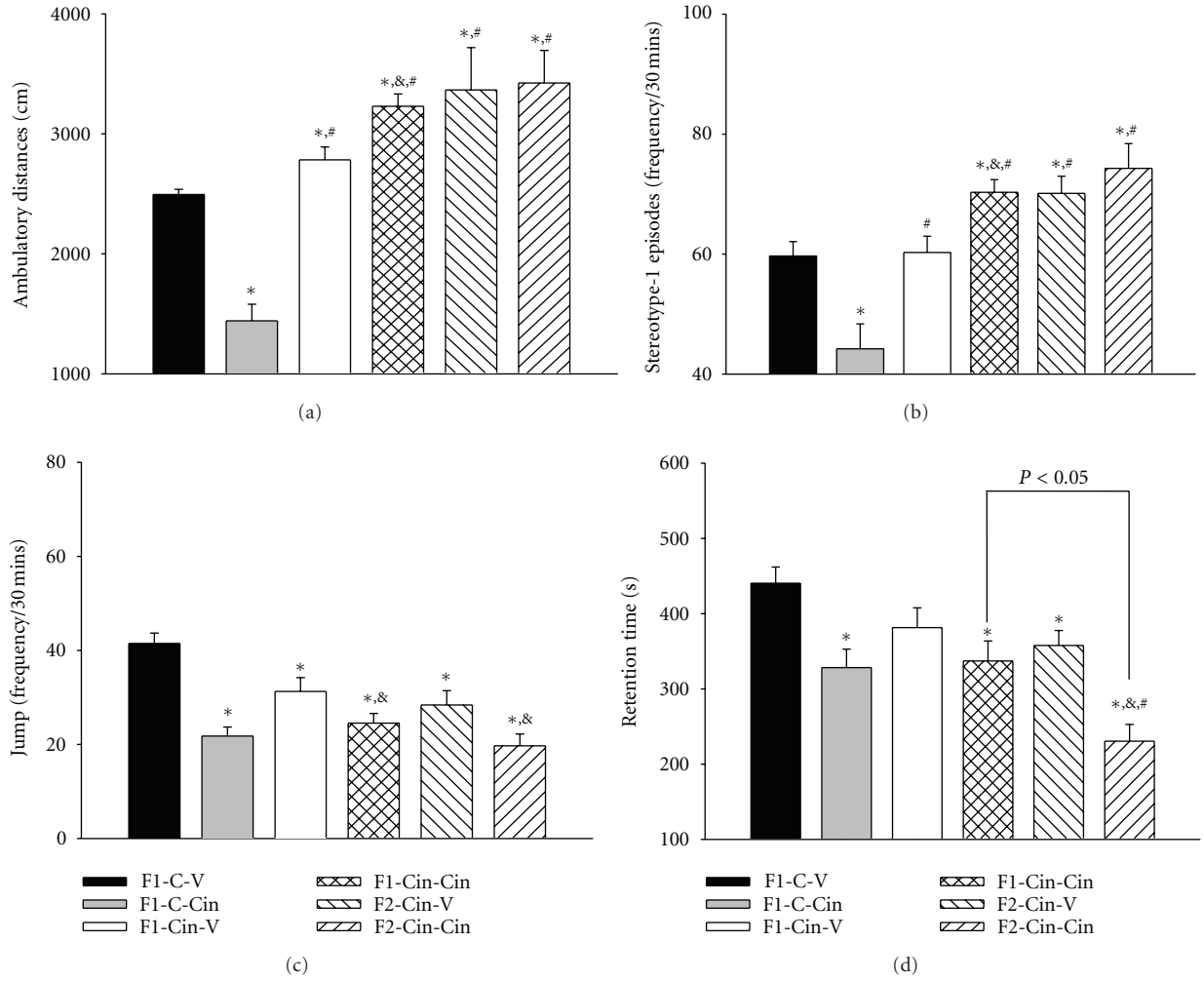


Figure 4: Changes in locomotor activities and motor equilibrium performance of offspring mice treated with cinnabar. Experimental mice were treated with cinnabar (10 mg/kg/day) or distilled water as described in Figure 1. Spontaneous locomotor activities (ambulatory distance (a), stereotypy-1 episodes (b) and Jump (c)) and motor equilibrium performance (retention times on rotating rod, 60 rpm (d)) of all groups were recorded and analyzed as described of Materials and Methods. Data are presented as mean ± SE (n = 12–15/group). $^{*}P < 0.05$ as compared with F1-C-V group; $^{\&}P < 0.05$ as compared with F1- or F2-Cin-V group; $^{\#}P < 0.05$ as compared with F1-C-Cin group, respectively.

in the cerebral and cerebellar cortex ($^{*}P < 0.05$ as compared with F1-C-V group). In F1- and F2-Cin-V groups, Na^{+}/K^{+}-ATPase activities were significantly enhanced in the cerebral and cerebellar cortex of F1-Cin-V group, but unaffected that in F2-Cin-V group. In F1- and F2-Cin-Cin groups, Na^{+}/K^{+}-ATPase activities were altered more intensively with an increase in the cerebral cortex of both groups and a decrease in the cerebellar cortex of the F1-Cin-Cin group and, most notably, an increase in the cerebellar cortex of the F2-Cin-Cin group ($^{\&}P < 0.05$ as compared with F1- or F2-Cin-V group, resp.; $^{\#}P < 0.05$ as compared with F1-C-Cin group). In the brainstem (the main organ of central auditory system), Na^{+}/K^{+}-ATPase activities were increased in F1-C-Cin group and also changed in the mice exposed during the perinatal and weaning stages (decreased in F1-Cin-V group; increased in F2-Cin-V group). In F1- and F2-Cin-Cin groups, there was a gradual but clear increase in Na^{+}/K^{+}-ATPase activities of the brainstem compared with F1-C-Cin group. Furthermore, the changes of Na^{+}/K^{+}-ATPase activities of the brain in F2-Cin-Cin group were more significant than those observed in F1-Cin-Cin group ($P < 0.05$; Figure 8).

4. Discussion

Cinnabar has been used as a TCM for the management of various diseases for more than 2000 years, especially as a tranquilizer for infants or adults, and is still used in Asia countries [3, 5, 8]. The reputed insolubility of cinnabar, or its counterpart HgS, has led to the assumption or disregard that it is not significantly absorbed from the G-I tract following oral administration, and thus it is generally considered to have a low toxicity in vivo. However, many studies have

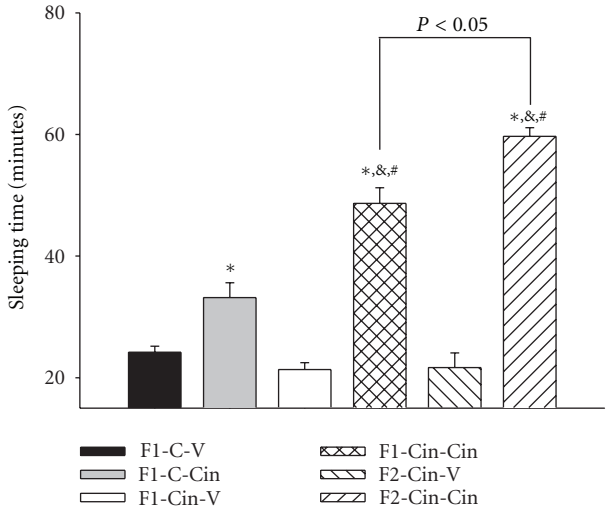


Figure 5: Prolongation of pentobarbital-induced sleeping time by cinnabar in offspring mice. Offspring mice were administered with distilled water or cinnabar (10 mg/kg/day, oral application by gavage) as described in Figure 1. Sleeping time was measured at the end of 49 days after weaning as described in Materials and Methods. Data are presented as mean ± S.E. (n = 12–15/group). $^{*}P < 0.05$ as compared with F1-C-V group; $^{\#}P < 0.05$ as compared with F1- or F2-Cin-V group; $^{\&}P < 0.05$ as compared with F1-C-Cin group, respectively.

found that mercury content of cinnabar, or HgS, can still be significantly absorbed from G-I tract, and transported and accumulated to various tissues after oral administration at the high dose or sufficient levels in the experimental animals to induce neurotoxicity [10–12, 22]. Subsequently, it has been revealed that oral administration of cinnabar (0.01 g/kg/day) for 11 consecutive weeks produced neurobehavioral abnormalities [13]. In addition, many studies have documented that mercury could be transferred to the fetus through the placenta and to newborn offspring through maternal milk, which caused the high level mercury accumulation in brain and the severe deficit of neurobehavioral and learning disability in offspring [1, 31, 33, 35]. Based on these findings, we considered that expectant women, infants, or children might encounter overdoses of cinnabar through long-term therapy with TCM leading to mercury poison. Therefore, it was necessary to clarify whether a low dose (an actual clinical dose of 5–25 mg/kg/day) of cinnabar could be significantly absorbed from G-I tract and exert its toxic effects by interfering with the integrity and functional performance of the central nervous system (CNS) following exposure during the perinatal and/or developmental periods. To investigate the extent of Hg absorbed from G-I tract and the subsequent neurobiological effects of low dose of cinnabar, we first investigated the effects of cinnabar (10 mg/kg/day) in offspring exposed to cinnabar during differential developmental stages. Our results showed that: (1) exposure to low dose of cinnabar from weaning for 7 consecutive weeks (F1-C-Cin group) induced neurotoxic responses (Figures 4, 5, and 6) and (2) exposure to cinnabar during the perinatal and weaning stages, and then continued exposure for a further 7 consecutive weeks after weaning (F1- and F2-Cin-Cin groups) caused more significant abnormalities of spontaneous locomotor activities (hyperactivities), disruption of motor equilibrium performance, prolonged pentobarbital-induced sleeping time, and dysfunction of the auditory system (elevated hearing thresholds and delayed absolute latency of wave V and interwave latencies of I–V, III–V), particularly in F2-Cin-Cin group more severe than F1-Cin-Cin group, which were accompanied by significant Hg accumulation in the brain regains. The cerebrum and cerebellum offered several unique advantages as the controlled motor coordination. If those regions were injuries by toxic insults, it would induce neurobehavioral abnormalities, such as hyperactivities in the ambulatory distances and stereotype-1 episodes of the spontaneous locomotor activities and the disruption of rotarod performance [21, 36]. Due to this, our results not only agree with previous findings that the exposure to low dose mercurial compounds (MeHgCl and/or mercuric chloride ($HgCl_2$)) for 3 to 7 consecutive weeks caused hyperactivity and disruption of motor equilibrium performance and auditory function [21, 34], but also verify that offspring were much more severe and susceptible to mercurial compounds-induced neurotoxicological injuries during the perinatal and/or developmental periods exposed.

Furthermore, results of this study also found that offspring treated with low dose of cinnabar (F1- and F2-Cin-Cin groups) exhibited more abnormal prolonged wave (V) and interwave (I–V and III–V) latencies in the ABR, indicating an abnormality at a late phase of the ABR at the higher center (Figure 6(b) and Figure 6(c)), which was in accordance with the clinical effects of mercury upon the brainstem auditory pathway of children or the occupational workers in the mercury contaminated area with a discernible prolongation of interwave latency I–III and III–V and the higher levels of Hg accumulation [18, 19, 21, 22]. These phenomena might be due to the fact that I–V and III–V interwave latencies relate to the central auditory pathway of the brainstem, and cinnabar (as well as MeHg) could be absorbed and passed through the BBB, accumulate in the brainstem, and cause central neurotoxicity. Moreover, our results also showed that offspring that were exposed to low dose of cinnabar only during the perinatal and weaning stages (in F1- and F2-Cin-V groups) still revealed irreversible neurotoxicological damage (Figures 4 and 6) despite the finding that their brain Hg levels (about 30 ppb) were lower than, or equal to, levels measured in brain tissue from victims of mercury-contaminated areas or experimental animals [37–39]. These findings indicate that exposure to low dose of cinnabar can still be absorbed Hg from the G-I tract, transported to brain regions where they can cause dysfunction of the neurobehavioral abnormalities and auditory system following a continuous long-term exposure regime of more than 7 consecutive weeks. In addition, exposure to cinnabar during perinatal and weaning stages can cause irreversible impairments. It was also observed through this work that the ABR system may provide a sensitive and powerful tool for detecting subclinical central hearing impairment induced by cinnabar.

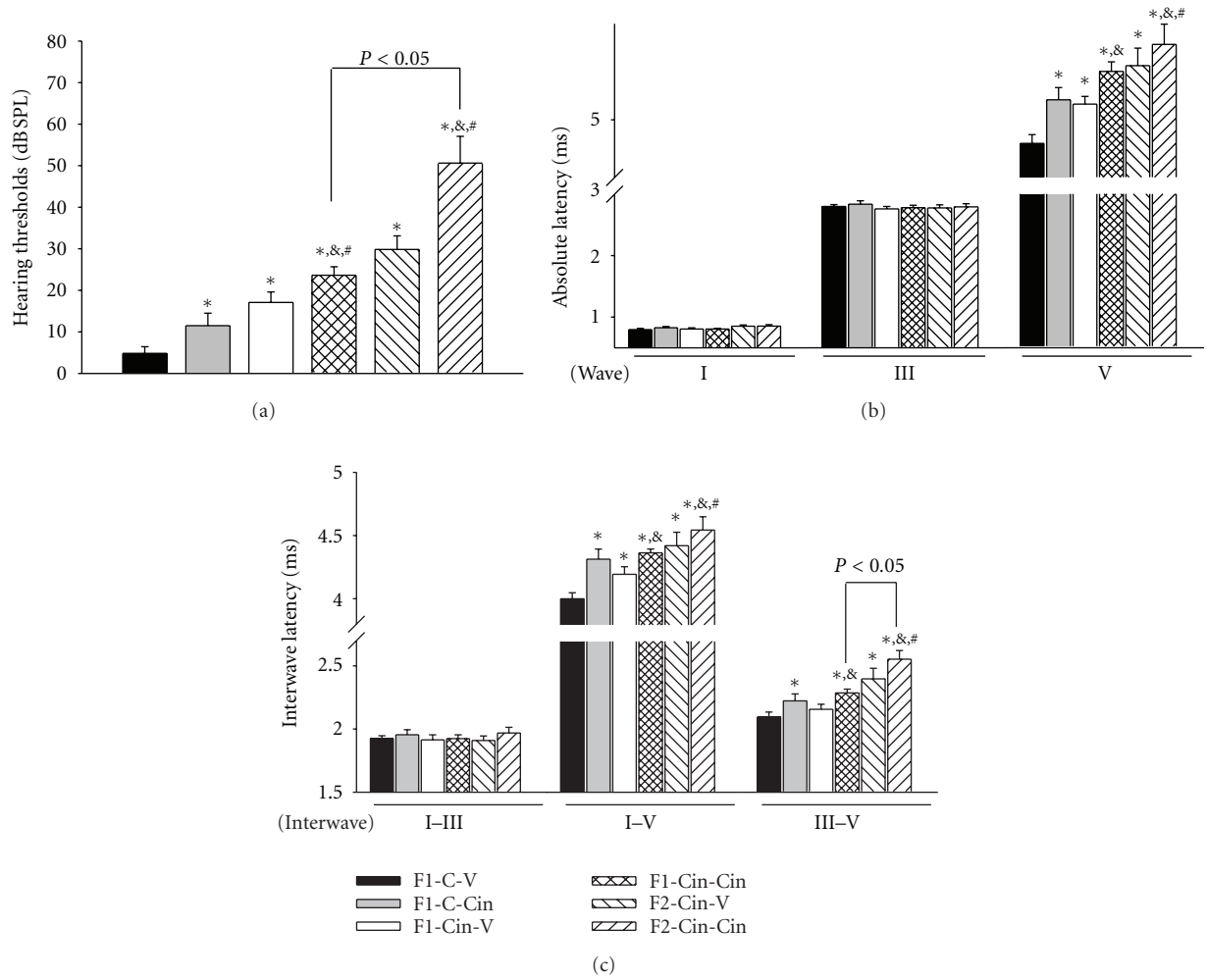


Figure 6: Chronological change of hearing thresholds and the absolute and the interwave latencies of ABRs waveforms in offspring mice treated with cinnabar. Offspring mice were orally gavaged with either cinnabar (10 mg/kg/day) or distilled water as described in Figure 1. The hearing thresholds (a), absolute wave (I, III, and V) (b), and the interwave latencies (I–III, I–V, and III–V) (c) of ABRs waveforms were recorded as described in Materials and Methods. Data are presented as mean ± SE (n = 12–15/group). $^{*}P < 0.05$ as compared with F1-C-V group; $^{\&}P < 0.05$ as compared with F1- or F2-Cin-V group; $^{\#}P < 0.05$ as compared with F1-C-Cin group, respectively.

Arito et al. [32] showed that short-term exposure of adult rat to MeHg (total doses of 10 and 30 mg/kg) led to a marked sleep disorder (an increase in both slow-wave sleep and paradoxical sleep in the dark phase, as well as long-lasting sleep-waking changes) and that was accompanied by high levels of Hg in the brain. In this study, the results showed that offspring exposed to low dose of cinnabar (10 mg/kg/day) for 7 consecutive weeks beginning after weaning (in F1-C-Cin group) had a significantly prolonged pentobarbital-induced sleeping time, and this was even more severe in all experimental stage exposed to low dose of cinnabar, particularly in F2-Cin-Cin group more than F1-Cin-Cin group ($P < 0.05$; Figure 5). These changes were accompanied by notable Hg accumulation in the brain (Table 2). Thus, these results suggest that offspring exposed to low dose of cinnabar during the prenatal and weaning stages and followed by continued exposure for further 7 consecutive weeks can suffer disturbances of the sleep-waking pattern (a severe sleep disorder).

The membrane bound Na^+/K^+-ATPase is essential for the generation or maintenance of basic cellular Na^+ and K^+ ion homeostasis and the functioning of specialized tissues, such as the nervous system. The inhibition of this enzyme could result in membrane depolarization, leading to the suppression of neuronal and excitatory transmission [40, 41]. It has been reported that Na^+/K^+-ATPase activities are very sensitive totoxic agents, which are the significant alteration (increase or inhibition) during mercurial compounds-induced neurological injuries *in vivo* or *in vitro* especially in brain regions and the cochlear lateral wall, which is accompanying with the significant increase in hearing loss [10, 11, 21–23, 42–44]. Furthermore, a recent study has indicated that low dose and long-term exposure to MeHgCl and $HgCl_2$ in offspring during the prenatal,

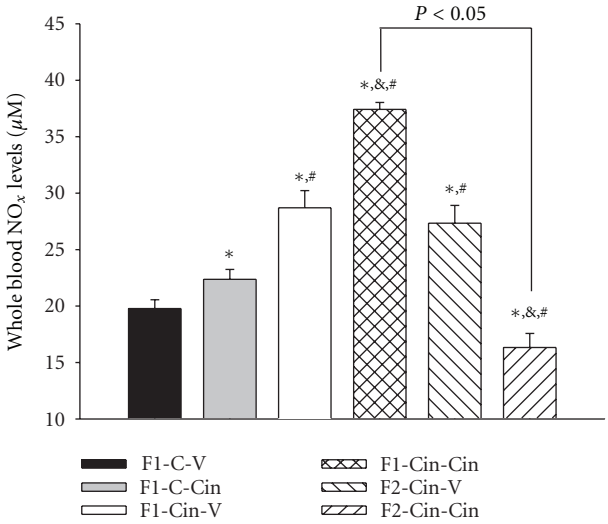


Figure 7: Nitric oxide (NO_x) levels of the whole blood of offspring mice treated with cinnabar. Experimental mice were orally gavaged with cinnabar (10 mg/kg/day) as described in Figure 1. Whole blood samples were acquired, de-proteinized, and NO_x levels were determined as described in Materials and Methods. All data are presented as mean ± SE (n = 12–15/group). $^*P < 0.05$ as compared with compared with F1-C-V group; $^{\&}P < 0.05$ as compared with F1- or F2-Cin-V group; $^{\#}P < 0.05$ as compared with F1-C-Cin group, respectively.

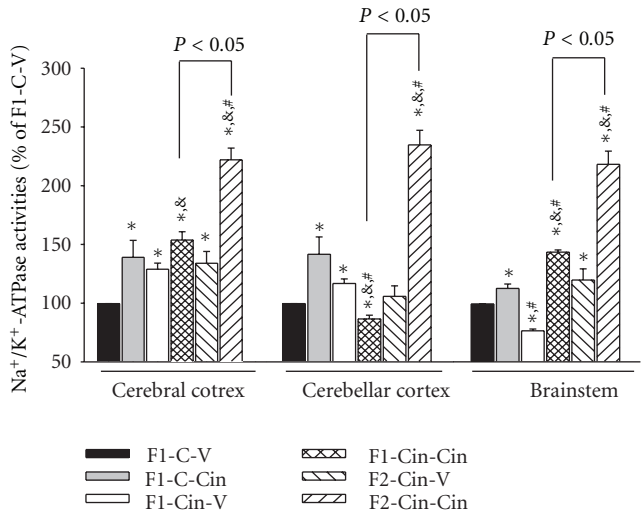


Figure 8: Alteration of Na^+/K^+-ATPase activities in the brain tissues of offspring mice treated with cinnabar. Experimental mice were orally administrated with distilled water or cinnabar (10 mg/kg/day) as described in Figure 1. Na^+/K^+-ATPase activities of the cerebral cortex, cerebellar cortex and brainstem were determined as described in the section of Materials and Methods. All data are presented as mean ± S.E. (n = 12–15/group). $^*P < 0.05$ as compared with F1-C-V group; $^{\&}P < 0.05$ as compared with F1- or F2-Cin-V group; $^{\#}P < 0.05$ as compared with F1-C-Cin group, respectively.

neonatal, and/or postnatal periods cause neurotoxicological effects, which is accompanied with the marked increase Na^+/K^+-ATPase activities in the cerebral cortex, cerebellar cortex, and brainstem [34]. Nevertheless, an important role of Na^+/K^+-ATPase activities in the neurotoxic effects induced by long-term exposure to low dose of cinnabar in differential offspring remained unclear. Moreover, nitric oxide (NO_x) is also an important signaling molecule that not only mediates several physiological functions, including the regulation of neurotransmission, but also regulates many pathological processes [45, 46]. NO_x plays a crucial factor in the regulation of Na^+/K^+-ATPase activities in brain, and less or excess of NO_x production can result in neurotoxicity [47, 48]. Acute exposure to high-dose of toxic metals has been indicated to be capable of inhibiting NO_x levels *in vivo* and *in vitro* [22, 49, 50]. Recently, accumulated evidence has reported that the chronic exposure to toxic insults induces significant alteration of Na^+/K^+-ATPase activities in brain accompanied with the whole blood and/or brain NO_x changes [12, 51, 52] and increased or decreased the whole blood NO_x levels, which closely correlates with the exposure to low dose of mercurial compounds-induced neurotoxicity, has been revealed [13, 21, 34]. Here, our results demonstrated that Na^+/K^+-ATPase activities in the cerebral cortex, cerebellar cortex, and brainstem of offspring were significantly increased after the administration of a low dose of cinnabar for 7 consecutive weeks (the F1-C-Cin group), which were related to the increase of NO_x levels in the whole blood. Furthermore, offspring that were only exposed during the perinatal and weaning stages (F1- and F2-Cin-V groups) had markedly altered Na^+/K^+-ATPase activities in all of the three brain regions tested and elevated NO_x levels in the whole blood, which more severely changed following further exposure to cinnabar for further 7 consecutive weeks (alterant effect: F2-Cin-Cin > F1-Cin-Cin group; $P < 0.05$; Figures 7 and 8). These findings implicate that cinnabar can alter Na^+/K^+-ATPase activities of the brain and NO_x levels of the whole blood, which maybe responsible for inducing the dysfunctions of nervous (abnormalities of locomotor activities and motor equilibrium performance) and central auditory system (elevation of hearing thresholds, and delay of absolute latency and interwave latency). In addition, recent studies have suggested that changes in Na^+/K^+-ATPase activities and/or NO_x levels could be the useful biochemical markers for chemical-induced neuronal injuries or subclinical disease, especially in mercuric compounds-induced neurotoxicity [21, 53, 54]. Based on these suggestions and our findings, we suggest that Na^+/K^+-ATPase activities and NO_x levels appear to serve as an important and useful biochemical marker of lower dose of cinnabar-induced neurotoxicity.

5. Conclusion

In conclusion, our results provide a toxicological basis for cinnabar-induced neurotoxic and ototoxic effects in offspring mice, which may be extrapolated to adults and children exposed to therapeutic dosage in TCM. Changes in NO_x levels and Na^+/K^+-ATPase activities appear to

be the underlying mechanism of the toxicological effects of cinnabar, which may supply an important and useful biomarker in offspring exposure to low dose and long-term mercuric compounds-induced neurotoxicity.

Funding

This study was supported by research grants from Committee on Chinese Pharmacy, Department of Health, Executive Yuan (CCMP92-RD-044, CCMP100-RD-046) and also supported in part by Taiwan Department of Health Clinical Trial and Research Center of Excellence (DOH101-TD-B-111-004).

References

[1] J. R. O'Kusky, B. E. Boyes, and E. G. McGeer, "Methylmercury-induced movement and postural disorders in developing rat: regional analysis of brain catecholamines and indoleamines," *Brain Research*, vol. 439, no. 1-2, pp. 138–146, 1988.

[2] M. Uchino, Y. Tanaka, Y. Ando et al., "Neurologic features of chronic minamata disease (organic mercury poisoning) and incidence of complications with aging," *Journal of Environmental Science and Health—Part B*, vol. 30, no. 5, pp. 699–715, 1995.

[3] B. Tie, W. Qi, and G. Chen, "Determination of soluble mercury contents in Chinese traditional patent medicines for children," *Zhongguo Zhong Yao Za Zhi*, vol. 15, no. 10, pp. 602–639, 1990.

[4] E. O. Espinoza, M. J. Mann, and B. Bleasdell, "Arsenic and mercury in traditional Chinese herbal balls," *The New England Journal of Medicine*, vol. 333, no. 12, pp. 803–804, 1995.

[5] A. D. Hardy, H. H. Sutherland, R. Vaishnav, and M. A. Worthing, "A report on the composition of mercurials used in traditional medicines in Oman," *Journal of Ethnopharmacology*, vol. 49, no. 1, pp. 17–22, 1995.

[6] Pharmacoeia Comittee, *Pharmacopoeia of China*, Chemical Industry Press, Beijing, China, 2005.

[7] E. Ernst and J. Thompson Coon, "Heavy metals in traditional Chinese medicines: a systematic review," *Clinical Pharmacology and Therapeutics*, vol. 70, no. 6, pp. 497–504, 2001.

[8] E. Kang-Yum and S. H. Oransky, "Chinese patent medicine as a potential source of mercury poisoning," *Veterinary and Human Toxicology*, vol. 34, no. 3, pp. 235–238, 1992.

[9] C. C. Yen, S. H. Liu, W. K. Chen, R. H. Lin, and S. Y. Lin-Shiau, "Tissue distribution of different mercurial compounds analyzed by the improved FI-CVAAS," *Journal of Analytical Toxicology*, vol. 26, no. 5, pp. 286–295, 2002.

[10] J. J. Chuu, S. H. Liu, and S. Y. Lin-Shiau, "Effects of methyl mercury, mercuric sulfide and cinnabar on active avoidance responses, Na^+/K^+-ATPase activities and tissue mercury contents in rats," *Proceedings of the National Science Council, Republic of China. Part B*, vol. 25, no. 2, pp. 128–136, 2001.

[11] J. J. Chuu, S. H. Liu, and S. Y. Lin-Shiau, "Differential neurotoxic effects of methylmercury and mercuric sulfide in rats," *Toxicology Letters*, vol. 169, no. 2, pp. 109–120, 2007.

[12] Y. H. Young, J. J. Chuu, S. H. Liu, and S. Y. Lin-Shiau, "Neurotoxic mechanism of cinnabar and mercuric sulfide on the vestibulo-ocular reflex system of guinea pigs," *Toxicological Sciences*, vol. 67, no. 2, pp. 256–263, 2002.

[13] C. F. Huang, S. H. Liu, and S. Y. Lin-Shiau, "Neurotoxicological effects of cinnabar (a Chinese mineral medicine, HgS) in mice," *Toxicology and Applied Pharmacology*, vol. 224, no. 2, pp. 192–201, 2007.

[14] A. H. Liang, Y. J. Xu, and M. F. Shang, "Analysis of adverse effects of cinnabar," *Zhongguo Zhong Yao Za Zhi*, vol. 30, no. 23, pp. 1809–1811, 2005.

[15] S. A. Counter, M. Vahter, G. Laurell, L. H. Buchanan, F. Ortega, and S. Skerfving, "High lead exposure and auditory sensory-neural function in Andean children," *Environmental Health Perspectives*, vol. 105, no. 5, pp. 522–526, 1997.

[16] M. F. Wu, J. R. Ison, J. R. Wecker, and L. W. Lapham, "Cutaneous and auditory function in rats following methyl mercury poisoning," *Toxicology and Applied Pharmacology*, vol. 79, no. 3, pp. 377–388, 1985.

[17] S. A. Counter, L. H. Buchanan, G. Laurell, and F. Ortega, "Blood mercury and auditory neuro-sensory responses in children and adults in the nambija gold mining area of ecuador," *NeuroToxicology*, vol. 19, no. 2, pp. 185–196, 1998.

[18] K. Murata, P. Weihe, S. Araki, E. Budtz-Jorgensen, and P. Grandjean, "Evoked potentials in Faroese children prenatally exposed to methylmercury," *Neurotoxicology and Teratology*, vol. 21, no. 4, pp. 471–472, 1999.

[19] K. Murata, P. Weihe, E. Budtz-Jorgensen, P. J. Jorgensen, and P. Grandjean, "Delayed brainstem auditory evoked potential latencies in 14-year-old children exposed to methylmercury," *The Journal of Pediatrics*, vol. 144, no. 2, pp. 177–183, 2004.

[20] F. E. Musiek and D. P. Hanlon, "Neuroaudiological effects in a case of fatal dimethylmercury poisoning," *Ear and Hearing*, vol. 20, no. 3, pp. 271–275, 1999.

[21] C. F. Huang, C. J. Hsu, S. H. Liu, and S. Y. Lin-Shiau, "Neurotoxicological mechanism of methylmercury induced by low-dose and long-term exposure in mice: oxidative stress and down-regulated Na^+/K^+-ATPase involved," *Toxicology Letters*, vol. 176, no. 3, pp. 188–197, 2008.

[22] J. J. Chuu, C. J. Hsu, and S. Y. Lin-Shiau, "Abnormal auditory brainstem responses for mice treated with mercurial compounds: involvement of excessive nitric oxide," *Toxicology*, vol. 162, no. 1, pp. 11–22, 2001.

[23] I. Hajek, J. Teisinger, and E. Sykova, "The effect of opioids and of naloxone on Na^+,K^+-adenosine triphosphatase activity in frog spinal cord membrane fractions," *Neuroscience Letters*, vol. 59, no. 3, pp. 291–295, 1985.

[24] B. M. Anner and M. Moosmayer, "Mercury inhibits Na-K-ATPase primarily at the cytoplasmic side," *The American Journal of Physiology*, vol. 262, no. 5, pp. F843–F848, 1992.

[25] L. Ji, A. Chauhan, W. T. Brown, and V. Chauhan, "Increased activities of Na^+/K^+-ATPase and Ca^{2+}/Mg^{2+}-ATPase in the frontal cortex and cerebellum of autistic individuals," *Life Sciences*, vol. 85, no. 23-26, pp. 788–793, 2009.

[26] L. V. Karpova, E. R. Bulygina, and A. A. Boldyrev, "Different neuronal Na^+/K^+-ATPase isoforms are involved in diverse signaling pathways," *Cell Biochemistry and Function*, vol. 28, no. 2, pp. 135–141, 2010.

[27] Z. Xie and T. Cai, "Na^+-K^+-ATPase-mediated signal transduction: from protein interaction to cellular function," *Molecular Interventions*, vol. 3, no. 3, pp. 157–168, 2003.

[28] S. P. Yu, "Na^+, K^+-ATPase: the new face of an old player in pathogenesis and apoptotic/hybrid cell death," *Biochemical Pharmacology*, vol. 66, no. 8, pp. 1601–1609, 2003.

[29] S. Bhattacharya, S. Bose, B. Mukhopadhyay et al., "Specific binding of inorganic mercury to Na^+- K^+-ATPase in rat liver plasma membrane and signal transduction," *BioMetals*, vol. 10, no. 3, pp. 157–162, 1997.

[30] X. Wang and J. D. Horisberger, "Mercury binding site on Na^+/K^+-ATPase: a cysteine in the first transmembrane segment," *Molecular Pharmacology*, vol. 50, no. 3, pp. 687–691, 1996.

[31] J. L. Franco, A. Teixeira, F. C. Meotti et al., "Cerebellar thiol status and motor deficit after lactational exposure to methylmercury," *Environmental Research*, vol. 102, no. 1, pp. 22–28, 2006.

[32] H. Arito, N. Hara, and S. Torii, "Effect of methylmercury chloride on sleep-waking rhythms in rats," *Toxicology*, vol. 28, no. 4, pp. 335–345, 1983.

[33] M. Sakamoto, A. Kakita, K. Wakabayashi, H. Takahashi, A. Nakano, and H. Akagi, "Evaluation of changes in methylmercury accumulation in the developing rat brain and its effects: a study with consecutive and moderate dose exposure throughout gestation and lactation periods," *Brain Research*, vol. 949, no. 1-2, pp. 51–59, 2002.

[34] C. F. Huang, S. H. Liu, C. J. Hsu, and S. Y. Lin-Shiau, "Neurotoxicological effects of low-dose methylmercury and mercuric chloride in developing offspring mice," *Toxicology Letters*, vol. 201, no. 3, pp. 196–204, 2011.

[35] S. Goulet, F. Y. Doré, and M. E. Mirault, "Neurobehavioral changes in mice chronically exposed to methylmercury during fetal and early postnatal development," *Neurotoxicology and Teratology*, vol. 25, no. 3, pp. 335–347, 2003.

[36] K. L. Marquis, N. C. Paquette, R. P. Gussio, and J. E. Moreton, "Comparative electroencephalographic and behavioral effects of phenylcyclidine, (+)-SKF-10,047 and MK-801 in rats," *Journal of Pharmacology and Experimental Therapeutics*, vol. 251, no. 3, pp. 1104–1112, 1989.

[37] P. Grandjean, P. Weihe, R. F. White et al., "Cognitive deficit in 7-year-old children with prenatal exposure to methylmercury," *Neurotoxicology and Teratology*, vol. 19, no. 6, pp. 417–428, 1997.

[38] P. Li, X. Feng, and G. Qiu, "Methylmercury exposure and health effects from rice and fish consumption: a review," *International Journal of Environmental Research and Public Health*, vol. 7, no. 6, pp. 2666–2691, 2010.

[39] T. Takeuchi and K. Eto, *The Pathology of Minamata Disease*, Kyushu University Press, Hakozaki, Japan, 1999.

[40] M. Balestrino, J. Young, and P. Aitken, "Block of (Na^+,K^+)ATPase with ouabain induces spreading depression-like depolarization in hippocampal slices," *Brain Research*, vol. 838, no. 1-2, pp. 37–44, 1999.

[41] A. T. S. Wyse, C. S. Bavaresco, E. A. Reis et al., "Training in inhibitory avoidance causes a reduction of Na^+,K^+-ATPase activity in rat hippocampus," *Physiology and Behavior*, vol. 80, no. 4, pp. 475–479, 2004.

[42] J. Albrecht and W. Hilgier, "Similarities of the in vivo and in vitro effects of mercuric chloride on [^{3}H]ouabain binding and potassium activation of Na^+/K^+-ATPase in isolated rat cerebral microvessels," *Toxicology Letters*, vol. 70, no. 3, pp. 331–336, 1994.

[43] G. Szumanska, R. Gadamski, and J. Albrecht, "Changes of the Na/K ATPase activity in the cerebral cortical microvessels of rat after single intraperitoneal administration of mercuric chloride: histochemical demonstration with light and electron microscopy," *Acta Neuropathologica*, vol. 86, no. 1, pp. 65–70, 1993.

[44] P. W. Cheng, S. H. Liu, C. J. Hsu, and S. Y. Lin-Shiau, "Correlation of increased activities of Na^+, K^+-ATPase and Ca^{2+}-ATPase with the reversal of cisplatin ototoxicity induced by D-methionine in guinea pigs," *Hearing Research*, vol. 205, no. 1-2, pp. 102–109, 2005.

[45] S. Moncada and E. A. Higgs, "Endogenous nitric oxide: physiology, pathology and clinical relevance," *European Journal of Clinical Investigation*, vol. 21, no. 4, pp. 361–374, 1991.

[46] A. G. Estévez and J. Jordán, "Nitric oxide and superoxide, a deadly cocktail," *Annals of the New York Academy of Sciences*, vol. 962, pp. 207–211, 2002.

[47] N. F. Avrova, K. I. Shestak, I. O. Zakharova, T. V. Sokolova, and V. G. Leont'ev, "The difference in the effect of glutamate and NO synthase inhibitor on free calcium concentration and Na^+,K^+-ATPase activity in synaptosomes from various brain regions," *Neurochemical Research*, vol. 24, no. 9, pp. 1101–1106, 1999.

[48] A. Conti, M. Miscusi, S. Cardali et al., "Nitric oxide in the injured spinal cord: synthases cross-talk, oxidative stress and inflammation," *Brain Research Reviews*, vol. 54, no. 1, pp. 205–218, 2007.

[49] C. K. Mittal, W. B. Harrell, and C. S. Mehta, "Interaction of heavy metal toxicants with brain constitutive nitric oxide synthase," *Molecular and Cellular Biochemistry*, vol. 149-150, pp. 263–265, 1995.

[50] H. Yanagisawa, M. Nodera, M. Sato, K. Moridaira, G. Sato, and O. Wada, "Decreased expression of brain nitric oxide synthase in macula densa cells and glomerular epithelial cells of rats with mercury chloride-induced acute renal failure," *Toxicology and Applied Pharmacology*, vol. 184, no. 3, pp. 165–171, 2002.

[51] C. F. Huang, R. S. Yang, S. H. Liu, P. C. Hsieh, and S. Y. Lin-Shiau, "Evidence for improved neuropharmacological efficacy and decreased neurotoxicity in mice with traditional processing of Rhizoma Arisaematis," *The American Journal of Chinese Medicine*, vol. 39, no. 5, pp. 981–998, 2011.

[52] H. Zha, Z. Cheng, J. Chen et al., "The toxicological effects in brain of mice following exposure to cerium chloride," *Biological Trace Element Research*, vol. 144, no. 1–3, pp. 872–884, 2011.

[53] G. Nicolini, S. Balzan, R. Colzani, M. Scarlattini, M. C. Taddei, and G. Iervasi, "Erythrocyte Na^+/K^+-ATPase is increased in subjects with subclinical hypothyroidism," *Clinical Endocrinology*, vol. 60, no. 6, pp. 705–710, 2004.

[54] D. Pałecz, R. Komuński, and T. Gabryelak, "Na^+K^+-ATPase activity as a biomarker of toxaphene toxicity in *Unio tumidus*," *Toxicology in Vitro*, vol. 19, no. 5, pp. 707–712, 2005.

Hindawi Publishing Corporation
Journal of Biomedicine and Biotechnology
Volume 2012, Article ID 359879, 9 pages
doi:10.1155/2012/359879

Research Article

Biomarkers of Adverse Response to Mercury: Histopathology versus Thioredoxin Reductase Activity

Vasco Branco,[1,2] Paula Ramos,[3] João Canário,[2] Jun Lu,[4] Arne Holmgren,[4] and Cristina Carvalho[1]

[1] *Research Institute for Medicines and Pharmaceutical Sciences (iMed.UL), Faculty of Pharmacy, University of Lisbon, Avenue Professor Gama Pinto, 1649-003 Lisbon, Portugal*
[2] *Marine Environment and Biodiversity Unit, Institute for Sea and Atmospheric Research (IPIMAR/IPMA), Avenue Brasília, 1440-006 Lisbon, Portugal*
[3] *Aquaculture Unit, Institute for Sea and Atmospheric Research (IPIMAR/IPMA), Avenue Brasília, 1440-006 Lisbon, Portugal*
[4] *Department of Medical Biochemistry and Biophysics, Karolinska Institutet, 17177 Stockholm, Sweden*

Correspondence should be addressed to Cristina Carvalho, cristina.carvalho@ff.ul.pt

Received 20 April 2012; Accepted 25 May 2012

Academic Editor: João B.T. Rocha

Exposure to mercury is normally assessed by measuring its accumulation in hair, blood or urine. Currently, the biomarkers of effect that have been proposed for mercurials, such as coproporphyrines or oxidative stress markers, are not sensitive enough and lack specificity. Selenium and selenoproteins are important targets for mercury and thioredoxin reductase (TrxR) in particular was shown to be very sensitive to mercury compounds both *in vitro* and *in vivo*. In this study we looked into the relation between the inhibition of thioredoxin reductase (TrxR) activity and histopathological changes caused by exposure to mercurials. Juvenile zeabra-seabreams were exposed to Hg^{2+} or MeHg for 28 days and histopathological changes were analyzed in the liver and kidney as well as TrxR activity. Both mercurials caused histopathological changes in liver and kidney, albeit Hg^{2+} caused more extensive and severe lesions. Likewise, both mercurials decreased TrxR activity, being Hg^{2+} a stronger inhibitor. Co-exposure to Hg^{2+} and Se fully prevented TrxR inhibition in the liver and reduced the severity of lesions in the organ. These results show that upon exposure to mercurials, histopathological alterations correlate with the level of TrxR activity and point to the potential use of this enzyme as a biomarker of mercury toxicity.

1. Introduction

Adverse health effects of mercury include neurotoxicity, nephrotoxicity, cardiotoxicity, teratogenicity and immunotoxicity. However, the molecular mechanisms underlying mercury toxicity remain unclear with detrimental consequences on the development and validation of appropriate biomarkers of predictive toxic effects. Neurotoxic symptoms are the most visible aspect of mercury poisoning [1]. Nevertheless, the liver and kidney also accumulate high amounts of mercurials [2–4] that may impair their regular functioning. As happens with most xenobiotics, mercury compounds are mainly metabolized in the liver, where demethylathion [5, 6] and conjugation with Se [7, 8] or glutathione can occur [1]. In the liver of animals exposed to high levels of mercurials, hepatocytes are frequently hypertrophied with large-size vacuoles and widespread areas of necrosis can often be observed [3, 9]. The proximal tubule is the kidney structure most affected by mercurials; the cellular changes include swelling of the mitochondrial matrix and endoplasmatic reticulum, loss of membrane integrity and eventual cellular necrosis [10]. Nephrotoxicity caused by to Hg^{2+} accumulation, is well recognized but may also arise from MeHg exposure [10, 11].

In risk assessment, biomarkers are important tools to assess the exposure, effect or susceptibility of individuals to a given xenobiotic. In the case of mercury compounds, only the use of biomarkers of exposure, such as the determination of mercury levels in hair [12, 13], blood [14, 15] and urine [16, 17] is generalized. However, the correlation between

symptoms and Hg levels in hair, blood or urine is not always evident due to inter-individual variability in susceptibility to mercury [17] and to the delayed onset of effects that characterizes mercury poisoning [1]. Mercury toxic effects are frequently evaluated in humans and animals by conducting psychological and motor tests to assess the degree of neurological damage [17–19] although, changes in mental and motor skills might signify that mercury impairment of biological functions is already established. Therefore, the imposing challenge is still to identify a biomarker predictive of effect for mercury. Coproporphyrines levels and their excretion pattern in urine were proposed to evaluate early effects of mercurials [20, 21], but they have not proven to be a sensitive and useful indicator of mercury toxicity [19] and its use has been quite limited.

The affinity of mercury to thiol groups (–SH) makes peptides and proteins vulnerable to its presence, especially when sulfhydryl groups are in the active site of enzymes. Changes in the activity of several enzymes involved in antioxidant action, such as glutathione reductase (GR) [22], superoxide dismutase (SOD) [23], and catalase (CAT) [24] are indicative of mercury induced oxidative stress. Glutathione depletion [25, 26], resulting from complexation with mercurials is also a good but unspecific indicator of mercury effects. Metallothionine induction [27] is a biochemical change that has been previously related to mercury exposure. Nevertheless, these changes are not specific and do not allow to distinguish mercury related effects in a multi-contaminant context.

Selenols (–SeH) have a lower pKa than thiols (5.3 versus 8.5) and under physiological conditions are fully ionized to selenolates ($–Se^-$) and thus are more reactive and can easily interact with mercury [28]. Selenoenzymes such as glutathione peroxidases (GPxs) are good targets for mercury [29–32] but, recently [28], the involvement of the thioredoxin system-comprising thioredoxin (Trx), the selenoenzyme thioredoxin reductase (TrxR) and NADPH-on the molecular mechanism of mercury toxicity was proven. The inhibitory effects of mercurials on the thioredoxin system have been shown both *in vitro* [28, 33] and *in vivo* [4, 34, 35]. Thioredoxin reductase is particularly sensitive to mercurials which results from its highly nucleophylic structure. Reduced TrxR has two active sites in each homodimer that include a dithiol in the FAD domain and a selenolthiol in the interface domain [34, 36]. By contrast, the homologous enzyme GR, which differs from TrxR by lacking Se in the C-terminal active site, is not inhibited in the presence of mercury compounds [4, 28]. Given the importance of the TrxR and the thioredoxin system to several cellular functions such as protein repair and regulation of the cellular cycle [36], we hypothesize that TrxR inhibition might be a key mechanism by which mercury toxicity develops. Thus, this work investigates the incidence of histopathological changes in the liver and kidney of zeabra-seabreams and its correlation with the decrease in TrxR activity caused by MeHg or Hg^{2+} exposure. The influence of Se co-exposure and post-exposure treatment on enzyme activity and on the alterations observed is also discussed.

2. Materials and Methods

2.1. In Vivo Assays. Zeabra-seabreams (*Diplodus cervinus*), were used as a model. This species is easy to handle in captivity and, since the organs of fishes are similar to those of mammals, they constitute a good alternative to rodents as a model [37]. A total of 63 fishes were divided into 6 experimental groups: control (C; $n = 12$); selenium (Se, provided as sodium selenite; $n = 9$); exposure to Hg^{2+} (HgII; $n = 12$); exposure to MeHg (MeHg; $n = 12$); co-exposure to Hg^{2+} and Se (HgSe; $n = 12$); co-exposure to MeHg and Se (MeHgSe; $n = 12$). Juvenile zeabra-seabreams were kept in tanks at a density of 2.5 g of fish per liter. Oxygen saturation, in water was kept close to 100% and ammonia and pH were kept within normal limits. Exposure concentrations were set at $2\,\mu g\,L^{-1}$ for both Hg^{2+} and MeHg and at $10\,\mu g\,L^{-1}$ for Se. Exposure lasted 28 days and was followed by 14 days of depuration. During the depuration, fishes from HgII and MeHg groups were divided into two sub-groups, one kept in clean water and the other one kept in water supplemented with Se (HgRSe and MeHgRSe). The experiment was described in detail in Branco et al. [34].

2.2. Organ Collection. Sampling of fish took place at days 14, 28 and 42 (hereafter referred as d14, d28 and d42). Three fishes were taken from each group at each sampling day. The liver and kidney were collected and rinsed with 0.9% NaCl. Sub-samples of these organs (were immediately fixed in 10% neutral buffered formalin (diluted in 10% salt water) for histopathological observation and the remaining organs frozen at $-80^\circ C$ until analysis.

2.3. Histopathology Analysis. Following fixation in 10% neutral buffered formalin, samples were washed with distilled water and dehydrated in a progressive series of ethanol, embedded in paraffin and then cut into $3\,\mu m$ thick sections further stained with Harris haematoxylin (Merck) and counterstained with Eosin (Merck) according to the standard method described by Lillie and Fullmer [39]. Histopathological observations were carried out by using a Olympus BX 51light microscope linked to a digital camera (Olympus DP-20).

The Organ Damage Index (ODI) was calculated for each exposure group at d28 and d42 according to the formula:

$$\mathrm{ODI} = \sum_{n=1}^{4} \mathrm{Pi} \times N_{\mathrm{OL}}, \tag{1}$$

where Pi is the pathological importance factor following the proposal of Bernet et al. [38] for a given organ lesion (OL) observed in the number of fishes (N) tested at each time-point.

2.4. Total Protein Determination. Organ samples were homogenized with a glass mortar and Teflon pestle in TE buffer (pH 7.5) containing a protease inhibitor cocktail (Roche), followed by centrifugation for 7 min at 12,000 rpm and $4^\circ C$. The pellet was discarded and supernatants used for enzyme activity assays. The total amount of protein in samples

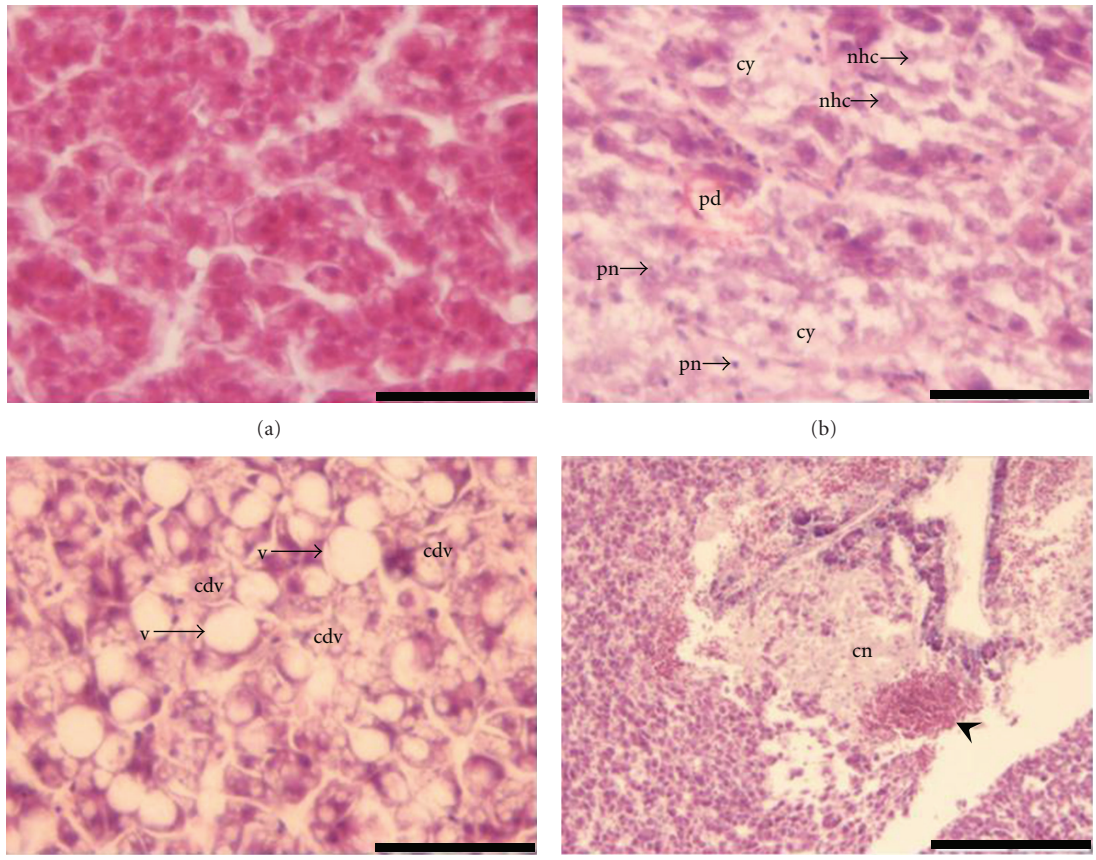


Figure 1: Histopathological observations in the liver of *Diplodus cervinus* after 28 days of exposure. (a) control group: section of polygonal hepatocytes cords (bar = 50 μm); (b) exposure to Hg^{2+}: extensive necrosis with congestion of sinusoids and pigment deposition (pd), nuclear hypercromatose (nhc), picnotic nucleus (pn) and cytolysis (cy) (bar = 50 μm); (c) exposure to MeHg: extensive cytoplasmic degenerative vacuolization (cdv) with large, smooth-edged vacuoles (v) (bar = 50 μm); (d) co-exposure to MeHg and Se: focal coagulative necrosis (cn) associated to blood congestion (arrowhead) (bar = 200 μm).

was determined in the supernatant fraction by measuring absorption at 595 nm in a microplate reader, according to Bradford [40], using Coomassie Brilliant Blue G-250 dye (Bio-Rad). Concentration of protein was quantified by using a calibration curve prepared by sequential dilution of a BSA standard solution.

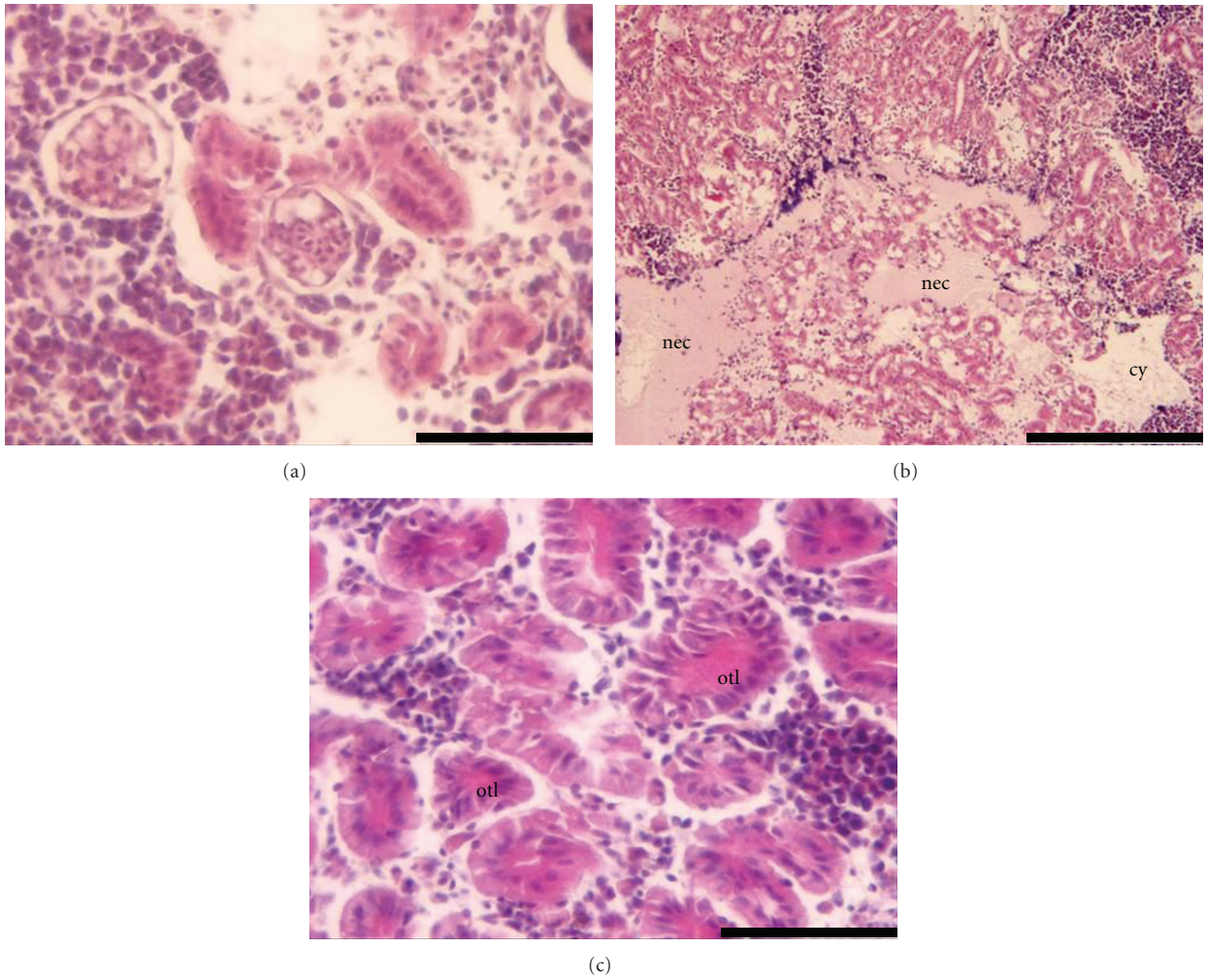
2.5. Thioredoxin Reductase Activity. The activity of TrxR was determined using the insulin reduction endpoint assay proposed by Arnér and Holmgren [41]. Samples were incubated with TE buffer, fully reduced human Trx (3 μM; IMCO Corp. Sweden), insulin (0.3 mM), NADPH (2.5 mM), EDTA (2.5 mM) and HEPES (85 mM; pH 7.6) for 20 min at room temperature. Control wells containing the same mixture but without added Trx were simultaneously prepared. After incubation, the reaction was stopped by adding 250 μL of a 1 mM DTNB solution in 6 M guanidine·HCl and absorbance at 412 nm was measured in a microplate reader.

2.6. Glutathione Reductase Activity. For GR activity, supernatants were incubated in 96-well plates with phosphate buffer (100 mM; pH 7.0), NADPH (1 mM), and GSSG (200 μM). The reaction was monitored for 5 min at 30°C in a microplate reader and the decrease in absorption at 340 nm, resulting from NADPH oxidation was registered [42].

2.7. Statistical Analysis. Differences between groups were assessed by computing the Mann-Whitney test for independent samples. Differences were considered significant at a P value below 0.05 [43].

3. Results and Discussion

3.1. Liver Histopathology. Fish dissection showed that the liver of fishes from MeHg and HgII groups had softer consistency, when compared with the C group. No lesions or alterations were observed in the liver of controls (Figure 1(a)). The liver of fishes exposed for 28 days to MeHg and Hg^{2+} showed signs of hepatocyte alterations, namely, degenerative cytoplasm alterations, architectural pattern changes, loss of typical polygonal cell shape and undefined cell limits. Additionally, vacuolar degeneration with lateral migration of the



(a) (b)

(c)

Figure 2: Histopathological observations in the kidney of *Diplodus cervinus* after 28 days of exposure to Hg^{2+}. (a) control group: posterior kidney section (bar = 50 μm); (b) exposure to Hg^{2+}: posterior kidney section showing a massive necrosis area (nec) and cytolysis (cy) (bar = 200 μm). (c) exposure to Hg^{2+}: occlusion of the tubular lumen (otl) (bar = 50 μm).

nuclei, hydropic degeneration (Figure 1(b)), vacuolization within the hepatocytes with lipid-type vacuoles, which can be infiltrated fats, and appearance of some typical globular bodies may result from an increase in the lipid, water and/or glycogen content [3]. Hypertrophied hepatocytes were also disseminated at the parenchyma in fish from both HgII and MeHg groups, which is in good agreement with alterations described in the literature [3, 44, 45]. Liver extensive focal necrosis associated to congestion and pigments deposition was observed in two fishes belonging to the HgII group (Figure 1(c)). The MeHg group displayed three cases of little focal liver necrosis. During exposure to both mercurials the type of lesions was the same, but the necrotic lesions observed in the liver of fishes exposed to Hg^{2+} were more predominant and severe, leading to higher ODI values (Table 1). Despite the fact that, the accumulation of Hg^{2+} was lower than MeHg (Table 2), the ODI was higher. This result contrasts with the observations by Ribeiro et al. [45] where Hg^{2+} failed to cause any significant liver change in the artic charr, in comparison to MeHg. However, it should be stressed that Ribeiro et al. [45], used oral administration of mercurials with food and in those circumstances Hg^{2+}, is much less absorbed in the GI tract than MeHg [1]. Although fishes from HgSe and MeHgSe groups (Figure 1(d)) showed at d28 the same kind of lesions observed in fish exposed only to mercurials (i.e, focal cellular vacuolization, megalocytic hepatocytes focal necrosis and congestion of the hepatic parenchyma), these lesions were observed side by side with normal cells.

After the depuration phase at d42, fish exposed to mercurials still exhibited extensive coagulative necrotic changes. Hypertrophy of the hepatocytes was clear in the parenchyma outside necrotic areas. Fishes depurating in water containing Se presented the same type of necrotic lesion although liver parenchyma beyond the necrotic zones appeared normal, resulting in a lower ODI than the index attained by fishes depurating in clean water (Table 1).

3.2. Kidney Histopathology. Kidney changes observed in fish exposed to both mercurials at days 28 and 42 were

Table 1: Values of the organ damage index* (ODI) calculated for the liver and kidney of zeabra-seabreams exposed to the different treatments. No lesions were observed in control fishes. MeHg: exposure to Methylmercury; HgII: exposure to Hg^{2+}; MeHgSe: coexposure to MeHg and Se; HgSe: coexposure to Hg^{2+} and Se; MeHgRSe: exposure to MeHg followed by exposure to Se during depuration; HgRSe: exposure to Hg^{2+} followed by exposure to Se during depuration; Se: exposure to selenium.

	Organ lesion (OL)	Pi	MeHg		HgII		MeHgSe		HgSe		MeHgRSe	HgRSe	Se	
			d28	d42	d28	d42	d28	d42	d28	d42	d42	d42	d28	d42
			N_{OL}	N_{OL}	N_{OL}	N_{OL}	N_{OL}	N_{OL}	N_{OL}	N_{OL}	N_{OL}	N_{OL}	N_{OL}	N_{OL}
Liver	Architectural pattern lost	1	4	3	4	4	3	2	4	0	3	3	2	0
	Vacuolar degeneration	1	3	1	4	1	2	0	1	2	3	3	2	0
	Hydropic degeneration	1	4	2	3	1	1	2	1	1	0	3	0	0
	Hypertrophy of hepatocytes	2	2	3	1	4	4	0	4	1	0	0	0	0
	Extensive focal necrosis	3	0	3	2	4	0	2	1	3	0	2	0	0
	Focal necrosis	2	3	0	0	0	1	2	2	0	3	1	0	0
	Parenchymal congestion	1	0	1	2	0	1	0	3	0	0	0	0	0
	Pigment deposition	1	0	0	2	0	0	0	0	0	0	0	0	0
	ODI		21	22	23	26	17	14	24	14	12	17	4	0
Kidney	Vacuolar degeneration	1	1	0	1	2	1	1	0	1	1	2	0	0
	Hydropic degeneration	1	3	2	1	0	1	3	0	3	0	2	0	0
	Pigment deposition	1	3	1	1	1	0	2	0	1	1	0	0	0
	Parenchymal necrosis	3	0	0	0	3	0	0	1	0	0	0	0	0
	ODI		7	3	3	12	2	6	3	5	2	4	0	0

Pi: Pathological index of each lesion according to their importance to organ function [38].
N_{OL}: number of fishes displaying one type of organ lesion (OL).
*ODI: sum of lesions observed for each organ taking into account their relative severity and the number of fishes (N_{OL}).

Table 2: Quantification of total mercury (HgT) and selenium (Se) values ($\mu g\ g^{-1}$) in liver and kidney of zeabra-seabreams exposed to different treatments at days 28 and 42. Results were previously reported in Branco et al. [34]. MeHg: exposure to Methylmercury; HgII: exposure to Hg^{2+}; MeHgSe: co-exposure to MeHg and Se; HgSe: co-exposure to Hg^{2+} and Se; MeHgRSe: exposure to MeHg followed by exposure to Se during depuration; HgRSe: exposure to Hg^{2+} followed by exposure to Se during depuration; Se: exposure to selenium; C: control group.

		MeHg		HgII		MeHgSe		HgSe		MeHgRSe	HgRSe	Se		C	
		d28	d42	d28	d42	d28	d42	d28	d42	d42	d42	d28	d42	d28	d42
Liver	HgT ($\mu g\ g^{-1}$)	10.2 ± 4.8	6.2 ± 1.4	1.4 ± 0.2	2.8 ± 0.4	5.8 ± 1.7	4.8 ± 1.3	1.5 ± 0.1	2.3 ± 0.5	5.8 ± 1.5	2.4 ± 0.5	0.07 ± 0.02	0.09 ± 0.02	0.10 ± 0.06	0.07 ± 0.03
	Se ($\mu g\ g^{-1}$)	0.6 ± 0.1	0.9 ± 0.2	0.5 ± 0.2	1.3 ± 0.2	0.9 ± 0.02	1.3 ± 0.3	1.1 ± 0.3	1.6 ± 0.3	1.3 ± 0.2	1.0 ± 0.1	1.3 ± 0.2	1.3 ± 0.2	1.0 ± 0.14	1.0 ± 0.21
Kidney	HgT ($\mu g\ g^{-1}$)	28.7 ± 8.3	17.3 ± 6.3	5.8 ± 1.9	8.3 ± 4.1	15.1 ± 1.9	11.9 ± 3.9	8.6 ± 3.3	10.0 ± 3.5	23.6 ± 1.1	8.8 ± 1.7	0.11 ± 0.03	0.15 ± 0.02	0.16 ± 0.04	0.14 ± 0.01
	Se ($\mu g\ g^{-1}$)	1.6 ± 0.6	3.3 ± 0.6	0.4 ± 0.3	1.3 ± 0.2	2.7 ± 1.2	2.1 ± 1.1	1.0 ± 0.3	2.8 ± 0.8	2.3 ± 0.6	4.5 ± 1.2	1.7 ± 0.3	1.7 ± 0.5	1.3 ± 0.6	1.0 ± 0.2

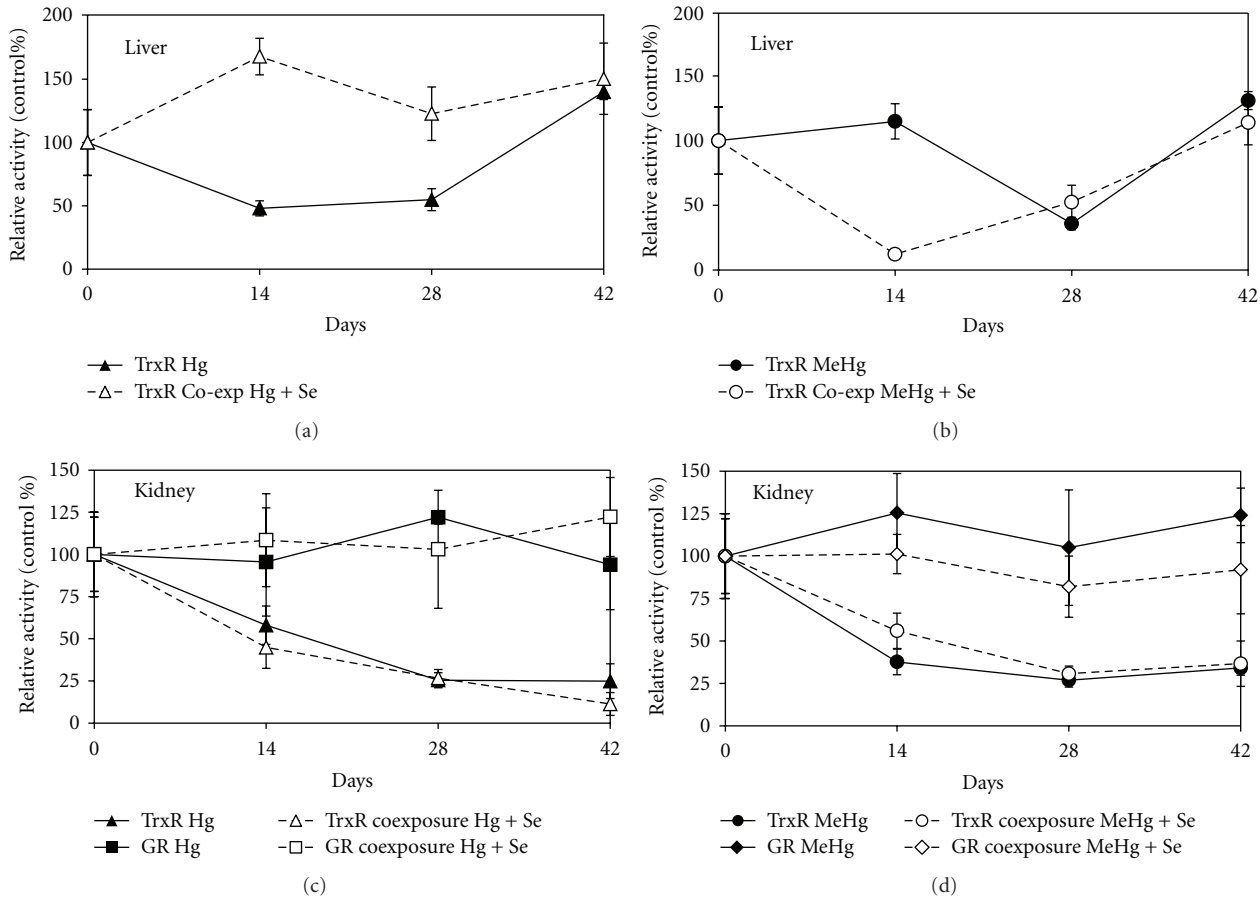


Figure 3: Enzymatic activities in liver and kidney of zeabra-seabreams exposed to mercurials and co-exposed to mercurials and Se (subset of results from a study previously reported in Branco et al. [34]). Exposure lasted 28 days and was followed by 14 days of depuration in clean or Se-supplemented water. (a) TrxR activity in the liver of seabreams exposed to Hg^{2+} and co-exposed to Hg^{2+} and Se; (b) TrxR activity in the liver of seabreams exposed to MeHg and co-exposed to MeHg and Se; (c) TrxR and GR activities in the kidney of seabreams exposed to Hg^{2+} and co-exposed to Hg^{2+} and Se; (d) TrxR and GR activities in the kidney of seabreams exposed to MeHg and co-exposed to MeHg and Se. GR activity in the liver did not show significant variation and was not represented to improve clarity.

vacuolar and hydropic degeneration of tubular epithelium and pigment deposits around the tubules. Comparatively, posterior kidney is quite more susceptible to Hg^{2+} which was evidenced by larger necrosis areas (Figure 2(b)) and occlusion of the tubular lumen with eosinophilic material (Figure 2(c)). Co-exposure to MeHg and Se seemed to delay the appearance of the more severe lesions in the kidney (Table 1). Co-exposure to Hg^{2+} and Se decreased the detrimental effects of mercury at the end of the depuration period (Table 1). This decrease in renal toxicity does not reflect a decrease in mercury concentration (Table 2) but instead it might be related to the formation of inorganic inert complexes.

3.3. Enzymatic Activities. The activity of TrxR in the liver and kidney of zeabra-seabreams is shown in Figure 3. In the liver, TrxR activity was decreased by 52% at d14 ($P < 0.05$; Figure 3(a)) during Hg^{2+} exposure, while in the case of MeHg, the inhibition was only observed by d28 ($P < 0.05$; Figure 3(b)). As previously shown and discussed [28, 34, 36] Hg^{2+} is a stronger inhibitor of TrxR than MeHg. Recovery of TrxR activity was complete at the end of depuration and was not influenced by Se supplementation during this period (data not shown). Co-exposure to Se besides reducing the ODI index, clearly prevented the inhibitory effect of Hg^{2+} over TrxR activity, that is, activity levels do not differ significantly from the control ($P > 0.05$; Figure 3(a)), but was not effective over MeHg inhibition (Figure 3(b)) whose toxic effects seem to be increased by Se. *In vitro* experiments also confirmed that the co-exposure to MeHg and Se increased the detrimental effects over the TrxR activity [34]. At the same time it was shown that Se can remove Hg^{2+} bound to the selenolthiol in the active site of TrxR restoring activity while MeHg showed no displacement from the active site [34]. Further studies to fully elucidate the contradictory role of Se in the presence of different mercury species are being conducted.

In the kidney, TrxR activity was significantly affected ($P < 0.05$) both in HgII (42% inhibition) and MeHg (62% inhibition) groups at d14 and the inhibitory effect remained throughout the entire experiment (Figures 3(c) and 3(d)). Selenium showed no protective effect during co-exposure or

on the recovery of TrxR activity during the depuration phase. As we have previously suggested [36], the protective Se over TrxR is organ specific and might be related to both the Se : Hg ratio and the capacity of selenoenzyme expression within the organ. In the liver, besides higher Se : Hg ratios [36] the level of selenoenzymes expression is assumed to be higher [44]. It should be stressed that, albeit TrxR activity did not recover, the citotoxic effects of MeHg decreased with Se co-administration in part due to the lower MeHg accumulation in both organs, liver and kidney, which reflected in the ODI values (Table 2). Since TrxR presents a high affinity for mercurials, any available mercury will primarily bound the selenolthiol of its active site and therefore it is normal that the inhibition of TrxR is observed in cases where cito-/organ toxicity is largely decreased (Table 1).

For the HgII group possible explanations for the decreased renal toxicity in the presence of Se include the formation of inorganic inert complexes and the participation of Se in antioxidant in cellular pathways. GR activity was not inhibited in the liver (results not shown) or in the kidney ($P > 0.05$; Figures 3(c) and 3(d)) in any group. On the contrary, the slight increase of GR activity seems to be a compensation mechanism for the loss of antioxidant protection provided by TrxR. Moreover, the fact that GR is homologous to TrxR, but lacks the Sec residue in the active site, reinforces the importance of TrxR active site as a main target for mercurials.

4. Conclusions

Mercury effects have been evaluated using different types of biomarkers. However, these are normally non-specific or are not predictive of toxicity but, instead, correspond to manifestations of toxicity itself. The thioredoxin system is responsible for several key cellular functions that range from anti-oxidant defense to regulation of the cellular cycle [33] and loss of activity will result in apoptosis [46]. Mercury (II) was shown to be a stronger inhibitor of TrxR than MeHg and also produced the most extensive array of organ lesions (Table 1). Also, when zeabra-seabreams were co-exposed to Se and Hg^{2+} the severity of the lesions in the liver decreased while TrxR activity was kept at normal levels. In the cases of exposure to Hg^{2+} and MeHg, although we observed full recovery of the activity of TrxR in the liver following depuration, the histopathological lesions reversion was not significant. Possibly, cellular mechanisms downstream of the thioredoxin system were affected to such a degree that recovery of TrxR activity does not reflect in an immediate organ recovery and time will be needed to replace damaged cellular structures. Overall, this work shows that the strong inhibition of TrxR activity is related with the histopathological alterations displayed in the liver and kidney of seabreams indicating the potential use of TrxR as a biomarker of effect of mercury toxicity.

Acknowledgments

The authors would like to thank Rui Silva and Pedro Pousão for assistance with the *in vivo* experiments. Vasco Branco was financed by a PhD fellowship (SFRH/BD/37388/2007) from the Fundação para a Ciência e Tecnologia (FCT) (http://www.fct.mctes.pt/). This study was financed by the MERTOX-TRX project (PTDC/QUI-BIQ/117281/2010) and by iMed.UL through FCT's strategic project: PEst-OE/SAU/UI4013/2011.

References

[1] T. W. Clarkson and L. Magos, "The toxicology of mercury and its chemical compounds," *Critical Reviews in Toxicology*, vol. 36, no. 8, pp. 609–662, 2006.

[2] C. W. J. Chang, R. M. Nakamura, and C. C. Brooks, "Effect of varied dietary levels and forms of mercury on swine," *Journal of Animal Science*, vol. 45, no. 2, pp. 279–285, 1977.

[3] C. Liao, J. Fu, J. Shi, Q. Zhou, C. Yuan, and G. Jiang, "Methylmercury accumulation, histopathology effects, and cholinesterase activity alterations in medaka (Oryzias latipes) following sublethal exposure to methylmercury chloride," *Environmental Toxicology and Pharmacology*, vol. 22, no. 2, pp. 225–233, 2006.

[4] V. Branco, J. Canário, A. Holmgren, and C. Carvalho, "Inhibition of the thioredoxin system in the brain and liver of zebra-seabreams exposed to waterborne methylmercury," *Toxicology and Applied Pharmacology*, vol. 251, no. 2, pp. 95–103, 2011.

[5] N. K. Mottet, M. E. Vahter, J. S. Charleston, and L. T. Friberg, *Metabolism of Methylmercury in the Brain and Its Toxicological Significance*, Marcel Dekker, New York, NY, USA, 1997.

[6] D. Yang, C. Yu-Wei, J. M. Gunn, and N. Belzile, "Selenium and mercury in organisms: interactions and mechanisms," *Environmental Reviews*, vol. 16, pp. 71–92, 2008.

[7] J. Parizek, I. Ostadalova, J. Kalouskva, A. Babichy, and J. Benes, *The Detoxifying Effects of Selenium. Interrelation Between Compounds of Selenium and Certain Metals*, Marcel Dekker, New York, NY, USA, 1971.

[8] M. A. K. Khan and F. Wang, "Mercury-selenium compounds and their toxicological significance: toward a molecular understanding of the mercury-selenium antagonism," *Environmental Toxicology and Chemistry*, vol. 28, no. 8, pp. 1567–1577, 2009.

[9] P. W. Wester and H. H. Canton, "Histopathological effects in *Poecilia reticulata* (guppy) exposed to methyl mercury chloride," *Toxicologic Pathology*, vol. 20, no. 1, pp. 81–92, 1992.

[10] R. K. Zalups, "Molecular interactions with mercury in the kidney," *Pharmacological Reviews*, vol. 52, no. 1, pp. 113–143, 2000.

[11] L. H. Lash, S. E. Hueni, D. A. Putt, and R. K. Zalups, "Role of organic anion and amino acid carriers in transport of inorganic mercury in rat renal basolateral membrane vesicles: influence of compensatory renal growth," *Toxicological Sciences*, vol. 88, no. 2, pp. 630–644, 2005.

[12] T. Giovanoli-Jakubczak, M. R. Greenwood, J. C. Smith, and T. W. Clarkson, "Determination of total and inorganic mercury in hair by flameless atomic absorption, and of methylmercury by gas chromatography," *Clinical Chemistry*, vol. 20, no. 2, pp. 222–229, 1974.

[13] H. Satoh, "Occupational and environmental toxicology of mercury and its compounds," *Industrial Health*, vol. 38, no. 2, pp. 153–164, 2000.

[14] J. C. Gage, "Distribution and excretion of methyl and phenyl mercury salts," *British Journal of Industrial Medicine*, vol. 21, no. 3, pp. 197–202, 1964.

[15] M. Berglund, B. Lind, K. A. Björnberg, B. Palm, Ö. Einarsson, and M. Vahter, "Inter-individual variations of human mercury

exposure biomarkers: a cross-sectional assessment," *Environmental Health: A Global Access Science Source*, vol. 4, article 20, 2005.

[16] J. C. Gage, "The distribution and excretion of inhaled mercury vapour," *British Journal of Industrial Medicine*, vol. 18, no. 4, pp. 287–294, 1961.

[17] S. Bose-O'Reilly, K. M. McCarty, N. Steckling, and B. Lettmeier, "Mercury exposure and children's health," *Current Problems in Pediatric and Adolescent Health Care*, vol. 40, no. 8, pp. 186–215, 2010.

[18] T. Iwata, M. Sakamoto, X. Feng et al., "Effects of mercury vapor exposure on neuromotor function in Chinese miners and smelters," *International Archives of Occupational and Environmental Health*, vol. 80, no. 5, pp. 381–387, 2007.

[19] A. P. M. dos Santos, M. L. Mateus, C. M. L. Carvalho, and M. C. C. Batoréu, "Biomarkers of exposure and effect as indicators of the interference of selenomethionine on methylmercury toxicity," *Toxicology Letters*, vol. 169, no. 2, pp. 121–128, 2007.

[20] J. S. Woods, *Porphyrin Metabolism as Indicator of Metal Exposure and Toxicity*, Springer, Berlin, Germany, 1995.

[21] J. S. Woods, M. D. Martin, B. G. Leroux et al., "Urinary porphyrin excretion in children with mercury amalgam treatment: findings from the Casa Pia children's dental amalgam trial," *Journal of Toxicology and Environmental Health A*, vol. 72, no. 14, pp. 891–896, 2009.

[22] S. Oh and M. Lee, "Interaction between inorganic mercury and selenium on tissue sulfhydryl groups and glutathione-linked enzymes in rats," *Yonsei Medical Journal*, vol. 22, no. 2, pp. 122–126, 1981.

[23] F. Livardjani, M. Ledig, P. Kopp, M. Dahlet, M. Leroy, and A. Jaeger, "Lung and blood superoxide dismutase activity in mercury vapor exposed rats: effect of N-acetylcysteine treatment," *Toxicology*, vol. 66, no. 3, pp. 289–295, 1991.

[24] R. Perrin-Nadif, M. Dusch, C. Koch, P. Schmitt, and J. M. Mur, "Catalase and superoxide dismutase activities as biomarkers of oxidative stress in workers exposed to mercury vapors," *Journal of Toxicology and Environmental Health A*, vol. 48, no. 2, pp. 107–119, 1996.

[25] T. Sarafian and M. A. Verity, "Oxidative mechanisms underlying methyl mercury neurotoxicity," *International Journal of Developmental Neuroscience*, vol. 9, no. 2, pp. 147–153, 1991.

[26] B. O. Lund, D. M. Miller, and J. S. Woods, "Studies on Hg(II)-induced H_2O_2 formation and oxidative stress in vivo and in vitro in rat kidney mitochondria," *Biochemical Pharmacology*, vol. 45, no. 10, pp. 2017–2024, 1993.

[27] M. G. Cherian and T. W. Clarkson, "Biochemical changes in rat kidney on exposure to elemental mercury vapor: effect on biosynthesis of metallothionein," *Chemico-Biological Interactions*, vol. 12, no. 2, pp. 109–120, 1976.

[28] C. M. L. Carvalho, E. Chew, S. I. Hashemy, J. Lu, and A. Holmgren, "Inhibition of the human thioredoxin system: a molecular mechanism of mercury toxicity," *The Journal of Biological Chemistry*, vol. 283, no. 18, pp. 11913–11923, 2008.

[29] O. Wada, N. Yamaguchi, T. Ono, M. Nagahashi, and T. Morimura, "Inhibitory effect of mercury on kidney glutathione peroxidase and its prevention by selenium," *Environmental Research*, vol. 12, no. 1, pp. 75–80, 1976.

[30] C. Watanabe, K. Yoshida, Y. Kasanuma, Y. Kun, and H. Satoh, "In utero methylmercury exposure differentially affects the activities of selenoenzymes in the fetal mouse brain," *Environmental Research*, vol. 80, no. 3, pp. 208–214, 1999.

[31] C. Bulato, V. Bosello, F. Ursini, and M. Maiorino, "Effect of mercury on selenium utilization and selenoperoxidase activity in LNCaP cells," *Free Radical Biology and Medicine*, vol. 42, no. 1, pp. 118–123, 2007.

[32] J. L. Franco, T. Posser, P. R. Dunkley et al., "Methylmercury neurotoxicity is associated with inhibition of the antioxidant enzyme glutathione peroxidase," *Free Radical Biology and Medicine*, vol. 47, no. 4, pp. 449–457, 2009.

[33] C. M. L. Carvalho, J. Lu, X. Zhang, E. S. J. Arnér, and A. Holmgren, "Effects of selenite and chelating agents on mammalian thioredoxin reductase inhibited by mercury: implications for treatment of mercury poisoning," *The FASEB Journal*, vol. 25, no. 1, pp. 370–381, 2011.

[34] V. Branco, J. Canário, J. Lu, A. Holmgren, and C. Carvalho, "Mercury and selenium interaction *in vivo*: effects on thioredoxin reductase and glutathione peroxidase," *Free Radical Biology and Medicine*, vol. 52, no. 4, pp. 781–793, 2012.

[35] C. Wagner, J. H. Sudati, C. W. Nogueira, and J. B. Rocha, "In vivo and in vitro inhibition of mice thioredoxin reductase by methylmercury," *BioMetals*, vol. 23, no. 6, pp. 1171–1177, 2010.

[36] C. H. Lillig and A. Holmgren, "Thioredoxin and related molecules—from biology to health and disease," *Antioxidants and Redox Signaling*, vol. 9, no. 1, pp. 25–47, 2007.

[37] J. C. Wolf and M. J. Wolfe, "A brief overview of nonneoplastic hepatic toxicity in fish," *Toxicologic Pathology*, vol. 33, no. 1, pp. 75–85, 2005.

[38] D. Bernet, H. Schmidt, W. Meier, P. Burkhardt-Holm, and T. Wahli, "Histopathology in fish: proposal for a protocol to assess aquatic pollution," *Journal of Fish Diseases*, vol. 22, no. 1, pp. 25–34, 1999.

[39] R. D. Lillie and H. M. Fullmer, *Histopathologic Technic and Practical Histochemistry*, McGraw- Hill, New York, NY, USA, 1976.

[40] M. M. Bradford, "A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein dye binding," *Analytical Biochemistry*, vol. 72, no. 1-2, pp. 248–254, 1976.

[41] E. S. J. Arnér and A. Holmgren, *Measurement of Thioredoxin and Thioredoxin Reductase*, John Wiley & Sons, New York, NY, USA, 1999.

[42] B. Mannervik, *Measurement of Glutathione Reductase Activity*, John Wiley & Sons, New York, NY, USA, 1999.

[43] J. H. Zar, *Biostatistical Analysis*, Prentice Hall, New Jersey, NJ, USA, 1999.

[44] M. Mela, M. A. F. Randi, D. F. Ventura, C. E. V. Carvalho, E. Pelletier, and C. A. O. Oliveira, "Effects of dietary methylmercury on liver and kidney histology in the neotropical fish Hoplias malabaricus," *Ecotoxicology and Environmental Safety*, vol. 68, no. 3, pp. 426–435, 2007.

[45] C. A. O. Ribeiro, L. Belger, E. Pelletier, and C. Rouleau, "Histopathological evidence of inorganic mercury and methyl mercury toxicity in the arctic charr (*Salvelinus alpinus*)," *Environmental Research*, vol. 90, no. 3, pp. 217–225, 2002.

[46] M. Conrad, "Transgenic mouse models for the vital selenoenzymes cytosolic thioredoxin reductase, mitochondrial thioredoxin reductase and glutathione peroxidase 4," *Biochimica et Biophysica Acta*, vol. 1790, no. 11, pp. 1575–1585, 2009.

Hindawi Publishing Corporation
Journal of Biomedicine and Biotechnology
Volume 2012, Article ID 472858, 10 pages
doi:10.1155/2012/472858

Research Article

Five Hundred Years of Mercury Exposure and Adaptation

Guido Lombardi,[1] Antonio Lanzirotti,[2] Clifford Qualls,[3] Francisco Socola,[4] Abdul-Mehdi Ali,[5] and Otto Appenzeller[6]

[1] *Laboratorio de Paleopatología, Cátedra Pedro Weiss, Universidad Peruana Cayetano Heredia, Lima, Peru*
[2] *CARS, The University of Chicago, Bulding 434A, 9700 South Cass Avenue, Argonne, IL 60439, USA*
[3] *Departments of Mathematics and Statistics, The University of New Mexico, Albuquerque, NM 87131, USA*
[4] *Department of Medicine, Jackson Memorial Hospital, University of Miami, Miami, FL 33124, USA*
[5] *Department of Earth and Planetary Sciences, Analytical Chemistry Laboratory, University of New Mexico, Albuquerque, NM 87131, USA*
[6] *Department of Neurology, New Mexico Health Enhancement and Marathon Clinics Research Foundation, Albuquerque, NM 87122, USA*

Correspondence should be addressed to Otto Appenzeller, ottoarun12@aol.com

Received 11 March 2012; Revised 13 May 2012; Accepted 15 May 2012

Academic Editor: João B. T. Rocha

Mercury is added to the biosphere by anthropogenic activities raising the question of whether changes in the human chromatin, induced by mercury, in a parental generation could allow adaptation of their descendants to mercury. We review the history of Andean mining since pre-Hispanic times in Huancavelica, Peru. Despite the persistent degradation of the biosphere today, no overt signs of mercury toxicity could be discerned in present day inhabitants. However, mercury is especially toxic to the autonomic nervous system (ANS). We, therefore, tested ANS function and biologic rhythms, under the control of the ANS, in 5 Huancavelicans and examined the metal content in their hair. Mercury levels varied from none to 1.014 ppm, significantly less than accepted standards. This was confirmed by microfocused synchrotron X-ray fluorescence analysis. Biologic rhythms were abnormal and hair growth rate per year, also under ANS control, was reduced ($P < 0.001$). Thus, evidence of mercury's toxicity in ANS function was found without other signs of intoxication. Our findings are consistent with the hypothesis of partial transgenerational inheritance of tolerance to mercury in Huancavelica, Peru. This would generally benefit survival in the Anthropocene, the man-made world, we now live in.

1. Introduction

The largest mercury processing in the Western Hemisphere was in Huancavelica in the Peruvian Andes, now a town of 30,000 people situated at 3676 m altitude. The extensive mercury deposits have been mined since pre-Hispanic times, they are just above the present town at ~4000 m. Although the mine closed 30 years ago, mining continues on small scales. The legacy of 500 years of mercury mining is now found in the city trenches and waterways [1], which are lined with mercury but this legacy is less evident in today's inhabitants of Huancavelica.

2. Historical Aspects

Mining is a dangerous, unhealthy occupation but nowhere was it more health threatening than in Colonial South America, especially in Huancavelica.

The widespread ecological impacts of mining in the Andes such as landscape destruction and pollution through the introduction of mercury and other toxicants into the biosphere were overshadowed by the population shifts to the then sparsely inhabited highlands. There, the challenges of survival in the ambient hypoxia of altitude, at which mining generally occurs in the Andes, were added to the deleterious health effects of mining.

In 1928, Carlos Monge of Lima, Peru, described chronic mountain sickness (CMS) in a native miner from Cerro de Pasco, a mining town in Peru situated at 4338 m above sea level [2]. This syndrome is attributed to ambient hypoxia. It occurs in high altitude natives (hypoxia adapted individuals) who for, as yet, unknown reasons lose their adaptation to their hypoxic homeland. Recently, CMS was found to be associated with deranged expression of hypoxia-related genes [3]. In colonial times, less so now, mining required heavy physical labor which predisposes to CMS. The toxicity of mercury, when added to the stress of inescapable hypoxia at high altitude and the dangers to health and predisposition to injury of mining in colonial Huancavelica, earned the mine the infamous reputation as the "mina de la muerte" (the mine of death) [4].

Because of its importance to the Spanish economy, mercury killed thousands of Andeans. Prior to the conquest, the locals used the Huancavelica cinnabar (mercury sulfide, HgS) deposits for body paint and cosmetics for their women. But, after the Spanish arrival, the lord of the local Angaraes people betrayed the cinnabar deposits to Amador de Cabrera in 1563. The riches of the deposits enticed newly arriving colonists to stake claims begin mining cinnabar, and distill mercury which they then sold to Mexico's silver refineries. Demand for mercury increased when the silver mines at Potosi began using mercury to amalgamate the ores in 1570 [4].

Francisco de Toledo, a Spanish viceroy, arrived in Peru in 1569. He expropriated the Huancavelica mine. According to the new law the subsoil now belonged to the crown which granted its use to miners upon payment of taxes.

Under the new rules the Huancavelica treasury contracted with former claim holders to keep the mine operating, setting the amount of mercury to be produced and the price to be paid. The mercury was then resold to silver miners, at a profit [4].

A change in the production of silver which required mercury for amalgamation of the ore increased the demand for labor in the mines. Toledo committed the colonial government to provide adequate manpower for the mercury producers. To do this, he copied Inca rules which required adult males to take turns on public projects called a "mit'a" (turn) and ordered neighboring provinces to send 900 "mitayos." This number was increased to 3280 in 1577 [5]. The population in the surrounding areas decreased because of disease and the demands of the mita. This forced the government to reduce the size of the mita to 620 by 1645 and expand the area from which mitayos were committed into the labor force [4]. Mitayos received a low wage but served only for 2 months at a time, taken here as evidence for governmental awareness of the devastating health effects of mercury mining.

Huancavelica became notorious as a dangerous workplace by the late 16th century and the Spanish crown received reports that the mita was abusive, unchristian, and unhealthy [6]. The high altitude of the mine added the stresses of ambient hypoxia, extreme temperatures and harsh weather to the hazards of mining, such as landslides and tunnel collapse. By 1600 the reputation of the Huancavelica mine as the "mine of death" was well deserved. The Franciscan Miguel Agia, in favor of the continuation of the mita, admitted that work in the mine was equivalent "to sending [Indians] to die." And the "protector" of Indians Damián de Jeria likened the tunnel of the mine to a "public slaughterhouse" [7]. Luis de Velasco, another viceroy, wrote to the king about the health aspects of the Huancavelica mine in 1600:

> *These quicksilver ores, when they extract them in the mines, they give out a dust that enters itself into the Indians as they breath and settles in the chest, of such evil quality, that it causes them a dry cough and light fever and at the end death without repair, because the doctors have it for an incurable evil.*

He wanted to close the mine but because of its economic importance, he pleaded for guidance from the crown [8, 9]. De Jeria wrote to the viceroy that although the mitayos had committed no crimes they were forced to endure conditions worse than those imposed on criminals in prisons and galleys. He argued that Huancavelica mining will eventually cause economic collapse because its continuance, costing so many lives of so many Indians… that which may be lost from not producing mercury is gained in the conservation of the Indians, for without them there will be neither quicksilver, nor silver, nor the common good, nor Peru [7].

By 1630, the Count of Chinchón, then viceroy, wrote to the king about the mita:

> *It is a most terrible matter because a free, innocent, defenseless, poor and afflicted people are condemned to a notorious risk of death their fear of the mita was such that trustworthy people who have seen it have reported to me that they take these miserable Indians by force against all their will from their houses and take them in iron collars and chains more than 100 leagues to put them in this risk… and from this has resulted their own mothers maiming and crippling their sons to preserve them [10].*

Pick-men and ore carriers developed silicosis (deposition of dust and silica particles in the lungs) rapidly because of enormous dust levels in the mine, but this also affected work in other mines. In Huancavelica, additionally, mercury killed by its inhalation. The miners remained in the mine throughout the week with little opportunity to wash. After returning to their huts, they transferred the dust to their living quarters and contaminated their families by prolonging exposure and mercury absorption through the skin and food.

The symptoms of mercury poisoning were known to the workers who called it the "Huancavelica sickness" or the "evil of Huancavelica." Although the usual treatment for poisoned miners was bloodletting to put "their bodily humors back into balance," this increased their susceptibility to intercurrent illnesses. Eventually the graves of mitayos were said to contain puddles of mercury after their bodies decayed [4, 7]. The threat of mercury poisoning was even greater to those

who worked the ovens to heat the ore and extract the mercury as the vapors cooled. And Indians were made to open the ovens before they had completely cooled to increase mercury extraction by reloading them with fresh ore. This practice enhanced their exposure to the toxic mercury vapors [4].

Those rendered unable to work because of mercury poisoning were known as *azogados* (azogue = quicksilver). The symptoms were easily recognized. Weight loss, tremors, excessive salivation, ulcers of the mouth, restlessness, anemia, and eventually severe depression which often lead to alcoholism. Many miners had to be fed by their relatives because of the violent tremors which could not be controlled to bring food to their mouths.

The Prince of Santo Buono (viceroy 1716–1720) urged the abolition of the mita. Although initially rejected by the crown and the Council of the Indies it was finally approved on humanitarian grounds. By 1740 the mining guild began using blasting powder to dislodge the ore. Skilled miners were now able to stand and swing sledge hammers in the mining shafts which, by now, had acquired improved ventilation.

Ulloa, governor of Huancavelica, wrote in 1760 [11] that miners were now free of mercury intoxication because the ore contained so little quicksilver but poisoning still occurred at the ovens when workers failed to let them cool completely before reloading them.

The miners had useful remedies for the symptoms of mercury poisoning. Ulloa reported that azogados descended to warmer lowlands, drank huge amounts of *chicha*, a beer made from maize, worked very hard in the fields, and "sweated" out the mercury. The documented improvement was, likely, due to the diuretic effect of the beer which expelled the mercury concentrated in the kidneys. After some weeks of heavy drinking and hard physical labor the shakes, excessive salivation and mouth ulcers disappeared and some miners returned to work in the Huancavelica mine.

Although the mine was especially deadly because of its mercury, most Spaniards at that time thought that the Indians were destined to die anyway if this was necessary to obtain the metal. Camargo wrote to the king in 1595 [12]:

> *They are barbarous people and without knowledge of God; in their lands they are only occupied in idolatries and drunkenness and other vices of great filthiness… These people going to work the mines first seems to me a service to God and to your Holy Catholic Royal Majesty and for the good of the natural Indians themselves, because the mines they teach them doctrine and make them hear mass and they deal with Spaniards by which they become ladinos [Hispanicized]…. losing their barbarity, and they have no place for the idolatries of their lands by being outside of those sites where they do them and being occupied, which is the principal thing.*

Camargo affirmed his "great desire to [also] die in the service of Your Holy Catholic Royal Majesty" [4].

Some improvements in health occurred with new mining methods but these failed to take into account the huge pollution of the biosphere of Huancavelica which persists to the present. The refining ovens were along the river where the escaping vapors settled on the ground, in the water, and blew across town depositing mercury on everything. These vapors were converted into methyl mercury by bacterial activity which then easily entered the food chain and which is especially injurious to the nervous system.

3. Epidemics of Mercury Poisoning

Outbreaks of mercury poisoning have occurred sporadically in more recent times. They were often associated with industrial pollution. These outbreaks were traced to methyl mercury, the highly toxic form of mercury [13]. The best-described mass poisoning occurred in Minamata Bay, Japan, in the 1950s [14]. But intoxications restricted to single families due to ingestion of mercury with longterm consequences have also been reported [15].

Mercury poisoning in infants (pink disease, also known as acrodynia) associated with the use of teething powders containing mercury, especially in England and Australia, has been eliminated by the removal of the metal from these remedies. Clinical features of acrodynia, were autonomic "hyperactivity" characterized by acral vasodilatation, excessive sweating and tachycardia. Pain, paresthesias, and hypotonia allowed the assumption of characteristic postures by the affected infants [16]. These symptoms reflect the especially injurious effects of mercury on the autonomic nervous system (ANS). The link between mercury and acrodynia is complex. The syndrome has not been found in recent epidemics of mercury poisoning in Japan, but it reemerged in an epidemic of methyl mercury (used as a fungicide) poisoning employed for the sterilization of diapers in Argentinean babies (for review see [16]).

4. Mercury in Human Hair

Mercury compounds occur in the environment and their levels are rising due to anthropogenic activities, especially the burning of fossil fuels. Coal contains large amounts of mercury, which is released into the atmosphere upon burning and it is then transported, possibly, around the globe. Even low concentrations of mercury can cause subclinical effects [17], and slightly increased levels of mercury in hair are associated with decreased academic performance [18]. The World Health Organization (WHO) places the level at which toxicity occurs at 50 ppm of mercury in hair and advises that healthy people should have no more than 5 ppm of mercury in their hair. However, normal mercury levels in hair are difficult to come by because they are dependent on the amount and type of fish eaten, on location (especially where the environmental mercury burden may be high) on the sampling methods, and on analytical methods [19]. Thus, in populations that have been exposed to high burdens of mercury for centuries, like those in Huancavelica, the effects of such exposure are difficult to assess.

5. Hair: Biologic Aspects

Hair is renewed throughout life; it has a well-defined variability in growth influenced by diet, age, blood flow to the skin, and hormonal and metabolic signals which are, ultimately, regulated by the ANS. But hair also reflects biologic rhythms because of clock-like signals that affect its growth through the intermediary of the ANS [20].

Recurrent circa-annual periods of slow and fast rhythms in the hydrogen isotope ratios of human and animal hair can be recognized. Using power spectral analysis of the ratios along the length of the hairs can reveal the effects of life at altitude and toxin accumulation on biologic rhythms [21].

Here we report a study of hydrogen isotope ratios in the hair and of heart rate variability in the same residents of Huancavelica, Peru, to assess their biologic rhythms and ANS function. We carried out this study to determine whether centuries of ancestral and present exposure to increased mercury in the biosphere could affect ANS function and give evidence for continuing deleterious effects on the health in modern Huancavelica residents.

6. Results

The demographics of the 5 subjects and mercury and copper levels in their hair are given in Table 1. Because the length of the hair reflects the duration of the stored information on biologic rhythms, our study was confined to women who generally have longer hair. The mercury levels varied from none detectable to 1.041 ppm. We confirmed these very low levels in the hair from two Huancavelica subjects using the X26A hard X-ray microprobe at the Brookhaven synchrotron. No detectable mercury in single hairs from each subject was reported by the X-ray microprobe.

To strengthen our conclusions about the surprisingly low mercury levels in the hair in Huancavelica residents we used a control from the USA.

In Table 1 cold vapor atomic absorption spectrometry was used to measure total mercury (organic + inorganic) and inductively coupled plasma-atomic emission spectrometry for the other element (one sample was misplaced by the analytical company performing the assays no values for Hg are available; here labeled lost).

To assess the impact of individual metals on the hydrogen isotope ratios of low frequency/high frequency (LF/HF) power bands, a measure of ANS control of biologic rhythms, we constructed "impact graphs" shown in Figure 1. These power bands were affected by both copper and mercury levels in the hair clearly separating the LF and HF power according to mercury and copper levels.

The annual growth rate reported for normal human hair is ~16 cm/year [21]. In our controls ($n = 4$) as determined from the sinusoidal variation in hydrogen isotope ratios in our samples from the USA and Europe, it was 14.2 ± 3.7 SD cm/year. The growth rate of hair from Huancavelica ($n = 5$) was 6.4 ± 1.7 SD cm/year ($P < 0.001$) (Figure 2).

The standard deviation of the variance of the power spectra of the hydrogen isotope ratios between controls from other parts of the world and that from Huancavelica is shown in Figure 3. Remarkably, this variance is significantly less in Huancavelica residents ($P = 0.01$).

Pooling of hydrogen isotope power spectra from Huancavelica and control hair is shown in Figure 4. The only statistically significant difference between these spectra is in the low-power frequencies ($P < 0.05$), though the power of high frequencies appears also to be less (NS).

The annual growth rates of hair can be shown to occur in fast and slow periods of weekly cycles (Figure 5). The shorter fast period, and very prolonged slow period which correspond to the rest phase of hair growth, are consistent with the reduced annual hair growth in Huancavelica residents.

We compared the power spectra derived from heart rate variability with those obtained from the hydrogen isotope ratios in the hair, in the same subjects (Figure 6). The low- and high-power frequency bands are of comparable length but, not surprisingly, the spectral power is different because of the different time scales—heart rate variability in milliseconds, hydrogen isotope ratios in days.

Table 1: Analyses of metals in hair from control and subjects in Huancavelica, Peru.

Sample ID	Cu (mg/K)	Hg (μg/L)	Hg (mg/Kg) (ppm)
Control age 82	3.72	0.673	*0.002*
Number 1 age 26	29.0	*ND*	*ND*
Number 2 age 26	8.86	6.23	*0.129*
Number 3 age 36	9.68	90.9	*1.041*
Number 4 age 48	7.48	lost	*lost*
Number 5 age 53	5.29	2.73	*0.058*

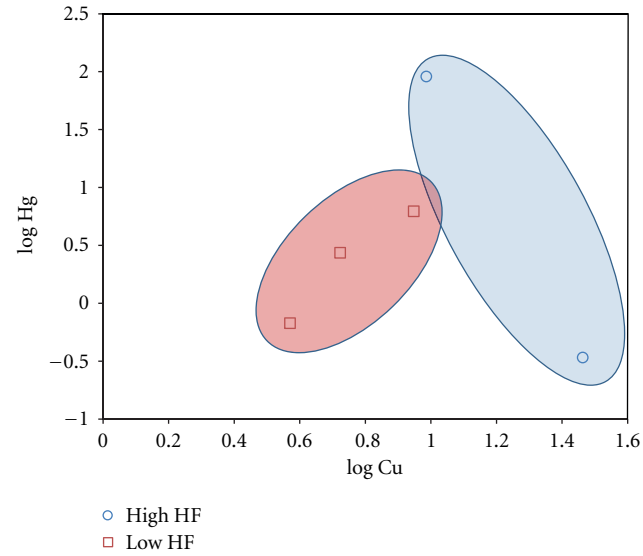


Figure 1: The high-frequency/low-frequency power bands of the hydrogen isotope ratios along the hair reflect the control by the autonomic nervous system (ANS) of biologic rhythms. Hg and Cu both influence the power bands. Note that Cu is associated with low-HF power of the spectra of the hydrogen isotope ratios along the length of the hairs.

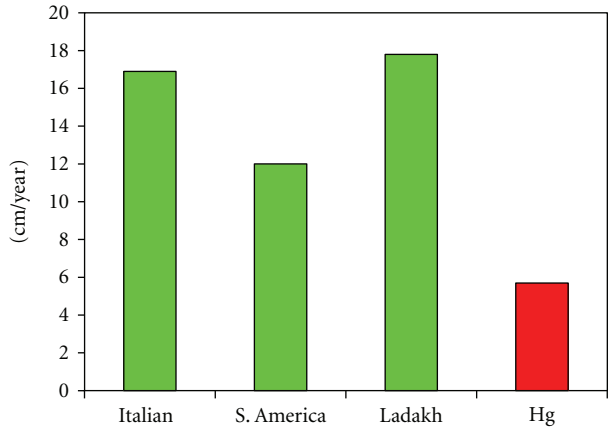


Figure 2: Growth rates of human scalp hair from different locales and from Huancavelica (Hg). Significantly slower yearly growth (~6 cm/year) is evident in modern residents of Huancavelica.

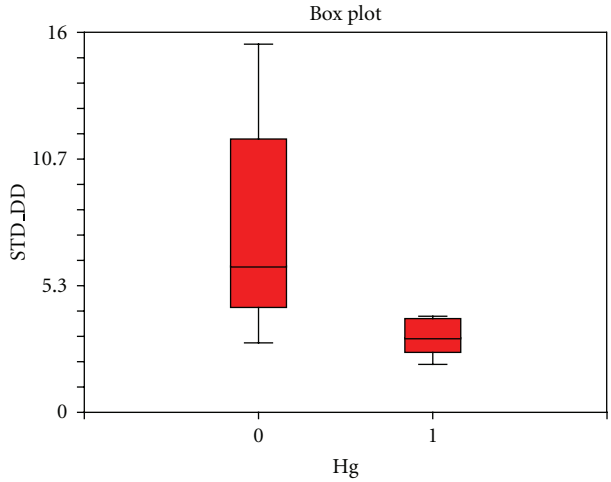


Figure 3: Box plots showing the variance (standard deviation; STD) of hydrogen isotope ratios along the length of the hair in Huancavelica residents (1) and controls (0). The statistically significant reduced variance in Hg exposed individuals ($P = 0.01$) signifies deranged biologic rhythms with significant reduction of variance in the system's control by the autonomic nervous system.

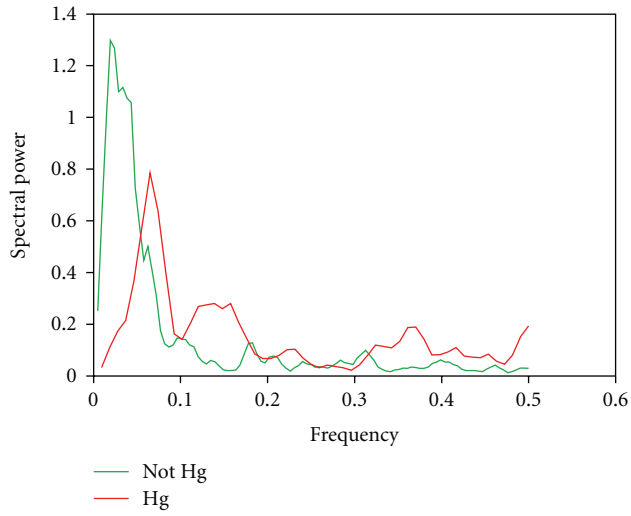


Figure 4: Pooled power spectra derived from hydrogen isotope ratios. There is statistically significant less low power ($P < 0.05$) in the mercury (Hg) spectrum (red) derived from Huancavelica hair.

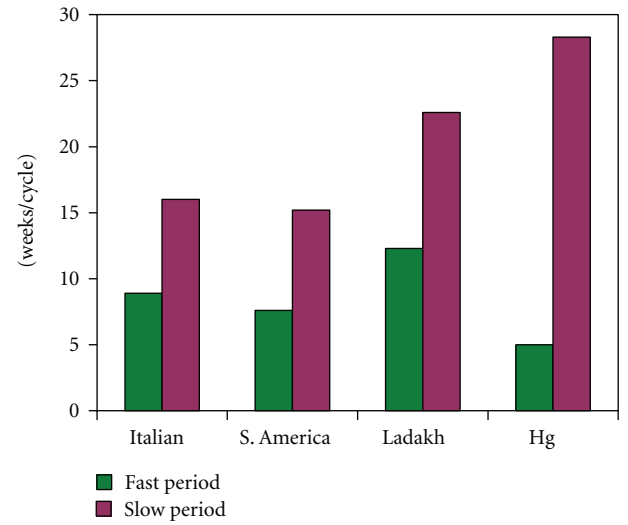


Figure 5: Fast and slow periods of hair growth in weekly cycles. The hairs from Huancavelica (Hg) show a shorter fast period and very prolonged slow period which correspond to the rest phase of hair growth.

Because of the complexity of the interactions between mercury and biologic rhythms as derived from hydrogen isotope ratios along the length of the hairs we used fractal analysis to illustrate the differences between those living in a mercury-polluted environment and a control (Figure 7).

These results are consistent with severe dysfunction of the ANS evidenced also by the reduced annual hair growth in Huancavelica residents.

7. Discussion

The most famous mercury poisoned character was the "Mad Hatter," the one from the Mad Tea Party in *Alice in Wonderland*. He was partner to the March Hare, mad as only a March hare can be (though the hare was not poisoned by mercury). Lewis Carroll created the characters. However, the phrases "mad as a hatter" and "mad as a March hare" were in common use in 1837, well before the publication of Alice in Wonderland in 1865.

Hatters did go mad because felt used for making hats was cured by mercurious nitrate and the mercury vapors inhaled by hatters gave them the characteristic symptoms reported also by azogados (quicksilver poisoned miners from Huancavelica) centuries earlier who noted the shakes, hallucinations, and psychosis as found in hatters centuries later.

Mercury was used in Europe throughout medieval times for medicinal purposes or as mercury sulphide, an ingredient

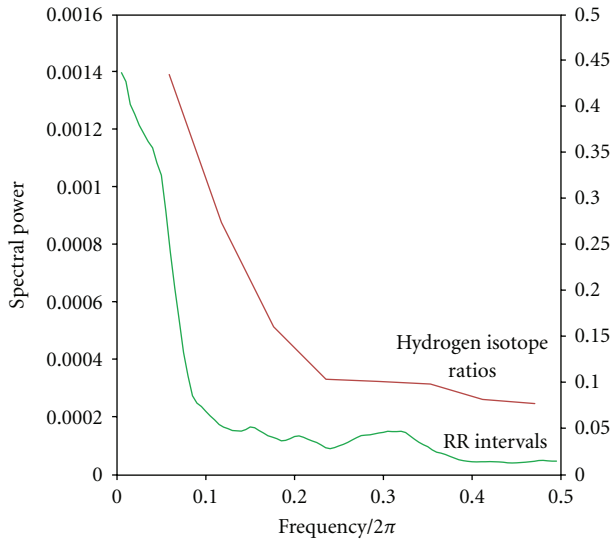


FIGURE 6: Comparison of power spectra derived from hydrogen isotope ratios along the length of the hairs and power spectra derived from heart rate intervals (RR) in the same subjects. Note that the low- and high-frequency power bands are of comparable lengths but the spectral power appears different (NS) ("stacked" averaged spectra from all subjects). Red line hydrogen isotope ratios; green line heart rate variability (RR intervals). Spectral power, on the y-axis, is hydrogen isotope ratios (right) and RR intervals (left).

of the bright red ink used by scribes in monasteries. Increased levels were found in medieval bones of those who had either employed it for the treatment of leprosy or syphilis, or with increased occupational exposure such as pharmacists [22].

Mercury is a neurotoxin but its damaging effects on humans are unpredictable and dependent, in part, on its chemical speciation and interactions with other metals in the environment and in the diet. Organic mercury which is the primary source of mercury exposure is principally derived from the diet.

Huancavelica is listed amongst the ten most polluted places in the world [1] and despite its listing, our measurement of mercury levels in the hair of current Huancavelica residents was noticeably low (Table 1) and, clinically, Huancavelica residents showed no signs of mercury poisoning as recorded in hatters and azogados centuries earlier. However, measurements of mercury levels in the hair are not good indicators of mercury's toxicity. While levels of mercury can be accurately measured in the blood, the hair has no blood supply and the levels derived from hair represent metabolism over time and reflect the chronobiology of the individuals rather than the blood levels of this metal. In modern times, hair is also frequently washed with shampoos and dried with hair driers; these manipulations can add or remove mercury and thus compound the difficulties of assaying the mercury levels in the hair [23].

Here we show that people living in a heavily mercury-polluted environment, in Huancavelica, may have acceptable mercury levels, by international standards, in their hair and no overt clinical symptoms of intoxication. Nevertheless, the effects of mercury exposure are evident from the reduced rate of hair growth and disturbed biologic rhythms in these people. Taken together these findings give unequivocal evidence of the toxicity of mercury to the human autonomic nervous system the most vulnerable part of the nervous system to mercury's damage, in modern Huancavelica, Peru.

The high-frequency/low-frequency (HF/LF) ratios are measures of the control by the autonomic nervous system (ANS) of biologic rhythms [21]. We find that Hg and Cu both affected the power bands of the HF and LF. Mercury does interact with other metals to produce its effect on biologic rhythms in Huancavelica and such interactions might account for the unpredictable effects of Hg on individual physiology in other parts of the world too (Figure 1).

The growth rate of human scalp hair is variable but on average ~16 cm/year. The growth rate of hair in Huancavelica is significantly reduced compared to our controls. We suggest that the altered biologic rhythms in modern Huancavelica residents may have contributed to this failure to renew the hair at an appropriate rate (Figure 2).

The standard deviation of the variance of the power spectra of the hydrogen isotope ratios along the length of the hairs between our controls from other parts of the world and that from Huancavelica is shown in Figure 3. This variance is significantly less in Huancavelica residents ($P = 0.01$). Normal rhythmic oscillations in biology are marked by high variability (large variances); less variability degrades performance of the system [24]. The reduction in variance we observed is yet another expression of the deranged biologic rhythms in our Huancavelica subjects.

The low-frequency peak of the power spectra reflects primarily the sympathetic nervous system's control of autonomic variability. A decrease or absence of this peak in heart rate variability and in the power spectra derived from hydrogen isotope ratios in the hair in the same individuals is seen in ANS diseases such as pure autonomic failure (PAF), manifested by widespread failure of sympathetic nervous system function [21]. Our identification of significant reduction in low-frequency power in the hydrogen isotope ratios in Huancavelica subjects (Figure 4) further supports ANS impairment in these individuals.

Long-term rhythms recurring at weekly, monthly, or yearly intervals are well known in animal and human physiology. The recurring annual fast periods reflect growth while the slow periods reflect the rest stages in hair growth. The shortened fast period rhythms in Huancavelica residents' hair (Figure 5) are in keeping with the reduced hair growth rate and further support the altered ANS function (and biologic rhythms) in these people.

To confirm our findings from hydrogen isotope derived biologic rhythms, we measured heart rate variability using 2 minutes of recording of cardiac action (~150 heart beats) in the same subjects (Figure 6). Averaged power spectra showed comparable durations of low- and high-frequency bands and spectral power did not differ significantly. We have previously shown that spectra obtained from human heart rate variability were comparable to those derived from power spectra obtained from hydrogen isotope ratios in the hair and from growth lines from teeth [25]. Our results

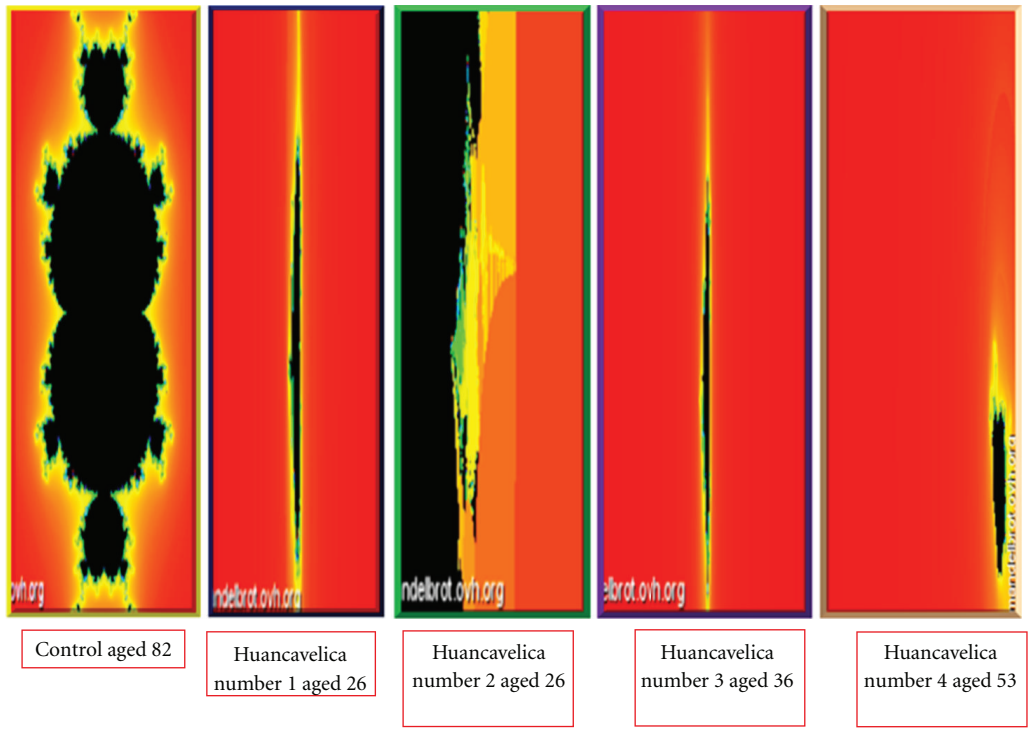


Figure 7: Mandelbrot sets showing the complex interactions between Hg and biologic rhythms. Notable is the lack of structural features in the sets derived from all Huancavelica residents in keeping with the lack of variability found in the power spectra derived from the hydrogen isotope ratios of the same individuals (see Figure 3).

confirmed that biologic rhythms as gleaned from power spectral analysis reflected the pulsing of life, the varying rhtymicity of physiologic functions even when the ANS has been affected by disease or mercury as in Huancavelica residents.

Fractals are evident throughout nature. They reflect recurrent relations at each point in a space. They can show complex natural phenomena in graphic form such as ice crystals on a window pane and the intricate contours of mountains and ridges, and river branching. In biology they illustrate the 3/4 scaling of metabolism with body size, the complexity of heart rate control, of blood circulation, growth, development, and lifespan [26]. The term fractal was introduced by Benoit Mandelbrot in 1982. Here we used Mandelbrot fractal sets to show the complex relationships between Hg, its effects on the ANS's control of human biologic rhythms, and the variability of hydrogen isotope ratios along the length of the hair in our subjects (Figure 7). The fractals derived from all Huancavelica subjects clearly reflect derangement in biologic rhtymicity and the lack of variance which characterizes their exposure to Hg, supporting our contention of continuing influence of this metal on human life in modern Huancavelica.

Taken together our results show that survival in a heavily, mercury, polluted environment is possible without overt signs of mercury intoxications such as those found during mercury epidemics of the past or in miners during the Spanish exploitation of Peru's mineral riches. Nevertheless, careful analyses of the function of the most mercury vulnerable part of the nervous system gives evidence of continued effects in contemporaneous Huancavelica residents.

How can we reconcile the clear evidence of Hg's effects on biologic rhythms and ANS function with the unexpected low levels of the metal in the hair of these same individuals and their apparent lack of overt signs of intoxication? First, there is the fact that Hg in hair is a poor indicator of its possible toxic effects. Secondly, we propose a parallel between how human height and life expectancy increase in a short span of ~300 years after the industrial revolution [27]. This well-documented phenomenon has been explained by several contributing factors such as technological advances and physiological adaptation [28].

The history of mining in the high Andes is replete with anecdotes of how technical advances in the extraction of ores improved miners' survival [11] and at the same time, in Huancavelica, increased the yield of Hg extraction. While the cause of the shortened lifespan in Huancavelica miners remained a mystery for long, nevertheless, the miners themselves devised useful adaptations which allowed them to return to work after total incapacitation, extending their lifespan [11]. Just like in the early years of the industrial revolution in Europe, improved health, better nutrition through obtaining the means of continuing to work, increased productivity of the mines and of the miners themselves, implied greater resilience, increase in longevity, and improved fertility in the high Andes. Human genetic potentials that could produce drastic changes in the relatively short span of 12–15 generations in Europeans during

the industrial revolution may also have been operative in the mining towns of the high Andes for more than 500 years. The Andean miners had the opportunity to adapt to enormous environmental pollution and now their descendants tolerate, relatively, but not entirely, unscathed, the persistent Hg in the Huancavelica biosphere. Nevertheless, our multilevel analyses clearly showed that even 21 generations is insufficient for complete adaptation to the horrendous mercury pollution.

How could such adaptation have occurred? We propose that an experimental model may have recently been described in fruit flies [29]. The microbiome, the symbiotic bacteria in the gut of the flies, profoundly affected their physiology. The molecular mechanisms that underlie the interactions of gut bacteria with their host are beginning to be unraveled in the flies [29].

In humans the microbiome is also closely linked to physiology and disease. Mercury resistant bacteria have been well recognized [30]. Since one route of entry of mercury in humans is through the gut, the microbiome might be the first to encounter the toxin and the bacteria adapt to increasing levels of ingested mercury. The survival of a functioning microbiome in the human gut like in the flies [29] is, in turn, closely linked to the survival of the host. Adaptation over a period of some 500 years to enormous environmental mercury loads in Huancavelica may have ultimately been the results of bacterial (microbiome) adaptation to mercury.

For this proposal it is necessary to invoke so-called transgenerational effects. These have been found in humans and animals [31]. In Sweden, for example, the nutritional and smoking habits of paternal grandparents could influence the health of their grandchildren and in Australia the mercury sensitivity of paternal grandparents who had pink disease as infants, affected their grandchildren [32]. This hypothesis is eminently testable in contemporaneous Huancavelica residents.

A third possible explanation is found in the theory of tolerance to disease or to environmental stressors such as toxins [33]. This theory has the most support from infectious diseases, where tolerance to bacterial invaders has been shown to offer evolutionary advantages by avoiding the costly means to eliminate the infectious agents. In this setting, tolerance to invaders offers a defense strategy that favors survival in heavily contaminated surroundings. Similarly, cellular responses to stress can offer survival advantages in stressful environments such as the hypoxia of altitude or excessive heat by inducing reactive oxygen species (ROS) or activating numerous heat-shock proteins, respectively.

Resistance and tolerance are thought to be complimentary strategies for living [33] also in heavily polluted environments such as in Huancavelica. Analyzing the molecular pathways which allow relatively normal survival in a heavily mercury, polluted environment after five hundred years of mercury exposure may offer insights into strategies of coping with the climate and environmental changes wrought by the anthropocene, the man-made world, we now live in.

8. Materials and Methods

The ethics committee of the New Mexico Health Enhancement and Marathon Clinics (NMHEMC) Research Foundation reviewed and approved the study. Informed written consent was obtained from all participants.

The hair was obtained from five Huancavelica females.

8.1. Study Limitations. This was a field study, and as such suffers from limitations common to such studies such as small number of subjects, poor adherence to study protocols, and low number of controls. Nevertheless, wherever statistical analyses were possible these were performed and gave clear and meaningful results. (See Text S1 in Supplementary Material available online at doi:10.1155/2012/472858).

8.2. Microfocused Synchrotron X-Ray Fluorescence Analyses. Microfocused synchrotron X-ray fluorescence analyses were performed at beamline X26A at the National Synchrotron Light Source, Brookhaven National Laboratory, Upton, NY, USA. Beamline X26A utilizes a bending magnet source on the NSLS 2.8 GeV electron storage ring, which operates at a current of 300 mA. For these experiments a monochromatic X-ray beam was used, monochromatized using a Si(111) channel-cut monochromator and tuned to an incident beam energy of 12.5 keV. The beam was focused to a spot size of 5 μm in the vertical x 8 μm in the horizontal using a pair of dynamically bent, grazing-incidence mirrors, each 100 mm long, arranged in a Kirkpatrick-Baez (KB) geometry. X-ray fluorescence from the human hairs was measured using a combined set of three energy dispersive detectors; a Canberra 9-element HPGe array detector placed 90° to the incident beam within the plane of the storage ring to minimize backgrounds from Compton scattering. The other two detectors are single element Radiant Vortex-EX silicon drift diode detectors also at 90° to the incident beam, but each sitting 45° above and below the plane of the storage ring, respectively. The 9-element HPGe array provides an active area of 900 mm^2 and each silicon drift diode detector has an active area of 50 mm^2. All eleven detector elements are integrated simultaneously using the XMap series of compact PCI-based digital spectrometers produced by X-ray Instrumentation Associates (XIA) interfaced through EPICS and controlled through in-house client software written in IDL. Incident beam intensity was monitored using an ion-chamber upstream of the focusing optics and all images were corrected for changes in incident beam flux through normalization to the change in ion-chamber counts over time.

8.3. Hydrogen Isotope Ratios. Hydrogen isotope ratios were determined using the continuous-flow high-temperature-reduction technique [34]. Hair was wrapped in silver foil and placed into the combustion chamber of a mass spectrometer using a Carlo Erba AS 200-LS autosampler. Three mm long sections of hair were sampled beginning at the proximal end. The stable isotopic compositions of low-mass elements such as hydrogen are reported as "delta" (Đ) values in parts per

thousand (‰). Đ values are calculated as follows: in (‰) = (R sample/R standard − 1) 1000, where "R" is the ratio of the heavy to the light isotope in the sample. The stable isotope standard for hydrogen is reported relative to the Standard Mean Ocean Water (SMOW). Isotope composition is reported in relation to this standard which has been defined as 0‰ [33].

8.4. Heart Rate Intervals. These were obtained from 2 minute EKG recordings using standard leads. Approximately 150 RR intervals were measured for each subject. The statistical analysis was similar to that used for the hydrogen isotope power spectra analysis (see below) and the frequency bands were identical to those chosen for the hydrogen isotope bands of the power spectra derived from the hair of the same subject.

8.5. Mandelbrot Sets. Data derived from each hair were entered into the Mandelbrot set-online generator by Dawid Makiela (http://www.Mandelbrot.ovh.org/)

X_1: mercury content; Y_1: low-frequency/high-frequency ratio of power spectrum; X_2: elemental content (Cu); Y_2: total power of low, mid- and high frequencies; X: total low power; Y: total high power; R: sum of low, mid, and high frequencies of the power spectrum obtained from the hydrogen isotope ratios along the length of each hair.

The functions were $Z_{n+1} = Z_n^2 + Z_0$. Maximum number of iterations was 100.

To strengthen our conclusions about biologic rhythms derived from the hair in Huancavelica residents we used an aged control from the USA. The variance of biologic rhythms decreases with advancing age [35] thus an old control subject, US resident, should serve better as a standard for comparison to the biologic rhythms derived from the hair of young mercury exposed subjects from Huancavelica.

8.6. Statistical Methods. In order to determine the relationship between metals in hair and the hydrogen isotope spectra in hair, we computed Pearson and Spearman correlations of each metal with measures of the spectra. The log-transformed quantities of Hg and Cu were found to be related to the spectral power in the high-frequency band We identify two clusters of high-frequency power (low and high power) in the Hg-Cu scatter plot of the 5 Huancavelica subjects (see Figure 1).

8.6.1. Growth Rate of Hair Calculations. There is a clear annual sinusoid in hydrogen isotope ratios in hair. The fact that periodicity of this sinusoid is 52 weeks allows us to compute annual growth rates in centimeters. Fitting the annual sinusoid for Huancavelica subject #3 by nonlinear regression yielded the function of length along the hair in cm:

$$\text{Predicted dD} = -91.1315 + 1.5832^{*} \sin(.8850^{*}\text{cm} + 1.3243). \quad (1)$$

The frequency of the sinusoid is 0.885 radians/cm. One can use the frequency of the fitted sinusoid to estimate the growth rate that matches the 0.0193 cycles/week = 1 cycle/52 weeks, whose periodicity is 52 weeks. So (freq/2π)*(growth/year)/(52 weeks/year) set equal to 1 cycle/52 weeks implies the following:

$$\frac{\text{growth}}{\text{year}} = \frac{2\pi}{(\text{frequency of fitted annual sinusoid})}. \quad (2)$$

Here growth/year = 2π/.885 = 7.1 cm/year. The average growth rate for the 5 Huancavelica subjects was 6.4 ± 1.7 SD cm/year. This is compared to the average growth rate of ~16 cm/year in human hair by a one-sample t-test (see Figure 2).

8.6.2. Spectral Variance. All power spectra were computed using the finite Fourier transform, decomposes time series into a sum of sine and cosine waves of different amplitudes and wavelengths (PROC SPECTRA from SAS). The spectral variance for a given time series of hydrogen isotope ratios in hair is the total power (area under the periodogram). The comparison of these standard deviations (square root of the variance) for the 5 Huancavelica subjects to our sample of controls are made by a two-sample t-test (see Figure 3).

The spectral power in the low-frequency band and their standard errors are computed as described in Priestly [36]. These are then compared by t-test (see Figure 4).

8.6.3. Periodicities. Fast and slow periods of hair growth in weekly cycles is computed from the frequencies of higher power observed in the periodogram. The form of the calculation is as follows:

$$\text{Period} = \frac{dt}{(\text{freq})\,(\text{growth})}, \quad (3)$$

where dt is the increment of the series (see Figure 5).

For Figure 6, the power spectra for both heart rate interval (RR) and hydrogen isotope ratio time series measured in the same 5 Huancavelica subjects (the 5 series are stacked) are superimposed.

Acknowledgments

Thanks are due to Dr. Uriel García, Cáceres, Universidad Peruana Cayetano Heredia Lima, Peru, for encouraging this work. The authors are grateful to the directors of the Hospital Regional de Huancavelica, Drs. Juan Gomez Límaco and Julio Alvarez León for allowing the study. The authors thank Ms. Isidora Cauchos Espinoza, at the HRH's Epidemiology and Public Health Department for her support. Especial thanks to the volunteers who participated in the study and to the miners from the distant past of the Villa Rica de Huancavelica who inspired this study. The study was funded through a grant provided by the New Mexico Health Enhancement and Marathon Clinics Research Foundation (NMHEMC Research Foundation) # 15–2010. The authors have declared that no conflicts of interests exist.

References

[1] The Blacksmith Institute, "The World's Worst Polluted Places," The Top Ten of the Dirty Thirty, New York: 50, 2007, http://www.blacksmithinstitute.org/.

[2] M. C. Monge, "La enfermedad de los Andes," *Anales de la Facultad de Medicina de Lima*, vol. 11, pp. 76–151, 1928.

[3] G. Xing, C. Qualls, L. Huicho et al., "Adaptation and mal-adaptation to ambient hypoxia; Andean, Ethiopian and Himalayan patterns," *PLoS ONE*, vol. 3, no. 6, Article ID e2342, 2008.

[4] K. W. Brown, "Workers' health and colonial mercury mining at Hhuancavelica, Peru," *Americas*, vol. 57, no. 4, pp. 467–496, 2001.

[5] V. Lohmann, *Minas de Huancavelica*, 1577.

[6] G. de Carvajal, A. de la Cerda, and M. Adrián, "To the King, AGI, Indiferente General 314," 1575.

[7] D. de Jeria, "Report of Damián de Jeria," AGI Lima 34, 1604.

[8] L. de Velasco, "Velasco to the King," AGI, Lima 34, 1600.

[9] L. de Velasco, "Velasco to the King," AGI, Lima 34, 1601.

[10] L. J. de Chinchón C, "Chinchón to the King AGI," 1630, Indiferente General 1777.

[11] A. Ulloa, "Noticias americanas," p.222, 1760.

[12] P. Camargo, "Pedro Camargo to the king," AGI, Lima 35, 1595.

[13] J. G. Omichinski, "Toward methylmercury bioremediation," *Science*, vol. 317, no. 5835, pp. 205–206, 2007.

[14] T. Tsuda, T. Yorifuji, S. Takao, M. Miyai, and A. Babazono, "Minamata disease: catastrophic poisoning due to a failed public health response," *Journal of Public Health Policy*, vol. 30, no. 1, pp. 54–67, 2009.

[15] L. E. Davis, M. Kornfeld, H. S. Mooney et al., "Methylmercury poisoning: long-term clinical, radiological, toxicological, and pathological studies of an affected family," *Annals of Neurology*, vol. 35, no. 6, pp. 680–688, 1994.

[16] O. Appenzeller and E. Oribe, *The Autonomic Nervous System; An Introduction to Basic and Clinical Concepts*, Elsevier, Amsterdam, The Netherlands, 5th edition, 1997.

[17] L. Friberg and J. Vostal, "General discussion and conclusions need for further research," in *Mercury in the Environment*, L. Friberg and J. Vostal, Eds., pp. 183–186, Chemical Rubber Company Press, Cleveland, Ohio, USA, 1972.

[18] A. A. Gordus, C. C. Maher, and G. C. Bird, "Human hair as an indicator of trace metal environmental exposure," Tech. Rep., National Laboratory, Oak Ridge, Tenn, USA, 1974, Proceeding of the 1st Annual NSF Trace Contaminants Conference, August 1973.

[19] D. Airey, "Mercury in human hair due to environment and diet: a review," *Environmental Health Perspectives*, vol. 52, pp. 303–316, 1983.

[20] Anonymous, Nature 442: VII, 2006.

[21] O. Appenzeller, C. Qualls, F. Barbic, R. Furlan, and A. Porta, "Stable isotope ratios in hair and teeth reflect biologic rhythms," *PLoS ONE*, vol. 2, no. 7, article e636, 2007.

[22] K. L. Rasmussen, J. L. Boldsen, H. K. Kristensen et al., "Mercury levels in Danish Medieval human bones," *Journal of Archaeological Science*, vol. 35, no. 8, pp. 2295–2306, 2008.

[23] S. Barrett, "Commercial hair analysis: acardinal sign of quackery," *Quackwatch*, 2001.

[24] M. D. McDonnell and L. M. Ward, "The benefits of noise in neural systems: bridging theory and experiment," *Nature Reviews Neuroscience*, vol. 12, no. 7, pp. 415–425, 2011.

[25] O. Appenzeller, H. C. Gunga, C. Qualls et al., "A hypothesis: autonomic rhythms are reflected in growth lines of teeth in humans and extinct archosaurs," *Autonomic Neuroscience*, vol. 117, no. 2, pp. 115–119, 2005.

[26] G. B. West, J. H. Brown, and B. J. Enquist, "A general model for the origin of allometric scaling laws in biology," *Science*, vol. 276, no. 5309, pp. 122–126, 1997.

[27] M. Sargent, "The growth of nations," *Nature*, vol. 474, article 448, 2011.

[28] R. Floud, R. W. Vogel, B. Harris, and S. C. Hong, *The Changing Body: Health, Nutrition and Human Development in the Western World Since 1700*, Cambridge University Press, 2001.

[29] S. C. Shin, S.-H. Kim, H. You, B. Kim, A. C. Kim et al., "Drosophila microbiome modulates host development and metabolic homeostasis via insulin signaling," *Science*, vol. 334, pp. 670–674, 2011.

[30] Y. Wang, I. Mahler, H. S. Levinson, and H. O. Halvorson, "Cloning and expression in Escherichia coli of chromosomal mercury resistance genes from a *Bacillus sp*," *Journal of Bacteriology*, vol. 169, no. 10, pp. 4848–4851, 1987.

[31] S. E. Mango, "Ageing: generations of longevity," *Nature*, vol. 479, no. 7373, pp. 302–303, 2011.

[32] K. Shandley and D. W. Austin, "Ancestry of pink disease (infantile acrodynia) identified as a risk factor for autism spectrum disorders," *Journal of Toxicology and Environmental Health A*, vol. 74, no. 18, pp. 1185–1194, 2011.

[33] R. Medzhitov, D. S. Schneider, and M. P. Soares, "Disease tolerance as a defense strategy," *Science*, vol. 335, no. 6071, pp. 936–941, 2012.

[34] USGS Menlo Park California Resources on Isotopes, "Fundamentals of Stable Isotope Geochemistry," 2006.

[35] O. Appenzeller and E. Oribe, *The Autonomic Nervous System; An Introduction to Basic and Clinical Concepts*, Elsevier, Amsterdam, The Netherlands, 5th edition, 1997.

[36] M. Priestly, *Spectral Analysis and Time Series*, Academic Press, 7th edition, 1981.

Hindawi Publishing Corporation
Journal of Biomedicine and Biotechnology
Volume 2012, Article ID 815186, 9 pages
doi:10.1155/2012/815186

Research Article

The Effects of Chronic Ingestion of Mercuric Chloride on Fertility and Testosterone Levels in Male Sprague Dawley Rats

John C. Heath,[1] Y. Abdelmageed,[1] Tim D. Braden,[2] and Hari O. Goyal[1]

[1] *Department Biomedical Sciences, College of Veterinary Medicine, Nursing and Allied Health, Tuskegee University, Tuskegee, Al 36088, USA*
[2] *Department of Anatomy, Physiology and Pharmacology, College of Veterinary Medicine, Auburn University, Auburn, Al 36049, USA*

Correspondence should be addressed to John C. Heath, jheath@mytu.tuskegee.edu

Received 21 March 2012; Accepted 6 May 2012

Academic Editor: João B. T. Rocha

Although male infertility is well researched, the effects of inorganic mercury on male reproduction and fertility are less well known. Studies pertaining to mercury and male fertility identified reduced concentration of testosterone in the serum of male workers, a toxic influence on fertility of organic mercury compounds within concentrations at the workplace, and increased days to pregnancy. We evaluated the effect of chronic mercuric chloride ($HgCl_2$) exposure in male rats on reproductive endpoints. Thirty-day old male Sprague Dawley rats ($n = 31$) were exposed to 0.0, 1.0, or 2.0 mg/kg/day of $HgCl_2$ via gavage. After 60 days exposure, they were housed with nonexposed females for 21 days. A survivor analysis revealed the exposed animals took longer to impregnate the females and had a lower rate of impregnation. Further statistical analysis revealed a lower correlation between testicular testosterone levels and days to impregnate, and also lower sperm counts in the epididymis head and body of the exposed males. The results indicate that $HgCl_2$ exposure had significant adverse effects on male rat reproduction endpoints including fertility at a dose that was not clinically toxic.

1. Introduction

Although male infertility is well documented as a result of exposure to numerous toxicants, the effects of inorganic mercury on male reproduction and fertility are less well known. A 2008 study on the outcome of various heavy metals in relation to semen quality reported data on human nonoccupational exposure to mercury (Hg), and its reproductive outcomes are very sparse [1]. An earlier review of the consequences of mercury exposure in the workplace on fertility and related reproductive outcomes found only three studies pertaining to male fertility [2], which were ambiguous at best. Two studies found effects by establishing the toxic influence on fertility of organic mercury compounds within concentrations that can be seen at the workplace [3] and reduced concentration of testosterone in the serum of male workers, considered to be associated with exposure to inorganic mercury [4]. The third study, looking directly at mercury exposure, found no effects on male fertility; however, the authors relied on the memory of both workers and spouses to obtain data [5]. An additional study found no effects on fertility when workers chronically exposed to mercury vapor were assessed by questionnaire [3].

Studies in Hong Kong reported more than one-third of male partners of infertile couples had elevated blood mercury concentrations. Although the results were not statistically significant, a trend towards a decline in all aspects of semen quality when the blood mercury concentration was elevated was reported [6, 7]. Similarly, workers exposed to Hg vapor, which frequently exceeded the maximum allowable concentration, had hypospermia and a considerable increase in the number of abnormal sperm in the ejaculate [8].

An in vivo study using adult male mice found a reduction in sperm count in the testis, vas deferens, and cauda epididymis revealing the inhibitory effects of mercuric chloride ($HgCl_2$), on spermatogenesis [9]. Rats exposed to $HgCl_2$ for 81 days experienced a reduction in the weights of the left and right testes and prostate in a high-dose group, and the seminal vesicles weights, in mid and high-dose groups, were significantly decreased when compared to controls [10].

Male fertility can be impaired by various toxicants known to target Sertoli cells, which play an essential role in spermatogenesis. Sertoli cells from male Sprague-Dawley rats exposed in vitro to mercury had severely inhibited inhibin production [11]. Bull spermatozoa exposed to 50–300 μmol/L concentration range of $HgCl_2$ revealed reduced sperm membrane and DNA integrity [12]. A lipoperoxidation test performed on sperm membranes from normospermic individuals showed mercury in different concentrations and induced a significant peroxidation to the human sperm membranes in a concentration-dependent manner. Excessive amounts of mercury in the seminal plasma appear to be related to abnormal spermatozoal function [13]. Furthermore, epididymal spermatozoa obtained from adult rats and incubated with $HgCl_2$ for 3 hours showed markedly reduced motility, plus other characteristics, that point to oxidative stress by the $HgCl_2$ in a dose-dependent manner [14].

The above-cited research demonstrates that in human studies there is some evidence inorganic mercury has an effect on male fertility. This is supported by both in vivo and in vitro studies, which indicate strong evidence that $HgCl_2$ has an effect on the development of sperm together with the integrity of the sperm after spermogenisis [9–14]. However, what form the effect on fertility due to inorganic mercury has on males has not been adequately reported. The study described here reports the effects on fertility of exposure to mercuric chloride in male Sprague Dawley in terms of both the reduced probability of the male to impregnate a female in a dose-dependent fashion, plasma and testicular testosterone levels, and the correlation between the two.

2. Method

2.1. Subjects. Thirty-one male Sprague Dawley rats, 22 days of age, were purchased from Harlan Sprague-Dawley (Indianapolis, IN). Each subject was randomly assigned to one of three experimental groups, control, low $HgCl_2$, and high $HgCl_2$. They were housed in polycarbonate cages (two per cage) on beta chip bedding (Northeastern Products, Warrensburg, NY) with cage mates belonging to the same experimental group. Subjects were given access to Purina certified rodent chow 5001 (Purina Mills, St. Louis, MO) and water ad libitum. Room conditions were controlled at 22°-23°C ambient temperature, 50%–60% relative humidity, and 12 hour light/dark cycle. All conditions conformed to the regulations of, and were approved by, the Tuskegee University Animal Care and Use Committee.

2.2. Preparation of $HgCl_2$ Solutions. A concentration of 1.0 mg/mL was prepared using 1 gram of mercuric chloride ($HgCl_2$; SIGMA-Aldrich Inc, St. Louis, MO) added to a solution of 998.5 mL of de-ionized H_2O and 1.5 mL of nitric acid HNO_3 (to aid dilution). Similarly, a concentration of 0.5 mg/mL solution was prepared with 0.5 mg of $HgCl_2$. added to a solution of 998.5 mL of de-ionized H_2O and 1.5 mL of nitric acid HNO_3.

2.3. Administration of $HgCl_2$. At age 30 days, subjects in the $HgCl_2$ groups began exposure to the solutions of mercuric chloride ($HgCl_2$) in concentrations of 1.0 mg/kg/day (low Hg) or 2.0 mg/kg/day (high Hg) via gavage. Solution administered each day ranged from 0.48 to 7.2 mL dependent on the weight of the animal and the exposure concentration. Controls were given an equivalent amount of de-ionized water (with no nitric acid added) using the same administration method. The amount of $HgCl_2$ administered each day, to each subject, was calculated based upon its weight and exposure group. Each subject was weighed three times per week and the dose was adjusted accordingly. The solution was administered seven days a week via gavage until the subjects reached 90 days of age thus, total exposure was for 60 days. Concentration of exposure to $HgCl_2$, route of administration, and exposure period were selected to mimic exposure paradigms used in previous published studies from our lab [10, 15]. In Atkinson et al. [10], the maximum tolerated dose (MTD) was obtained from prior studies [16, 17] and the National Toxicology Program Technical report [18]. Based on these data concentrations of 1.0 mg/kg/day and 2.0 mg/kg/day were used in our study on female rats [15], the higher dose being the highest dose we found that did not show a manifestation of physical signs of mercuric chloride exposure. The exposure paradigm used in this study allows us to do a direct comparison to the effects of exposure in female rats.

2.4. Breeding Females. Thirty-one female Sprague Dawley rats aged 80 days were purchased from the same supplier as the males. The females were acclimated for 10 days and then housed in the same conditions as the males, two to a cage, and fed the same diet. None of the females were purposefully exposed to $HgCl_2$. No analysis of the females, other than period from cohabitation to fertilization, was performed, and all females were euthanized at the completion of the study.

2.5. Breeding Protocol. At 90 days of age, exposure to $HgCl_2$ ceased and each male was housed with an unexposed female for 21 days. All females were at the same stage of the estrous cycle at the beginning of their cohabitation with males. After 21 days, the breeding pairs were separated and the males were euthanized using CO_2 inhalation after which necropsies were performed. The females were observed for a further 21 days and all pregnant females were allowed to come to term.

2.6. Plasma and Testicular Testosterone. For plasma testosterone, one blood sample was collected by cardiac puncture from each animal just prior to necropsy. For intratesticular testosterone, a part of the right testis was collected from each animal at the time of necropsy. Both plasma and testicular parenchyma were frozen at −20°C until assayed. Testes were processed in accordance with the protocol described by Park et al. [19]. Briefly, 10–30 mg of testicular tissue was homogenized in phosphate-buffered saline (PBS; 0.01 M phosphate, pH 7.4). Eight volumes of ether were added to the homogenate and vortexed vigorously. The aqueous phase was snap-frozen, and the organic supernatant was transferred to a secondary tube and air-dried. Just prior to assay, samples were resuspended in phosphate-buffered saline. Testosterone

Table 1: Mean weights of subjects across exposure period.

	Days of $HgCl_2$ exposure																			
	1		6		13		20		27		34		41		48		55		59	
Exposure	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE
0.0 $HgCl_2$	85	2.0	144	3.3	194	4.4	240	4.6	280	5.3	315	5.7	341	6.8	366	7.4	369	6.8	364	4.9
1.0 $HgCl_2$	82	2.3	140	3.5	187	4.2	231#	4.1	266#	4.1	296#	5.8	319#	5.4	340#*	5.7	341#*	5.4	349#	5.4
2.0 $HgCl_2$	79	3.3	131	3.8	174*	5.0	212*	5.1	241*	6.4	271*	8.0	281*	7.7	307*	8.0	310*	8.3	319*	7.9

Mean columns: mean weight in grams for an exposure group on a particular day of exposure. SE: standard error of the mean. *: significant difference from controls. #: significant difference from 2.0 mg/kg $HgCl_2$ group. Post hoc test using fisher LSD test. $P < 0.05$.

in plasma and testicular tissue was measured using a COAT-A-COUNT testosterone radioimmunoassay (Diagnostic Products Corporation, Los Angeles, CA) according to manufacturer's protocol. The sensitivity of the assay was 0.2 ng/mL. The intra-assay and interassay coefficients of variations were 6% and 12%, respectively.

2.7. Preparation of Samples. The testes were homogenized in 50 mL PBS for 30 seconds, filtered, and 1.5 mL was placed in a separate container. The same process was performed on the epididymis head and body, as well as the epididymis tail.

2.8. Analysis of Samples. Samples were prepared for sperm count by mixing 100 μL of filtered homogenate with 100 μL of trypan blue and 50 μL of saline. The sample substance was kept overnight for better result. Eight to ten μL of the mixture was spread on a hemocytometer and the sperm count was conducted under a light microscope. Two samples of each were prepared and counted to ensure accurate data.

2.9. Data Analysis. Data on when the females were impregnated is incorporated in Stratified Kaplan-Meier estimates followed by log-rank tests, which were conducted to determine the probability of an exposed male impregnating a female. The independent variable was the period of time that the $HgCl_2$-exposed male cohabited with the female before she became pregnant. The day of impregnation was calculated by subtracting the length of pregnancy (22 days) from the total period from the beginning of cohabitation until birth of the offspring. Subjects were right censored if no birth occurred. Right censoring is required in survival analysis to indicate the subject was not in the experiment long enough to experience the measured variable, that is, the male did not impregnate the female.

Body weight was analyzed using repeated measures ANOVA 3 ($HgCl_2$) $\times$ 8 (length of exposure) factorial design, with duration of exposure as the repeated measure. The first weight taken and every subsequent fourth weight taken were used as the repeated measure. Other variables were analyzed using one-way ANOVA 3 ($HgCl_2$) $\times$ quantity or number of measured variable. When a significant difference was observed, a post hoc analysis, as described below, was performed to identify the differences between the groups. All results were considered significant at $\alpha = 0.05$. A Pearson product-moment correlation analysis with a Bonferroni correction for probability was performed between latency to impregnate the female by the male and testicular testosterone level.

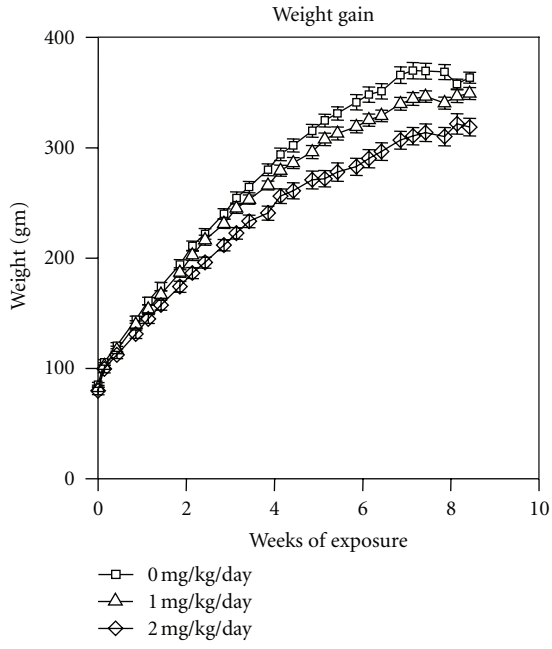
3. Results

3.1. Body Weight. A repeated measures ANOVA revealed both a main effect of $HgCl_2$ ($F_{(2,28)} = 14.07$, $P < 0.001$) and an interaction between $HgCl_2$ concentration and exposure duration ($F_{(16,252)} = 14.07$, $P < 0.001$). There was also a significant difference in weight at necropsy between the exposure groups ($F_{(2,27)} = 7.193$, $P = 0.003$). Post hoc analysis using a Bonferroni correction revealed a significantly lower weight for the high Hg compared to controls ($P = 0.003$), and the low Hg ($P = 0.045$), but not between the controls and the low Hg ($P = 0.525$), see Figure 1. Table 1 shows the weights and significant difference in weights during the progression of exposure. The high Hg group was significantly different from the control group after 13 days exposure but remained statistically equal to the low Hg exposure group until 20 days of exposure.

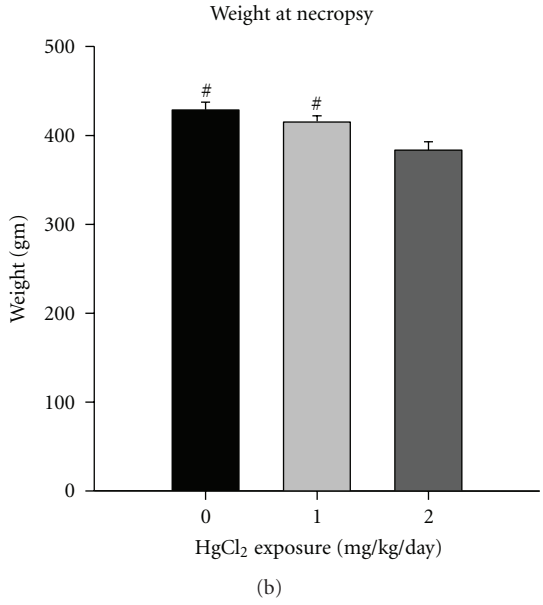
3.2. Latency to Impregnation. Within the three exposure groups 100% (10/10), unexposed females from the control $HgCl_2$ group gave live birth. In the low Hg and high Hg groups, 10/11 and 7/10 unexposed females, respectively, gave live birth. Figure 2 displays the Kaplan-Meier survival curves showing the probability of a male impregnating an unexposed female. Subjects were right censored if they failed to impregnate within 21 days of mating. The control group impregnated the females within 13 days of mating, (mean days to impregnation 5.9). The low Hg group had an impregnation rate of 91% (10/11) with one animal failing to impregnate (mean days to impregnation 7.5). The high Hg group had an impregnation rate of 70% (7/10), with a mean period to impregnate of 12.5 days. A log-rank test revealed the high Hg group had a significantly greater probability of not impregnating a female ($\chi^2_{(1,19)} = 5.29, P = 0.021$) than the control group.

3.3. Testosterone Levels

3.3.1. Testicular Testosterone. A one-way ANOVA 3 ($HgCl_2$) $\times$ testicular testosterone levels per gram of testes tissue revealed a significant difference ($F_{(2,26)} = 5.684, P = 0.009$). Post hoc analysis using the Tukey honest significance test (Tukey's HSD test) revealed that both exposure groups had significantly lower levels of testicular testosterone than



(a)



(b)

Figure 1: Body weight gain (a) and weight at necropsy (b) of male rats exposed to 0, 1.0, or 2.0 mg/kg/day of $HgCl_2$ by gavage for 60 days and housed with unexposed female rats for 21 days. Data are mean and standard error. (b) Weight at necropsy. #A significant difference from the $HgCl_2$ group 2.0 mg/kg ($P < 0.05$).

controls (low Hg, $P = 0.016$, high Hg, $P = 0.023$); however, the exposure groups did not differ from each other. Figure 3 shows the levels of testicular testosterone (a) and the distribution across exposure levels (b). While the controls show higher levels of testicular testosterone, both Hg groups are similar except 30% of the subjects in the high group had lower testicular testosterone levels than subjects in the low Hg group.

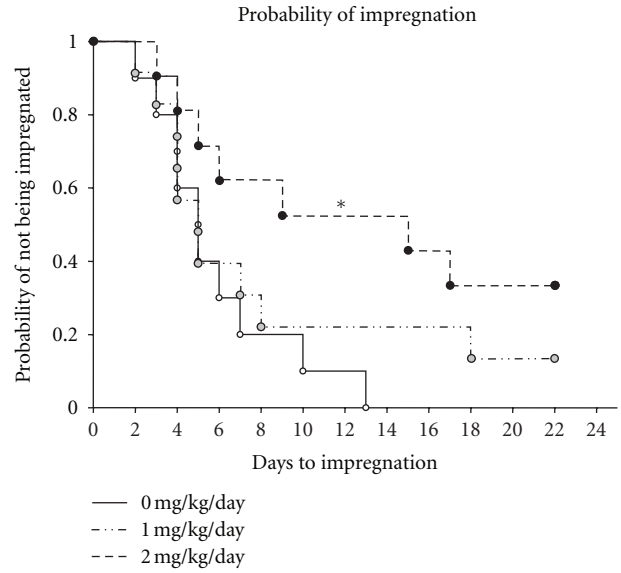


Figure 2: Kaplan-Meier survival curves. Probability of an exposed male impregnating an unexposed female. *Indicates a significant difference from the control group. $P < 0.05$.

3.3.2. Plasma Testosterone. A one-way ANOVA conducted on plasma testosterone levels across exposure groups revealed no significant difference in levels at $\alpha = .05$; however, P values were approaching significance ($F_{(2,26)} = 3.102$, $P = 0.062$). A post hoc test using the least square difference (LSD) method showed that the low Hg group had a significantly lower level of plasma testosterone than controls. However, it should be noted the LSD method is a somewhat less conservative test of analysis. Inspection of the distribution of plasma testosterone (see Figure 4) shows a marked higher level of testosterone in the control group, with both exposure groups in a similar lower range. Distribution of plasma testosterone across the Hg groups is very similar to the distribution of testicular testosterone.

3.4. Correlation of Testosterone and Latency to Impregnate. Pearson correlation coefficients, with Bonferroni corrected P values, were calculated between both testicular and plasma testosterone levels and days required to impregnate an unexposed female. For controls, testicular testosterone levels were inversely correlated to latency to impregnate ($r = -0.80$, $P = 0.012$) indicating that as testicular testosterone levels decreased the days taken to impregnate increased. However, for the low Hg group the Pearson coefficients were considerable lower, positive, and not significant ($r = .47$, $P = 0.411$), while for the high Hg group, there was no correlation ($r = .07$, $P = 1.0$), (Figure 5). A similar pattern was observed for plasma testosterone levels except the correlation coefficients were lower than for testicular testosterone, and none of the correlations were significant when using the Bonferroni correction, controls ($r = -.48$, $P = 0.32$), low Hg ($r = 0.29$, $P = 0.89$), high Hg ($r = .08$, $P = 1.0$). A further Pearson coefficient was calculated on the results of the testicular testosterone and plasma testosterone against latency to

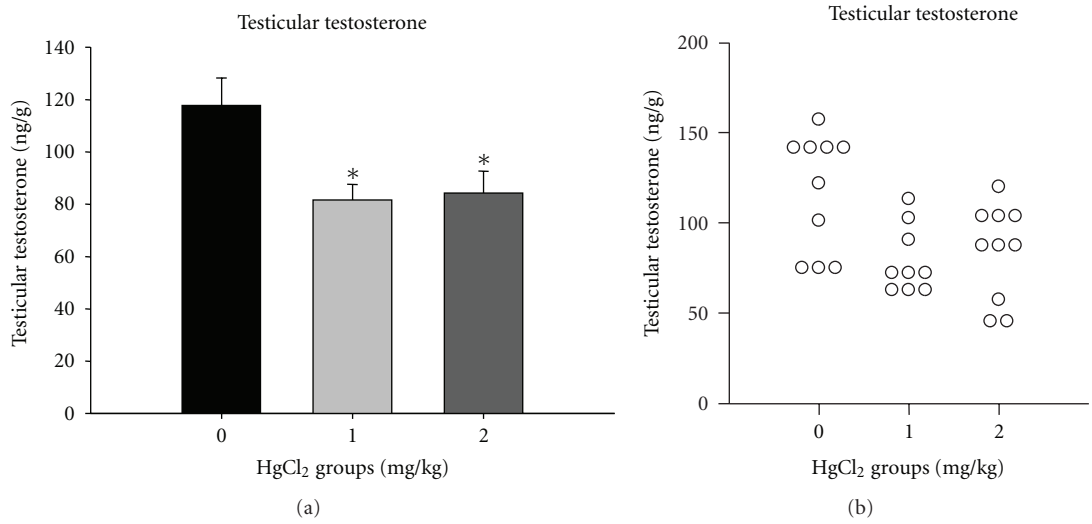


Figure 3: Testicular testosterone levels (a) levels of testes testosterone (ng/gm) for each exposure group. (b) Distribution of testes testosterone for each exposure group. Data are mean and SE. *Represents a significant difference from controls. $P < 0.05$.

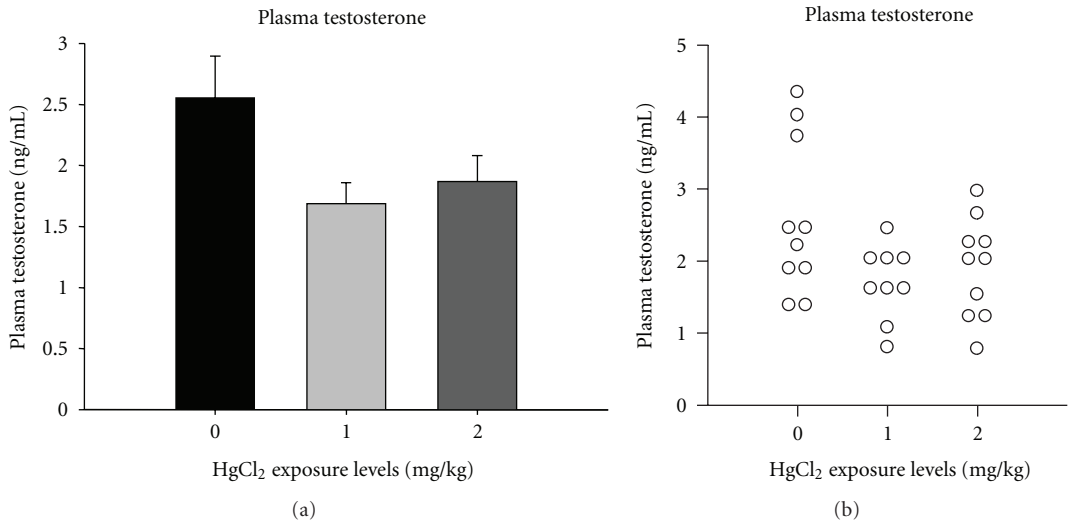


Figure 4: Plasma testosterone levels (a) levels of blood plasma testosterone (ng/g) for each exposure group. (b) Distribution of blood plasma testosterone for each exposure group. Data are mean and SE. No significant difference was found between groups.

impregnate. Results showed a high correlation ($r = 0.9996$, $P = 0.17$) between these results.

3.5. Sperm Count of Epididymus Head and Body. A one-way ANOVA conducted across the sperm count of the epididymis head and body for the three $HgCl_2$ concentrations showed a significant reduction in the sperm count ($F_{(2,27)} = 4.428$, $P = 0.022$) (Figure 6). Post hoc analysis revealed that both the low ($P = 0.048$) and the high ($P = 0.008$) $HgCl_2$ groups had significantly lower sperm counts than the controls.

4. Discussion

The present study was designed to address the effects on fertility of mercuric chloride ($HgCl_2$) toxicity in growing male rats. There were five main findings. There was an increased latency of the exposed males to impregnate the females as exposure concentrations increased. A decrease in the number of females impregnated was observed as $HgCl_2$ exposure concentrations increased. Both testicular and plasma levels of testosterone decreased in the exposed animals. Correlation between testicular testosterone levels and the latency to impregnate a female decreased and lost its significance as $HgCl_2$ exposure concentrations increased. Sperm count in the epididymis head and body showed a significant decrease with exposure to $HgCl_2$.

Previous studies have reported the effects of mercury (Hg) toxicity on fertility in the male human populations [2–5] and in various animal populations [9–12]; however, for inorganic mercury although the underlying mechanisms are relatively well understood, how inorganic mercury toxicity in male fertility manifests itself has remained relatively obscure.

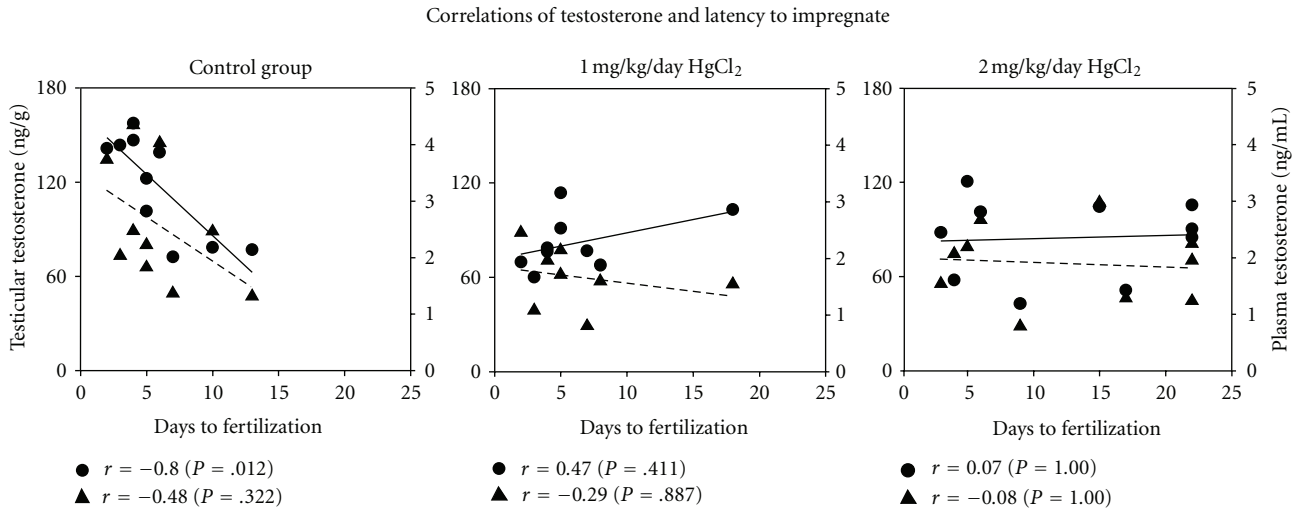


Figure 5: Correlation of testicular testosterone levels and plasma testosterone levels to days required to impregnate an unexposed female. (a) controls, (b) 1.0 mg/kg/day $HgCl_2$, (c) 2.0 mg/kg/day $HgCl_2$. • Indicates testicular testosterone levels; ▲ indicates plasma testosterone levels.

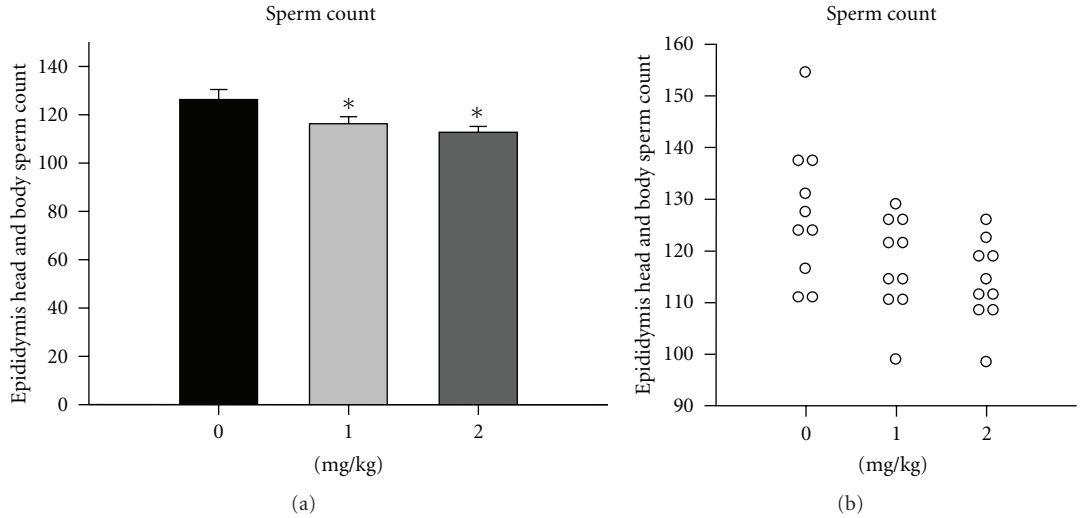


Figure 6: (a) Sperm count of the epididymis head and body at necropsy for each $HgCl_2$ exposure group. (b) Distribution of the epididymis head and body sperm count at necropsy for each exposure group. Data are mean and SE, *: a significant difference from the control group ($P < 0.05$). Scale on "y-axis" differs for each chart to give better representation of data.

The concentrations of mercuric chloride used in this study were selected to mirror the doses that we reported in a similar study using female rats [15] thus giving us the opportunity to show a direct comparison in effects across the sexes. As in the case of the females, no adverse physical signs of Hg toxicity were observed nevertheless, there was a decrease in weight gain in the exposed groups and significantly lower body weights for the high $HgCl_2$ group compared to controls at necropsy (see Table 1 and Figures 1(a) and 1(b)). This was in contrast to the females in which both exposure groups had significantly lower weight gains from controls [15]. Even though no overt physical signs were observed, the exposure concentrations used in this study cannot be construed as a safe level of intoxication, and cannot be extrapolated to a human population. A previous study reported that internal damage from mercury exposure can cause increased cardiovascular risk. Chronic exposure to low concentrations of mercury (1 dose 4.6 μg/kg, subsequent doses of 0.07 μg/kg/day for 30 days) in Wistar rats promotes endothelial dysfunction as a result of the decreased nitric oxide bioavailability induced by increases in oxidative stress [20]. However, they do tend to support, in rats, a concentration of $HgCl_2$ that does not convey any outward physical signs of toxicity, other than weight change, when administered as described in this experiment. This does not mean, however, that internal damage did not occur due to $HgCl_2$ toxicity.

4.1. Increased Latency of the Exposed Males to Impregnate the Females. Our results showed a significant increase in the length of time needed for exposed males to impregnate

unexposed females. Concurrently, as the exposure concentration increased, a decrease was observed in the number of females that were impregnated. These data support the findings that a lower mating index was found in a dose-response fashion in males rats exposed to $HgCl_2$ in their drinking water [21] as well as a lower fertility index for males [10].

4.2. Changes in Levels of Testosterone. While testicular testosterone levels were significantly different from each other, plasma testosterone levels were not. However, the distributions for both measures were remarkably similar and plasma testosterone levels were approaching significance (see Figures 3(a), 3(b), 4(a), 4(b)). One of the few measures that have been reported as an effect on the male fertility in human populations is a reduction in circulating testosterone levels. In male workers exposed to inorganic mercury and showing a mean blood mercury level of 46 nmol/L, a statistically significant positive correlation between total testosterone and the cumulative mercury dose index was observed even when other factors such as age and smoking were taken into account [4]. However, in a study on subfertile men in Hong Kong no statistical difference in circulating testosterone levels were found for subjects with 50 nmol/L ($\sim$10 μg/L) or higher blood Hg levels, though the authors attribute the lack of statistical significance to a small sample size, [7]. In a recent study, rats exposed orally to concentrations of 50 and 100 ppm $HgCl_2$ for 90 days recorded almost identical serum testosterone levels as our animals at 2.02, 1.72, and 1.62 ng/mL for controls, 50 and 100 ppm $HgCl_2$, respectively [21]. Similar results were seen in two other studies that exposed adult male albino mice to 1.25 mg/kg of $HgCl_2$ daily for 30 days [9] or 45 days [22] by gavage. In the first study, serum testosterone from controls was recorded at 4.50 ng/mL, while exposed animals had a level of 2.6 ng/mL. After a withdrawal from the $HgCl_2$ for 45 days, serum testosterone had recovered to 4.1 ng/mL [9]. In the second study, serum testosterone levels in controls were very similar (4.21 ng/mL), while the exposed animals showed a level of 1.64 ng/mL. Interestingly, in this study, after 45 days of recovery the testosterone level had only recovered to 2.2 ng/mL [22]. Although a different species of animal was used in these studies, our results tend to support the findings both in levels of testicular testosterone reported and in correlation to exposure to $HgCl_2$.

4.3. Correlation of Testosterone and Latency to Impregnate. A unique finding in our study was the correlation between testicular testosterone levels and the latency to impregnate. As far as we are aware, this is the first time this particular comparison has been made. For testicular testosterone, within the control group the correlation coefficient was very high and significant; however, as the dose of $HgCl_2$ increased the correlation became lower and lost its statistical significance. This is evident in the correlation of the low Hg group, which was lower than the controls and not significant, and the absence of any correlation in the high Hg group. Plasma testosterone levels, although having lower correlation coefficients' which were not significant, nevertheless, followed the same pattern as testicular testosterone results. This was confirmed by the high correlation between the two measures of testosterone and latency to impregnate indicating the results were very similar. This would suggest that while testosterone levels played an important factor in the time taken for the animals to mate in the control group, it became a less important factor as $HgCl_2$ concentrations increased. This information presents two possibilities. Either $HgCl_2$ reduces the amount of testosterone to the point that it no longer influences sexual activity, or the effect of $HgCl_2$ exposure has a large enough effect on the mechanisms of reproduction, that is, a lowering of the sperm count, as to overcome repeated copulation. As is discussed later, it appears that morphological damage to spermatozoa is the greater factor affecting male reproduction, when mercury exposure has occurred, rather than a loss of libido.

4.4. Sperm Count of Epididymis Head and Body. A second factor contributing to the lower fertility and the increase of latency to impregnate was the lower sperm count in the epididymis head and body. Both exposed groups had significantly lower counts than the control group, (see Figure 6). Inspection of the distribution for the three groups indicates that there may be a dose-response effect, although it should be emphasized that there was no statistical difference between the two mercury-exposed groups. Exposure of mice to 1.25 mg/kg of $HgCl_2$ by gavage for 45 days has been shown to significantly reduce the epididymis sperm count compared to controls while also reducing serum testosterone levels [22]. The same results were seen in another study administering the same dose for 30 days [9]. In both cases, the mating rate for the $HgCl_2$ treated mice was zero [22]. A study on the reproductive effects of methylmercury in male rats showed a decrease in testicular sperm count of 27% after 14 days exposure, while also recording a decrease in testosterone levels after 6 days [23]. These results tend to concur with our study and support our findings.

The present study was designed to look at the effects of $HgCl_2$ on fertility in growing male rats and not as a mechanistic analysis. However, close comparison with a recent report that does speculate on the mechanisms possibly responsible for the observed results is interesting.

Boujbiha, in his 2009 study, found very similar results to our study [21] including decreased testosterone, a decrease in mating index, and a lower viable implantation rate. We report a significantly longer period to impregnate control females by high exposure animals and a lower number of impregnations. Thus the data from the two studies tends to support each other even though there are significant methodological differences between the studies.

Studies of human populations into the effects of inorganic mercury on male fertility rates are very sparse. The majority of such studies focus on the mechanistic effects of mercury [4, 24, 25]; however, there is some evidence of reduced fertility in males due to mercury intoxication. More than one-third of male partners of infertile couples in Hong Kong had elevated blood mercury concentrations. As the authors of this study stated "From a public heath perspective, these findings confirm that mercury toxicity is a potentially serious problem affecting the local community," [7].

A study that looked at the environmental contaminant levels and fecundability on first pregnancies among nonsmoking couples reported that the partners of males who had blood Hg levels between 0.7–1.0 and 1.9–4.8 μg/L had a greater time to pregnancy (TTP), 9.9 and 8.1 months, respectively, than a reference group with blood Hg levels of 0–0.6 μg/L. Conversely, a group with levels of 1.1–1.8 μg/L had a mean TTP of 5.1 months [26]. The authors stated "Reduced fecundability at levels below the mercury reference dose warrants further research and prudent reduction in persistent toxic substances exposure among women and men of reproductive age" [26].

The paucity of studies into the effects of male inorganic Hg exposure and the resultant effects on male reproduction in human populations, coupled with the results found in our and other recent research, would suggest that a larger more extensive study is required. Many of the studies that have been conducted have either had a small number of subjects [26], or the results are hard to analyze because of other conflicting variables [3]. Also, while some studies have reported the mechanistic results of human male exposure [7], the resultant effects on male reproduction in terms of fertility were not reported. Our study tended to use lower exposure concentrations than other recent studies [21], and found significant effects of $HgCl_2$ exposure on male rat fertility.

The present study into the effects of $HgCl_2$ on male reproduction in rats found there were significant effects including greater latency to impregnate a female, lower impregnation rates, loss of correlation between testosterone levels and time to impregnate, and lower sperm count in the epididymis head and body. These negative effects on male fertility were identified in the face of doses that were too low to be clinically toxic.

Funding

This publication was made possible by Grant no. RCMI 5 G12 RR003059 from the Research Centers in Minority Institutions (RCMI) at the National Institutes of Health and Grant no. 1R15ES020066-01 from the National Institutes of Health, National Institute of Environmental Health Sciences. Its contents are solely the responsibility of the authors and do not necessarily represent the official views of RCMI, NIH, or NIEHS.

Acknowledgment

The authors wish to express their sincere thanks and appreciation to Dr. Liz Simmons for her invaluable help in editing this paper.

References

[1] J. D. Meeker, M. G. Rossano, B. Protas et al., "Cadmium, lead, and other metals in relation to semen quality: human evidence for molybdenum as a male reproductive toxicant," *Environmental Health Perspectives*, vol. 116, no. 11, pp. 1473–1479, 2008.

[2] B. Baranski, "Effects of the workplace on fertility and related reproductive outcomes," *Environmental Health Perspectives*, vol. 101, no. 2, pp. 81–90, 1993.

[3] S. Tas, R. Lauwerys, and D. Lison, "Occupational hazards for the male reproductive system," *Critical Reviews in Toxicology*, vol. 26, no. 3, pp. 261–307, 1996.

[4] L. Barregard, G. Lindstedt, A. Schutz, and G. Sallsten, "Endocrine function in mercury exposed chloralkali workers," *Occupational and Environmental Medicine*, vol. 51, no. 8, pp. 536–540, 1994.

[5] K. H. Alcser, K. A. Brix, L. J. Fine, L. R. Kallenbach, and R. A. Wolfe, "Occupational mercury exposure and male reproductive health," *American Journal of Industrial Medicine*, vol. 15, no. 5, pp. 517–529, 1989.

[6] C. M. Y. Choy, C. W. K. Lam, L. T. F. Cheung, C. M. Briton-Jones, L. P. Cheung, and C. J. Haines, "Infertility, blood mercury concentrations and dietary seafood consumption: a case-control study," *International Journal of Obstetrics and Gynaecology*, vol. 109, no. 10, pp. 1121–1125, 2002.

[7] T. Y. Leung, C. M. Y. Choy, S. F. Yim, C. W. K. Lam, and C. J. Haines, "Whole blood mercury concentrations in sub-fertile men in Hong Kong," *Australian and New Zealand Journal of Obstetrics and Gynaecology*, vol. 41, no. 1, pp. 75–77, 2001.

[8] H. I. Popescu, "Poisoning with alkylmercury compounds," *British Medical Journal*, vol. 1, no. 6123, p. 1347, 1978.

[9] A. K. Sharma, A. G. Kapadia, P. Fransis, and M. V. Rao, "Reversible effects of mercuric chloride on reproductive organs of the male mouse," *Reproductive Toxicology*, vol. 10, no. 2, pp. 153–159, 1996.

[10] A. Atkinson, S. J. Thompson, A. T. Khan et al., "Assessment of a two-generation reproductive and fertility study of mercuric chloride in rats," *Food and Chemical Toxicology*, vol. 39, no. 1, pp. 73–84, 2001.

[11] T. K. Monsees, M. Franz, S. Gebhardt, U. Winterstein, W. B. Schill, and J. Hayatpour, "Sertoli cells as a target for reproductive hazards," *Andrologia*, vol. 32, no. 4-5, pp. 239–246, 2000.

[12] M. Arabi, "Bull spermatozoa under mercury stress," *Reproduction in Domestic Animals*, vol. 40, no. 5, pp. 454–459, 2005.

[13] M. Arabi and M. S. Heydarnejad, "*In vitro* mercury exposure on spermatozoa from normospermic individuals," *Pakistan Journal of Biological Sciences*, vol. 10, no. 15, pp. 2448–2453, 2007.

[14] M. V. Rao and B. Gangadharan, "Antioxidative potential of melatonin against mercury induced intoxication in spermatozoa *in vitro*," *Toxicology In Vitro*, vol. 22, no. 4, pp. 935–942, 2008.

[15] J. C. Heath, Y. Abdelmageed, T. D. Braden, A. C. Nichols, and D. A. Steffy, "The effects of chronic mercuric chloride ingestion in female Sprague-Dawley rats on fertility and reproduction," *Food and Chemical Toxicology*, vol. 47, no. 7, pp. 1600–1605, 2009.

[16] A. T. Khan et al., "Mercury levels in target organs of male rats following multiple doses of mercuric cholride," in *Proceedings of the International Congress on the Health Effects of Hazardous Waste*, Washington, DC, USA, 1995.

[17] A. T. Khan, T. C. Graham, J. E. Webster, D. M. Forester, and J. A. Ferguson, "Mercury levels in target organs of female rats following multiple doses of mercuric chloride," *Toxicologist*, vol. 335, p. 12, 1996.

[18] National Toxiclogy Program, *Technical Report on the Toxicology and Carcinogenesis Studies of Mercuric Chloride in F344 Rats and B6C3F1 Mice*, NIH, 1993.

[19] J. D. Park, S. S. M. Habeebu, and C. D. Klaassen, "Testicular toxicity of di-(2-ethylhexyl)phthalate in young Sprague-Dawley rats," *Toxicology*, vol. 171, no. 2-3, pp. 105–115, 2002.

[20] G. A. Wiggers, F. M. Peçanha, A. M. Briones et al., "Low mercury concentrations cause oxidative stress and endothelial

dysfunction in conductance and resistance arteries," *American Journal of Physiology*, vol. 295, no. 3, pp. H1033–H1043, 2008.

[21] M. A. Boujbiha, K. Hamden, F. Guermazi et al., "Testicular toxicity in mercuric chloride treated rats: association with oxidative stress," *Reproductive Toxicology*, vol. 28, no. 1, pp. 81–89, 2009.

[22] M. V. Rao and P. S. N. Sharma, "Protective effect of vitamin E against mercuric chloride reproductive toxicity in male mice," *Reproductive Toxicology*, vol. 15, no. 6, pp. 705–712, 2001.

[23] S. Homma-Takeda, Y. Kugenuma, T. Iwamuro, Y. Kumagai, and N. Shimojo, "Impairment of spermatogenesis in rats by methylmercury: involvement of stage- and cell- specific germ cell apoptosis," *Toxicology*, vol. 169, no. 1, pp. 25–35, 2001.

[24] D. G. Ellingsen, J. Efskind, E. Haug, Y. Thomassen, I. Martinsen, and P. I. Gaarder, "Effects of low mercury vapour exposure on the thyroid function in chloralkali workers," *Journal of Applied Toxicology* , vol. 20, no. 6, pp. 483–489, 2000.

[25] E. M. Erfurth, A. Schutz, A. Nilsson, L. Barregard, and S. Skerfving, "Normal pituitary hormone response to thyrotrophin and gonadotrophin releasing hormones in subjects exposed to elemental mercury vapour," *British Journal of Industrial Medicine*, vol. 47, no. 9, pp. 639–644, 1990.

[26] D. C. Cole, B. Wainman, L. H. Sanin, J. P. Weber, H. Muggah, and S. Ibrahim, "Environmental contaminant levels and fecundability among non-smoking couples," *Reproductive Toxicology*, vol. 22, no. 1, pp. 13–19, 2006.

Hindawi Publishing Corporation
Journal of Biomedicine and Biotechnology
Volume 2012, Article ID 949048, 11 pages
doi:10.1155/2012/949048

Review Article

Toxic Effects of Mercury on the Cardiovascular and Central Nervous Systems

Bruna Fernandes Azevedo,[1] Lorena Barros Furieri,[1] Franck Maciel Peçanha,[2] Giulia Alessandra Wiggers,[3] Paula Frizera Vassallo,[4] Maylla Ronacher Simões,[1] Jonaina Fiorim,[1] Priscila Rossi de Batista,[1] Mirian Fioresi,[5] Luciana Rossoni,[6] Ivanita Stefanon,[1] María Jesus Alonso,[7] Mercedes Salaices,[8] and Dalton Valentim Vassallo[1,9]

[1] *Programa de Pós-Graduação em Ciências Fisiológicas, Universidade Federal do Espírito Santo, 29042-755 Vitória, ES, Brazil*
[2] *Curso de Fisioterapia, Universidade Federal do Pampa, 97050-460 Uruguaiana, RS, Brazil*
[3] *Programa de Pós-Graduação em Bioquímica, Universidade Federal do Pampa, 97050-460 Uruguaiana, RS, Brazil*
[4] *Hospital Universitário Cassiano Antônio de Morais, Universidade Federal do Espírito Santo, 29040-091 Vitória, ES, Brazil*
[5] *Departamento de Enfermagem, Universidade Federal do Espírito Santo, 29040-090 Vitória, ES, Brazil*
[6] *Departamento de Fisiologia e Biofísica, Universidade de São Paulo, 05508-900 São Paulo, SP, Brazil*
[7] *Departamento de Fisiologia, Universidad Rey Juan Carlos, 28922 Alcorcón, Spain*
[8] *Departamento de Farmacología, Universidad Autónoma de Madrid, 28029 Madrid, Spain*
[9] *Escola Superior de Ciências da Santa Casa de Misericórdia de Vitória, EMESCAM, 29045-402 Vitória, ES, Brazil*

Correspondence should be addressed to Bruna Fernandes Azevedo, brunafernandes.azevedo@gmail.com

Received 3 April 2012; Accepted 15 May 2012

Academic Editor: Marcelo Farina

Environmental contamination has exposed humans to various metal agents, including mercury. This exposure is more common than expected, and the health consequences of such exposure remain unclear. For many years, mercury was used in a wide variety of human activities, and now, exposure to this metal from both natural and artificial sources is significantly increasing. Many studies show that high exposure to mercury induces changes in the central nervous system, potentially resulting in irritability, fatigue, behavioral changes, tremors, headaches, hearing and cognitive loss, dysarthria, incoordination, hallucinations, and death. In the cardiovascular system, mercury induces hypertension in humans and animals that has wide-ranging consequences, including alterations in endothelial function. The results described in this paper indicate that mercury exposure, even at low doses, affects endothelial and cardiovascular function. As a result, the reference values defining the limits for the absence of danger should be reduced.

1. History

More than 2500 A.C., the prehistoric man used the cinabrio (mercury sulfide), due to its red-gold color, to draw on cave walls and perform face painting. Subsequently, mercury has been used in the amalgamation (direct burning of metallic mercury on the gravel, promoting the separation of gold), in photography and as an antiseptic in the treatment of syphilis [1, 2].

Exposure to mercury brought harmful effects to health of humans, but changes resulting from human exposure to mercury only called the attention of the scientific society after the accidents in Japan and Iraq [3]. In Japan, a serious accident occurred resulting from the deposition of industrial waste with large quantities of mercury in the Minamata Bay. Mercury was then ingested by human through fish intake, thus triggering signs and symptoms such as ataxia, speech impairment, visual field constriction, sensory disturbance, deafness, blindness, tremors, involuntary movements, mental retardation, coma, and death. Infants whose mothers were infected developed mental retardation, peripheral neuropathy, cerebral palsy, and blindness. These changes became

known as Minamata disease or Russell-Hunter syndrome [4, 5]. In Iraq, mercury poisoning occurred in 1971 when wheat grains were treated with fungicides containing organic mercury. This poisoning killed over 500 people who ate bread made with contaminated wheat [6, 7].

2. Mercury Characteristics

Mercury is characterized as a highly malleable liquid at normal temperature and pressure [8]. Its name is derived from the Latin word hydrargyrum, meaning metal that resembles liquid silver [8]. Mercury is classified into three main groups: elemental mercury, inorganic mercury, and organic mercury. Mercury exists in several forms: inorganic mercury, among which There have been the metallic mercury and mercury vapor (Hg^0) and mercurous mercury (Hg^+) or mercuric mercury (Hg^{++}) salts; organic mercury, also called organometallic, which results from a covalent bond between mercury and a carbon atom of an organic functional group such as a methyl, ethyl, or phenyl group. The biological behavior, pharmacokinetics, and clinical significance of the various forms of mercury vary according to its chemical structure [3].

2.1. Inorganic Mercury Compounds

2.1.1. Elemental Mercury or Metalic Mercury Compounds. In its liquid form, the elemental mercury (Hg^0) is poorly absorbed and presents little health risk. However, in the vapor form, metallic mercury is readily absorbed through the lungs and can produce body damage [9–11]. Because of its soluble characteristics, elemental mercury is highly diffusible and is able to pass through cell membranes as well as the blood-brain and placental barriers to reach target organs. Once in the bloodstream, mercury undergoes catalase and peroxidase-mediated oxidation in red blood cells and tissues and is transformed into inorganic mercuric mercury (Hg^{++}) and mercurous mercury (Hg^+), a process that limits its absorption [9, 12]. Inorganic mercury has low lipophilicity and thus has a limited ability to cross cell membranes [9].

Elemental mercury is used in thermometers and sphygmomanometers because of its uniform volumetric expansion, high surface tension, and lack of vitreous adherence to surfaces. Low electrical resistance and high thermal conductivity allow metallic mercury to be used in electrical and electronic materials. Because of its high oxidation power, metallic mercury is used in electrochemical operations in the chlorine and soda industries. Metallic mercury is also used in metallurgy, mining, and dentistry because of the easy amalgam formation with other metals. In addition, gold extraction with archaic and dangerous methods predispose miners to mercury poisoning. The burning of metallic mercury on the gravel promotes the separation of gold, a process called amalgamation, which causes emission of large amounts of mercury vapor that is inhaled immediately by the miner, since they do not use appropriate personal protective equipment [13, 14].

Occupational exposure to mercury vapor and the release of mercury from or during removal of amalgam dental fillings increase its blood and plasma concentration [15, 16] After exposure, blood concentrations attain 18 nmol/L [15], and after exposure to dental and removal of amalgam fillings plasma concentrations attain 5 nmol/L [17]. Occupational exposure also affects central nervous system [15] and amalgam tooth fillings impair sheep kidney function [18]. However, toxicological consequences are still a matter of debate [3, 19–23].

2.1.2. Mercurous Mercury and Mercuric Mercury Compounds. The mercurous mercury in the form of mercurous chloride (Hg_2Cl_2) is little absorbed in the body. It is believed that in the body the form of metallic mercury is changed to elemental mercury and mercuric mercury [24].

Mercuric mercury compounds, such as mercury salts, result from the combination of mercury with chlorine, sulfur, or oxygen. Mercuric mercury can be found in different states when combined with other chemical elements, including mercuric chloride ($HgCl_2$), which is highly toxic and corrosive; mercury sulfide (HgS), which is often used as a pigment in paints due to its red color; mercury fulminate ($Hg(CNO)_2$), which is used as an explosive detonator [8, 25]. Among the mercuric mercury compounds, mercuric chloride ($HgCl_2$) calls the attention. It was used as a preservative for development of photographic film and has been ingested accidentally or as a suicide measure [26]. As elemental mercury, the mercuric mercury in the blood stream binds to sulfhydryl groups on erythrocytes, glutathione, or metallothionein or is transported suspended in plasma [27]. Mercuric mercury accumulates in placenta, fetal tissues, and amniotic fluid, but it does not cross the blood-brain barrier efficiently [28]. Evidence exists showing the transport of mercuric mercury via one or more amino acid transporters [29]. Evidence also shows that the accumulation in the brain occurs through its binding to cysteine [24].

In the cardiovascular system, acute inorganic mercury exposition *in vivo* promotes reduction of myocardial force development [30] and inhibited myosin ATPase activity [31]. Chronic exposure increases vascular resistance and induces hypertension [32–34]. Numerous studies have also revealed that mercury generates oxygen free radicals mainly by activation of NAPHoxidase [35, 36].

2.2. Organic Mercury. Organic mercury compounds, also called organometallic, result from a covalent bond between mercury and the carbon [8] atom of an organic functional group such as a methyl, ethyl, or phenyl group. Methylmercury (CH_3Hg^+) is by far the most common form of organic Hg to which humans and animals are exposed. CH_3Hg^+ in the environment is predominantly formed by methylation of inorganic mercuric ions by microorganisms present in soil and water [37–39]. The expression methylmercury monomethylmercurial is used to denote compounds that contain the cation methylmercury (CH_3Hg^+). Some of these compounds were used as pesticides and had medical applications as antiseptics and diuretics.

The organomercury antiseptics still used are Merthiolate, Bacteran, and Thimerosal [40].

Thimerosal is an organomercurial compound that since 1930 has been widely used as a preservative in biological material such as vaccines and serums used to prevent microbiological growth [41]. Thimerosal is metabolized in the human body and degraded into ethylmercury and thiosalicylate. The chemical difference between these compounds is an important determinant of their toxicity [42, 43].

3. Forms of Mercury Exposure

Mercury is now considered an environmental pollutant of high risk to public health because of its high toxicity and mobility in ecosystems [11, 44]. Exposure to mercury can occur from both natural and artificial sources. Human activities that can result in mercury exposure include the burning of fossil fuels, chlor-alkali industries, mining, the burning of waste, and the use of coal and petroleum [10, 40, 45].

More natural sources of mercury include volcanic activity, earthquakes, erosion, and the volatilization of mercury present in the marine environment and vegetation [10, 46–48]. Mercury emitted both naturally or as a result of human activity is primarily found as inorganic metal vapor (Hg^0) [49]. Among the natural sources of mercury, the largest emissions are from the degassing of the earth's crust. More than five tons of mercury is estimated to be released into the sea every year as a result of erosion and geochemical cycles [50].

Mercury contaminates the environment through a cycle involving the initial emission, the subsequent atmospheric circulation of the vapor form, and the eventual return of mercury to the land and water via precipitation (Figure 3) [46]. The emission of mercury is an important part of this cycle of contamination and can occur through natural processes or as a result of human activities, as mentioned above [48].

Mercury present in seas and rivers after methylation can contaminate fish [51, 52]. The consumption of fish contaminated with mercury is a major source of mercury exposure in the Amazon basin. Studies show that the concentration of mercury in the muscles of fish that are widely consumed in the Amazon region are greater than the limit set by WHO (World Health Organization) as safe for human consumption (0.5 g/kg) [4, 10].

4. Transport and Elimination of Mercury

Inhaled elemental mercury vapor, for example, is readily absorbed through mucous membranes and the lung and is rapidly oxidized but not as quickly as to prevent the deposition of considerable amount in the brain [54]. Methylmercury is easily absorbed through the gut, and it is deposited in most tissue but does not cross the blood-brain barrier as efficiently as elemental mercury. However, to enter the brain, it is progressively demethylated to elemental mercury (Figure 2) [24]. Mercury salts, in contrast, tend to be insoluble, relatively stable, and hardly absorbed.

Then, toxicity for man varies depending on the form of mercury, dose, and rate of exposure. The target organ for inhalted mercury vapor is primarily the brain [24]. Mercurous and mercury salts especially damage the lining of the intestine and kidneys [5], and as methyl mercury, it is widely distributed throughout the body (Figure 1) [24]. Toxicity varies with dosage; a large acute exposure to elemental mercury vapor induces severe pneumonia, which in extreme cases can be fatal [24]. Low level of chronic exposure to elemental or other forms of mercury induces more subtle symptoms and clinical findings [3].

Oxidized mercury binds strongly to SH groups; this reaction can inactivate enzymes, lead to tissue damage and interfere with various metabolic processes [55–57]. Ingested methylmercury is almost completely absorbed and transported into the bloodstream [10]. Methylmercury enters cells mainly by forming a complex with L-cysteine and homocysteine and is eliminated in conjunction with glutathione [58]. After absorption, it is distributed primarily to the central nervous system and kidneys. Methylmercury elimination usually occurs in the urine and feces [59].

5. Doses of Mercury and Safety Legislation

The chemical form of mercury in the air affects its time of permanence and its dispersion in the atmosphere. The elemental mercury form can persist for more than four years in the air, while its compounds are deposited in a short time at locations near their origin. In the northern hemisphere, their average concentration in the atmosphere is estimated at 2 ng/m^3 and in the southern hemisphere is less than 1 ng/m^3. In urban areas, there is a great variability of these concentrations being found up to 67 ng/m^3 with a mean of 11 ng/m^3 in Japan [53]. FUNASA (Fundação Nacional de Saúde) standards of mercury in the air consider a mean of 1 ng/m^3 in the period of one year [60].

In 2004, the Joint FAO (Food and Agriculture Organization of the United National)/WHO Expert Committee on Food Additives (JECFA) established that the safe concentration of methylmercury intake, without the appearance of neurological disorders, is 1.6 mg/kg of body weight. However, in 2006, JECFA stated that this concentration is not safe for intrauterine exposure, because fetuses are more sensitive to the onset of neurological disorders after exposure to methylmercury [61].

Currently, the general population is exposed to mercury by the following main sources: the consumption of contaminated fish, the use and manipulation of dental amalgam, thimerosal contained in vaccines, workers in industries of chlorine, caustic soda, miners, and workers in industries of fluorescent lamps [62, 63]. Each of these sources of exposure contains specific toxicological characteristics [64].

In Brazil, the rules for vaccination of the Ministry of Health, published in June 2001, shows that thimerosal is used in many vaccines. These vaccines prevent flu (influenza vaccine), rabies (rabies vaccine), infection with meningococcus serogroup b, and hepatitis B [65].

The US Environmental Protection Agency's recommended a reference blood concentration of mercury to be

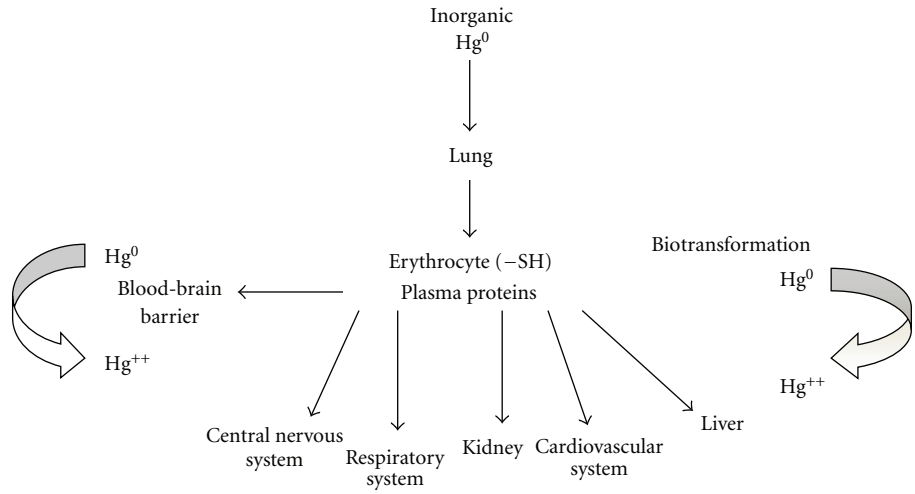


Figure 1: Scheme showing the entry of elemental mercury in organisms and their distribution in different organs.

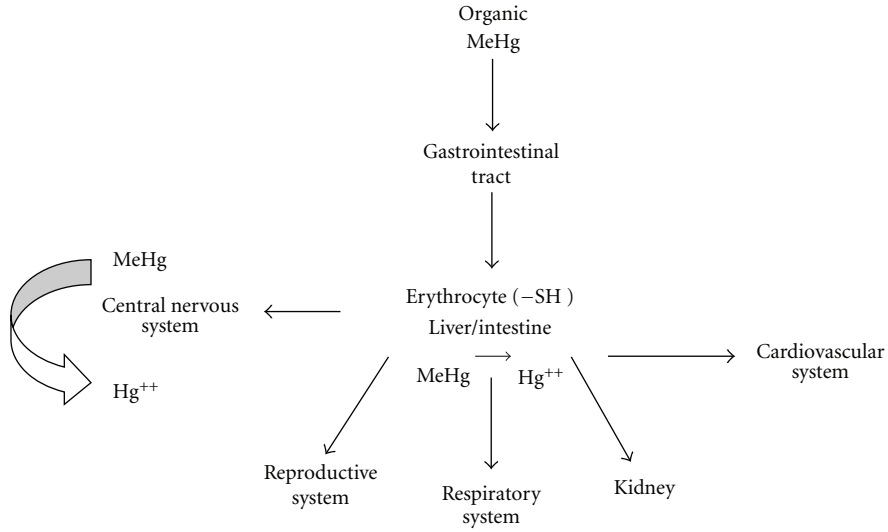


Figure 2: Scheme showing the entry of organic mercury in organisms and their distribution in different organs.

5.8 ng/mL; concentrations below this level are considered to be safe [66, 67]. Some studies have reported that the blood mercury concentration in the control population is approximately 1 ng/mL. On the other hand, levels of 7–10 ng/mL have been reported in workers exposed to mercury or in residents of Guizhou (China), an area that is known to suffer mercury contamination [68, 69]. In a recent biomonitoring study in New York City, the blood mercury concentration was found to be 2.73 ng/mL, and levels reached 5.65 ng/mL in adults that consumed fish regularly [70].

WHO [10] states that an allowable concentration of mercury in human hair is less than 6 μg/g. In the Amazon basin, where fish is the main source of dietary protein, mercury concentrations in hair reached up to 150 μg/g. Furthermore, only two of 40 cities studied have average mercury concentrations below the recommended amount [10, 71]. In individuals who have amalgam, the daily release of mercury amalgam is approximately 4-5 μg/day, and a positive correlation exists between the blood concentration of mercury and the number of amalgams. It is estimated that each dental amalgam releases 3–17 μg mercury vapor per day and that the blood concentration of mercury after removal of the restoration can reach 5 nmol/L [72–74]. However, even at concentrations below recommended levels, there is strong evidence that exposure to ethyl mercury, the major component of thimerosal, is associated with the onset of neurological and heart disorders in children [75].

In the following sections, we will describe results obtained from animals with chronic and acute exposure to mercury. Some of these studies were performed with mercury exposure protocols that led to blood concentrations slightly above the reference values. Nevertheless, these concentrations could be easily found in exposed populations and may even be considered low when compared with concentrations in humans who consume large amounts of fish or who live in areas contaminated with mercury.

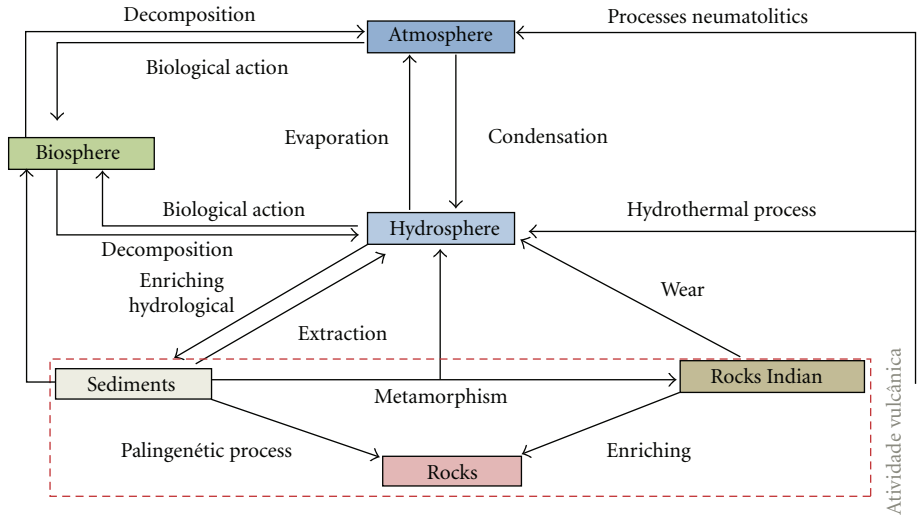


Figure 3: Cycle of mercury in the environment. Modified from Azevedo and Chasin 2003 [53]. Scheme demonstrating constant flow of mercury compounds in the hydrosphere, lithosphere, atmosphere, and biosphere.

6. Effect of Mercury on the Central Nervous System (CNS)

Among the compounds of mercury, the methylmercury is primarily responsible for the neurological alterations present in humans and experimental animals. It is believed that the mechanisms are related to the toxic increase in reactive oxygen species (ROS). Oxidative stress is associated with the etiology of neurodegenerative diseases such as amyotrophic lateral sclerosis, Parkinson's disease, and Alzheimer's disease [54, 55], but these mechanisms have yet to be fully recognized.

Reinforcing the hypothesis that the majority of injuries caused by methylmercury (MeHg) in the central nervous system are related to its ability to increase reactive oxygen species, Zhang et al. (2009) [20] reported that after pretreatment of bovine cells with pyrroloquinoline quinone (PQQ), an antioxidant, the cytotoxicity induced by MeHg is significantly attenuated. PQQ reduces the percentage of apoptotic cells, decreased significantly ROS production, suppressed lipid peroxidation, and increased antioxidant enzyme activity in cells exposed to MeHg. Furthermore, the protective effects elicited by an antioxidant (ebselen) strengthen the idea that seleno-organic compounds represent promising approaches to neutralize MeHg-induced neurotoxicity [19].

Studies also demonstrate that mercury has the ability to reduce the number of neuron and cytoarchitecture in individuals with prenatal exposure to mercury [76, 77]. In animal models, some of these symptoms are reproduced. Low-dose prenatal exposure to methylmercury during 10 gestational days impairs motor and mnemonic function in adult mice [23]. This hypothesis is supported by studies that describe methylmercury inhibition of cell division and migration both "*in vivo*" and "*in vitro*" [76–78].

In addition, because of its high affinity for sulfhydryl groups in tubulin, methylmercury inhibits the organization of microtubules that are important in CNS development [79–81]. The binding to SH groups also interferes with the intracellular signaling of multiple receptors (e.g., muscarinic, nicotinic, and dopaminergic) and promotes the blockade of Ca^{++} channels in neurons [82, 83]. In addition, inorganic mercury has the ability to increase the permeability of chloride channels of GABA A receptors in the dorsal root ganglion, which is associated with neuronal hyperpolarization [84].

Corroborating these findings, the study conducted by Maia et al., (2010) [21] demonstrates that the poisoning by methylmercury changes the nitrergic activities of adult mice, and the predominance of alterations may be related to different locations. Besides increasing the nitrergic activity methylmercury and mercuric chloride also have the ability to increase the release of neurotransmitters such as acetylcholine, dopamine, norepinephrine, and serotonin. Similar findings have also been reported to be a mechanism implicated in the effects of methylmercury and $HgCl_2$ on the central nervous system function [85–89].

Halbach et al. [90] studied a correlation in Iraqi children between the level of maternal exposure to methylmercury during pregnancy and psychomotor retardation. Sandborgh-Englund et al. [91] corroborated this finding in children from the Faroe Islands; they found that children exposed to mercury in the prenatal period had defects in attention, memory, language, and motor function. In addition, exposure to methylmercury in pregnant women or early childhood leads to changes in the CNS development of the fetus or child, respectively [50, 92, 93]. Thereupon, changes caused by mercury poisoning result in significant clinical deficit in motor skills, coordination, and general activity rate of cognitive and psychological disorders [23].

7. Effect of Mercury on the Cardiovascular System

For decades, the toxic effects of mercury were associated mainly with the central nervous system; however, inorganic mercury also produces profound cardiotoxicity [94–99]. Halbach and collaborators [100] showed that mercury concentrations in hair reached up to 150 μg/g in populations living in the Amazon basin. Furthermore, nearly all of the inhabitants of 40 cities studied have blood concentrations above the reference values. In this population, it has been demonstrated that exposure to mercury by frequent consumption of fish has a strong positive correlation with increased arterial blood pressure [101]. Other studies also correlate mercury exposure with increased risk of hypertension, myocardial infarction, coronary dysfunction, and atherosclerosis [102–105]. Data presented by Yoshizawa et al. [106] showed that mercury exposure was associated with the progression of atherosclerosis and an increased risk of developing cardiovascular disease. Houston [107] followed patients for approximately 13.9 years and found an association between the concentration of mercury in the hair and the risk of developing cardiovascular events or dying from cardiovascular disease and other causes.

Mercury levels are predictors of the levels of oxidized low-density lipoprotein (LDL) [106]. Oxidized LDL particles are frequently found in atherosclerotic lesions and are associated with the development of atherosclerotic disease [107, 108] and acute coronary insufficiency [109]. Another mechanism by which mercury exerts toxic effects on the cardiovascular system is through the inactivation of the "paraoxonase" [110], an enzyme that slows the LDL oxidation process and that has an important antiatherosclerotic action [101].

The mechanism by which mercury produces toxic effects on the cardiovascular system is not fully elucidated, but this mechanism is believed to involve an increase in oxidative stress. Exposure to mercury increases the production of free radicals, potentially because of the role of mercury in the Fenton reaction [111–113] and a reduction in the activity of antioxidant enzymes, such as glutathione peroxidase. The MeHg reaction with the glutathione peroxidase occurs via thiol (–SH) and/or selenol (–SeH) groups from endogenous molecules [114]. Even though there are 4 of glutathione molecules containing selene in their active sites, only the cytoplasmic glutathione peroxidase 1 (GPx 1) changes hydrogen peroxide to water [115, 116].

The reduction in glutathione peroxidase with selenium-dependent activity is the result of the decreased bioavailability of selenium, a molecule that is required for enzymatic activity [117–119]. The high affinity of mercury to the thiol group can lead to decreased glutathione peroxidase selenium-dependent activity. Other antioxidant enzymes which participate against reactive oxygen species due to mercury intoxication are catalase and superoxide dismutase. The increment of ROS and reduction of the antioxidant activity increase the risk of developing cardiovascular disease [118, 120, 121].

Sherwani et al. (2011) [122] showed that MeHg has the capacity to induce phospholipase D (PLD) activation through oxidative stress and thiol-redox alterations. They investigated the mechanism of the MeHg-induced PLD activation through the upstream regulation by phospholipase A_2 (PLA_2) and lipid oxygenases such as cyclooxygenase (COX) and lipoxygenase (LOX) in the bovine pulmonary artery endothelial cells. Their results showed that MeHg significantly activates both PLA_2 and PLD. MeHg also induces the formation of COX- and LOX-catalyzed eicosanoids in endothelial cells.

Cardiovascular changes resulting from mercury poisoning are also described in animal models. However, the mechanism involved in the effects of mercury on the cardiovascular system is not fully understood but seems to be dependent on both the dose and time of exposure. Raymond and Ralston [123] studied the hemodynamic effects of an intravenous injection of $HgCl_2$ (5 mg/kg) in rats and observed that mercury produced cardiac diastolic failure and pulmonary hypertension. Moreover, Naganuma et al. [124] reported that acute exposure to $HgCl_2$ (680 ng/kg) increased blood pressure, heart rate, and vascular reactivity to phenylephrine in rats; this increased reactivity seems to depend on an increased generation of free radicals. Perfused hearts from animals exposed acutely to $HgCl_2$ showed a reduction in left ventricular systolic pressure, heart rate, and atrioventricular conduction delay [125, 126].

Our group has found that chronic exposure to low doses of mercury (1st dose 4.6 μg/kg followed by 0.07 μg/kg/day for 30 days, im) attained a blood mercury concentration of approximately 8 ng/mL, a concentration similar to the levels found in exposed humans. This exposure produced a negative inotropic effect in perfused hearts, although increasing myosin ATPase activity. *Invivo*, arterial or ventricular pressures did not change [127]. The reduction in contractility was explained by alterations in calcium-handling mechanisms; protein expression of SERCA, Na^+ K^+ ATPase (NKA), and sodium/calcium exchanger (NCX) was diminished; phospholamban (PLB) expression was increased; the response to β-adrenergic stimulation was reduced following mercury exposure [128, 129].

The chronic exposure to low concentrations of mercury was also able to induce endothelial dysfunction in resistance and conductance vessels, most likely because of the decreased nitric oxide (NO) bioavailability due to the increased superoxide anion ($O_2^{\bullet -}$) production from NADPH oxidase [36, 130, 131]. This study was evidenced by the following effects of mercury treatment observed in aorta, coronary, and mesenteric arteries: (1) the endothelium-dependent vasodilator response induced by acetylcholine (ACh) was decreased [36, 130]; (2) vasoconstrictor responses to phenylephrine or serotonin were increased, and NO endothelial modulation of these responses was decreased [36, 130, 131]; (3) vascular superoxide anion production, the expression of SOD-2, NOX-1, and NOX-4 (two main isoforms of NADPH oxidase), plasmatic malondialdehyde levels, and plasmatic antioxidant status were all increased [36, 130]; (4) both the superoxide anion scavenger SOD and the NADPH oxidase inhibitor apocynin restored

the NO endothelial modulation of vasoconstrictor responses and the impaired ACh-induced vasodilatation in vessels from the mercury-treated rats [36, 130]. We also observed that mercury treatment increased the participation of COX-2-derived vasoconstrictor prostanoids in vasoconstrictor responses [132]. Other researchers have also observed a selective loss of NO-mediated vasodilatation with no effect on the EDHF-mediated component of relaxation, implying that chronic mercury exposure selectively impairs the NO pathway as a consequence of oxidative stress, while EDHF is able to maintain endothelium-dependent relaxation at a reduced level [133]. On the other hand, using this low dose of mercury, Blanco-Rivero et al. [134] observed an increase in vasoconstriction responses to electrical field stimulation mediated by alterations of adrenergic and nitrergic function in rat mesenteric arteries. $HgCl_2$ reduced neuronal NO bioavailability, most likely as a result of reduced nNOS (neuronal nitric oxide synthase) activity and increased O_2^- production as well as increased noradrenaline release and vasoconstrictor response. It is important to emphasize, regarding chronic low-dose exposure to mercury for 30 days, that although rats grow normally and have no changes in arterial blood pressure, endothelial function is already blunted affecting vascular reactivity [36, 131].

Taken together, these data show that chronic low doses of mercury have an important and deleterious effect on vascular function by reducing NO bioavailability. The degree of severity of mercury exposure is comparable to traditional cardiovascular risk factors, such as hypertension diabetes or hypercholesterolemia. Therefore, mercury could be considered an important risk factor for cardiovascular disease that could play a role in the development of cardiovascular events. The association between mercury exposure and an increased risk of developing cardiovascular and neurological diseases is apparent. Thus, continuous exposure to mercury can be dangerous, and current reference values, once considered to be without risk, should be reevaluated and reduced.

Acknowledgments

These studies were supported by Grants from CAPES and CNPq/FAPES/FUNCITEC (39767531/07), Brazil and MCINN and MECD (SAF 2009-07201, RD06/0014/0011, and PHB2011-0001-PC) and Banco Santander, Spain. All participants of the group who performed experiments that were used in this paper are listed as authors. Dr. M. Salaices and Dr. M. J. Alonso advised several students of the group during their Doctoral Sandwich Program in the Universidade Autonóma de Madrid, Spain.

References

[1] L. D. Hylander and M. Meili, "500 Years of mercury production: global annual inventory by region until 2000 and associated emissions," *Science of the Total Environment*, vol. 304, no. 1–3, pp. 13–27, 2003.

[2] S. Ekino, M. Susa, T. Ninomiya, K. Imamura, and T. Kitamura, "Minamata disease revisited: an update on the acute and chronic manifestations of methyl mercury poisoning," *Journal of the Neurological Sciences*, vol. 262, no. 1-2, pp. 131–144, 2007.

[3] R. A. Bernhoft, "Mercury toxicity and treatment: a review of the literature," *Journal of Environmental and Public Health*, vol. 2012, Article ID 460508, 10 pages, 2012.

[4] M. Gochfeld, "Cases of mercury exposure, bioavailability, and absorption," *Ecotoxicology and Environmental Safety*, vol. 56, no. 1, pp. 174–179, 2003.

[5] T. Takeuchi, K. Eto, Y. Kinjo, and H. Tokunaga, "Human brain disturbance by methylmercury poisoning, focusing on the long- term effect on brain weight," *NeuroToxicology*, vol. 17, no. 1, pp. 187–190, 1996.

[6] F. Bakir, S. F. Damluji, and I. Amin Zaki, "Methylmercury poisoning in Iraq: an interuniversity report," *Science*, vol. 181, no. 4096, pp. 230–241, 1973.

[7] T. W. Clarkson, "Molecular and ionic mimicry of toxic metals," *Annual Review of Pharmacology and Toxicology*, vol. 33, pp. 545–571, 1993.

[8] HSDB—Hazardous Substances Data Bank, "Mercury," in *Toxicology, Occupational Medicine and Environmental Series*, 2004, http://toxnet.nlm.nih.gov/.

[9] M. C. Houston, "The role of mercury and cadmium heavy metals in vascular disease, hypertension, coronary heart disease, and myocardial infarction," *Alternative Therapies in Health and Medicine*, vol. 13, no. 2, pp. S128–S133, 2007.

[10] Agency for Toxic Substances and Disease Registry (ATSDR), *Toxicological Profile for Mercury*, ATSDR, Public Health Service, US Department of Health and Human Services, Washington, DC, USA, 1999, http://www.atsdr.cdc.gov/toxprofiles/tp46.pdf.

[11] R. C. D. C. M. Mesquita, M. I. M. S. Bueno, W. D. F. Jardim, and W. D. F. Jardim, "Compostos de mercúrio. Revisão de métodos de determinação, tratamento e descarte," *Quimica Nova*, vol. 23, no. 4, pp. 487–495, 2000.

[12] M. Roulet, M. Lucotte, R. Canuel et al., "Distribution and partition of total mercury in waters of the Tapajos River Basin, Brazilian Amazon," *Science of the Total Environment*, vol. 213, no. 1–3, pp. 203–211, 1998.

[13] J. O. Nriagu, W. C. Pfeiffer, O. Malm, C. M. Magalhaes De Souza, and G. Mierle, "Mercury pollution in Brazil," *Nature*, vol. 356, no. 6368, article 389, 1992.

[14] M. H. D. Pestana and M. L. L. Formoso, "Mercury contamination in Lavras do Sul, south Brazil: a legacy from past and recent gold mining," *Science of the Total Environment*, vol. 307, no. 1–3, pp. 125–140, 2003.

[15] S. Langworth, "Exposure to mercury vapor and impact on health in the dental profession in Sweden," *Journal of Dental Research*, vol. 76, no. 7, pp. 1397–1404, 1997.

[16] S. Halbach, L. Kremers, H. Willruth et al., "Systemic transfer of mercury from amalgam fillings before and after cessation of emission," *Environmental Research*, vol. 77, no. 2, pp. 115–123, 1998.

[17] L. Björkman, G. Sandborgh-Englund, and J. Ekstrand, "Mercury in saliva and feces after removal of amalgam fillings," *Toxicology and Applied Pharmacology*, vol. 144, no. 1, pp. 156–162, 1997.

[18] N. D. Boyd, H. Benediktsson, M. J. Vimy, D. E. Hooper, and F. L. Lorscheider, "Mercury from dental "silver" tooth fillings impairs sheep kidney function," *American Journal of Physiology*, vol. 261, no. 4, pp. R1010–R1014, 1991.

[19] Z. Yin, E. Lee, M. Ni et al., "Methylmercury-induced alterations in astrocyte functions are attenuated by ebselen," *NeuroToxicology*, vol. 32, no. 3, pp. 291–299, 2011.

[20] P. Zhang, Y. Xu, J. Sun, X. Li, L. Wang, and L. Jin, "Protection of pyrroloquinoline quinone againts methylmercury-induced neurotoxicity via reducing oxidative stress," *Free Radical Research*, vol. 43, no. 3, pp. 224–233, 2009.

[21] C. D. S. F. Maia, V. M. M. Ferreira, R. L. Kahwage et al., "Adult brain nitrergic activity after concomitant prenatal exposure to ethanol and methyl mercury," *Acta Histochemica*, vol. 112, no. 6, pp. 583–591, 2010.

[22] A. F. Castoldi, N. Onishchenko, C. Johansson et al., "Neurodevelopmental toxicity of methylmercury: laboratory animal data and their contribution to human risk assessment," *Regulatory Toxicology and Pharmacology*, vol. 51, no. 2, pp. 215–229, 2008.

[23] K. S. Montgomery, J. Mackey, K. Thuett, S. Ginestra, J. L. Bizon, and L. C. Abbott, "Chronic, low-dose prenatal exposure to methylmercury impairs motor and mnemonic function in adult C57/B6 mice," *Behavioural Brain Research*, vol. 191, no. 1, pp. 55–61, 2008.

[24] T. W. Clarkson, J. B. Vyas, and N. Ballatori, "Mechanisms of mercury disposition in the body," *American Journal of Industrial Medicine*, vol. 50, no. 10, pp. 757–764, 2007.

[25] R. C. D. C. M. Mesquita, M. I. M. S. Bueno, and W. D. F. Jardim, "Compostos de mercúrio. Revisão de métodos de determinação, tratamento e descarte," *Quimica Nova*, vol. 23, no. 4, pp. 487–495, 2000.

[26] D. A. Geier and M. R. Geier, "An assessment of the impact of thimerosal on childhood neurodevelopmental disorders," *Pediatric Rehabilitation*, vol. 6, no. 2, pp. 97–102, 2003.

[27] N. Ballatori and T. W. Clarkson, "Biliary secretion of glutathione and of glutathione-metal complexes," *Fundamental and Applied Toxicology*, vol. 5, no. 5, pp. 816–831, 1985.

[28] S. Halbach, S. Vogt, W. Köhler et al., "Blood and urine mercury levels in adult amalgam patients of a randomized controlled trial: interaction of Hg species in erythrocytes," *Environmental Research*, vol. 107, no. 1, pp. 69–78, 2008.

[29] P. Grandjean, P. Weihe, R. F. White et al., "Cognitive deficit in 7-year-old children with prenatal exposure to methylmercury," *Neurotoxicology and Teratology*, vol. 19, no. 6, pp. 417–428, 1997.

[30] D. V. Vassallo, C. M. Moreira, E. M. Oliveira, D. M. Bertollo, and T. C. Veloso, "Effects of mercury on the isolated heart muscle are prevented by DTT and cysteine," *Toxicology and Applied Pharmacology*, vol. 156, no. 2, pp. 113–118, 1999.

[31] C. M. Moreira, E. M. Oliveira, C. D. Bonan, J. J. F. Sarkis, and D. V. Vassallo, "Effects of mercury on myosin ATPase in the ventricular myocardium of the rat," *Comparative Biochemistry and Physiology C*, vol. 135, no. 3, pp. 269–275, 2003.

[32] M. Carmignani, P. Boscolo, L. Artese et al., "Renal mechanisms in the cardiovascular effects of chronic exposure to inorganic mercury in rats," *British Journal of Industrial Medicine*, vol. 49, no. 4, pp. 226–232, 1992.

[33] Y. Wakita, "Hypertension induced by methyl mercury in rats," *Toxicology and Applied Pharmacology*, vol. 89, no. 1, pp. 144–147, 1987.

[34] M. C. Houston, "The role of mercury and cadmium heavy metals in vascular disease, hypertension, coronary heart disease, and myocardial infarction," *Alternative Therapies in Health and Medicine*, vol. 13, no. 2, pp. S128–S133, 2007.

[35] L. Massaroni, L. V. Rossoni, S. M. C. Amaral, I. Stefanon, E. M. Oliveira, and D. V. Vassallo, "Haemodynamic and electrophysiological acute toxic effects of mercury in anaesthetized rats and in Langendorff perfused rat hearts," *Pharmacological Research*, vol. 32, no. 1-2, pp. 27–36, 1995.

[36] L. B. Furieri, M. Fioresi, R. F. R. Junior et al., "Exposure to low mercury concentration *in vivo* impairs myocardial contractile function," *Toxicology and Applied Pharmacology*, vol. 255, no. 2, pp. 193–199, 2011.

[37] R. K. Zalups, "Molecular interactions with mercury in the kidney," *Pharmacological Reviews*, vol. 52, no. 1, pp. 113–143, 2000.

[38] T. W. Clarkson and L. Magos, "The toxicology of mercury and its chemical compounds," *Critical Reviews in Toxicology*, vol. 36, no. 8, pp. 609–662, 2006.

[39] J. P. K. Rooney, "The role of thiols, dithiols, nutritional factors and interacting ligands in the toxicology of mercury," *Toxicology*, vol. 234, no. 3, pp. 145–156, 2007.

[40] World Health Organization, *Methyl Mercury*, vol. 1, International Programme on Chemical Safety, Geneva, Switzerland, 1990.

[41] FDA- Food and Drug Administration. Thimerosal in Vaccines, *Center of Biologics Evaluation and Research*, 2008, http://www.fda.gov/Cber/vaccine/thimerosal.htm.

[42] G. Guzzi and C. A. M. La Porta, "Molecular mechanisms triggered by mercury," *Toxicology*, vol. 244, no. 1, pp. 1–12, 2008.

[43] T. W. Clarkson, "The three modern faces of mercury," *Environmental Health Perspectives*, vol. 110, no. 1, pp. 11–23, 2002.

[44] C. L. Johnson, "Mercury in the environment: sources, toxicities, and prevention of exposure," *Pediatric Annals*, vol. 33, no. 7, pp. 437–442, 2004.

[45] L. E. Davis, M. Kornfeld, H. S. Mooney et al., "Methylmercury poisoning: long-term clinical, radiological, toxicological, and pathological studies of an affected family," *Annals of Neurology*, vol. 35, no. 6, pp. 680–688, 1994.

[46] S. C. Alexandre, *Avaliação de área contaminada por mercúrio total em descoberto [Dissertação de Mestrado]*, Programa de Pós-Graduação em Engenharia Civil. Universidade Federal de Viçosa, Minas Gerais, Brazil, 2006.

[47] P. W. Davidson, G. J. Myers, B. Weiss, C. F. Shamlaye, and C. Cox, "Prenatal methyl mercury exposure from fish consumption and child development: a review of evidence and perspectives from the Seychelles Child Development Study," *NeuroToxicology*, vol. 27, no. 6, pp. 1106–1109, 2006.

[48] S. Lindberg, R. Bullock, R. Ebinghaus et al., "A synthesis of progress and uncertainties in attributing the sources of mercury in deposition," *Ambio*, vol. 36, no. 1, pp. 19–32, 2007.

[49] D. W. Boening, "Ecological effects, transport, and fate of mercury: a general review," *Chemosphere*, vol. 40, no. 12, pp. 1335–1351, 2000.

[50] E. B. Swain, P. M. Jakus, G. Rice et al., "Socioeconomic consequences of mercury use and pollution," *Ambio*, vol. 36, no. 1, pp. 45–61, 2007.

[51] Environmental Protection Agency, *Mercury Study Report to Congress*, U.S. Environmental Protection Agency, Washington, DC, USA, 1997.

[52] J. C. Hansen and G. Danscher, "Organic mercury: an environmental threat to the health of dietary-exposed societies?" *Reviews on Environmental Health*, vol. 12, no. 2, pp. 107–116, 1997.

[53] F. A. Azevedo and A. M. Chasin, *As Bases Toxicológicas da Ecotoxicologia*, São Carlos: RiMa, Atheneu/InterTox, São Paulo, Brazil, 2003.

[54] C. C. Bridges and R. K. Zalups, "Transport of inorganic mercury and methylmercury in target tissues and organs," *Journal of Toxicology and Environmental Health—Part B*, vol. 13, no. 5, pp. 385–410, 2010.

[55] M. Roulet, M. Lucotte, R. Canuel et al., "Distribution and partition of total mercury in waters of the Tapajos River Basin, Brazilian Amazon," *Science of the Total Environment*, vol. 213, no. 1–3, pp. 203–211, 1998.

[56] M. Roulet, M. Lucotte, J. R. D. Guimarães, and I. Rheault, "Methylmercury in water, seston, and epiphyton of an Amazonian river and its floodplain, Tapajos River, Brazil," *Science of the Total Environment*, vol. 261, no. 1–3, pp. 43–59, 2000.

[57] J. G. Dórea, R. C. Marques, and K. G. Brandão, "Neonate exposure to thimerosal mercury from hepatitis B vaccines," *American Journal of Perinatology*, vol. 26, no. 7, pp. 523–527, 2009.

[58] S. Asano, K. Eto, E. Kurisaki et al., "Acute inorganic mercury vapor inhalation poisoning," *Pathology International*, vol. 50, no. 3, pp. 169–174, 2000.

[59] R. K. Zalups and L. H. Lash, "Advances in understanding the renal transport and toxicity of mercury," *Journal of Toxicology and Environmental Health*, vol. 42, no. 1, pp. 1–44, 1994.

[60] F. A. Azevedo and A. A. M. Chasin, *Bases Toxicológicas da Ecotoxicologia*, São Carlos: RiMa, Atheneu/InterTox, São Paulo, Brazil, 2003.

[61] JECFA, "Methylmercury. Summary and conclusions of the 67thThe Joint FAO/WHO Expert Committee on Food Additives. Geneva, World Health Organization, International Programme on Chemical Safety," WHO Technical Report Series 940, 2006, http://www.who.int/foodsafety/chem/en/.

[62] K. R. Mahaffey, "Mercury exposure: medical and public health issues," *Transactions of the American Clinical and Climatological Association*, vol. 116, pp. 127–154, 2005.

[63] C. Zavariz and D. M. Glina, "Clinico-neuro-psychological evaluation of workers exposed to metallic mercury in the electric lamp industry," *Revista de Saude Publica*, vol. 26, no. 5, pp. 356–365, 1992.

[64] T. W. Clarkson, "The three modern faces of mercury," *Environmental Health Perspectives*, vol. 110, no. 1, pp. 11–23, 2002.

[65] C. Peterson, "Medical slang in Rio de Janeiro, Brasil," *Cadernos de saúde pública/Ministério da Saúde, Fundação Oswaldo Cruz, Escola Nacional de Saúde Pública*, vol. 14, no. 4, pp. 671–682, 1998.

[66] B. H. Choi, K. H. Cho, and L. W. Lapham, "Effects of methylmercury on human fetal neurons and astrocytes in vitro: a time-lapse cinematographic, phase and electron microscopic study," *Environmental Research*, vol. 24, no. 1, pp. 61–74, 1981.

[67] N. Ballatori and T. W. Clarkson, "Biliary secretion of glutathione and of glutathione-metal complexes," *Fundamental and Applied Toxicology*, vol. 5, no. 5, pp. 816–831, 1985.

[68] E. Brodkin, R. Copes, A. Mattman, J. Kennedy, R. Kling, and A. Yassi, "Lead and mercury exposures: interpretation and action," *CMAJ*, vol. 176, no. 1, pp. 59–63, 2007.

[69] National Academy of Sciences, *Toxicological Effects of Methylmercury*, National Research Council, Washington, DC, USA, 2000.

[70] A. H. Stern, "A revised probabilistic estimate of the maternal methyl mercury intake dose corresponding to a measured cord blood mercury concentration," *Environmental Health Perspectives*, vol. 113, no. 2, pp. 155–163, 2005.

[71] M. Gupta, J. K. Bansal, and C. M. Khanna, "Blood mercury in workers exposed to the preparation of mercury cadmium telluride layers on cadmium telluride base," *Industrial Health*, vol. 34, no. 4, pp. 421–425, 1996.

[72] C. Chen, L. Qu, B. Li et al., "Increased oxidative DNA damage, as assessed by urinary 8-hydroxy-2′-deoxyguanosine concentrations, and serum redox status in persons exposed to mercury," *Clinical Chemistry*, vol. 51, no. 4, pp. 759–767, 2005.

[73] W. R. Bastos, J. P. O. Gomes, R. C. Oliveira et al., "Mercury in the environment and riverside population in the Madeira River Basin, Amazon, Brazil," *Science of the Total Environment*, vol. 368, no. 1, pp. 344–351, 2006.

[74] S. Halbach, "Combined estimation of mercury species released from amalgam," *Journal of Dental Research*, vol. 74, no. 4, pp. 1103–1109, 1995.

[75] W. McKelvey, R. C. Gwynn, N. Jeffery et al., "A biomonitoring study of lead, cadmium, and mercury in the blood of New York City adults," *Environmental Health Perspectives*, vol. 115, no. 10, pp. 1435–1441, 2007.

[76] P. Grandjean, P. Weihe, R. F. White et al., "Cognitive deficit in 7-year-old children with prenatal exposure to methylmercury," *Neurotoxicology and Teratology*, vol. 19, no. 6, pp. 417–428, 1997.

[77] K. A. Graeme and C. V. Pollack, "Heavy metal toxicity, part I: arsenic and mercury," *Journal of Emergency Medicine*, vol. 16, no. 1, pp. 45–56, 1998.

[78] P. Grandjean, E. Budtz-Jørgensen, R. F. White et al., "Methylmercury exposure biomarkers as indicators of neurotoxicity in children aged 7 years," *American Journal of Epidemiology*, vol. 150, no. 3, pp. 301–305, 1999.

[79] P. R. Sager, M. Aschner, and P. M. Rodier, "Persistent, differential alterations in developing cerebellar cortex of male and female mice after methylmercury exposure," *Brain Research*, vol. 314, no. 1, pp. 1–11, 1984.

[80] T. Kishimoto, T. Oguri, and M. Tada, "Effect of methylmercury (CH_3HgCl) injury on nitric oxide synthase (NOS) activity in cultured human umbilical vascular endothelial cells," *Toxicology*, vol. 103, no. 1, pp. 1–7, 1995.

[81] R. A. Ponce, T. J. Kavanagh, N. K. Mottet, S. G. Whittaker, and E. M. Faustman, "Effects of methyl mercury on the cell cycle of primary rat CNS cells *in vitro*," *Toxicology and Applied Pharmacology*, vol. 127, no. 1, pp. 83–90, 1994.

[82] B. H. Choi, K. H. Cho, and L. W. Lapham, "Effects of methylmercury on human fetal neurons and astrocytes in vitro: a time-lapse cinematographic, phase and electron microscopic study," *Environmental Research*, vol. 24, no. 1, pp. 61–74, 1981.

[83] A. F. Castoldi, S. Barni, I. Turin, C. Gandini, and L. Manzo, "Early acute necrosis, delayed apoptosis and cytoskeletal breakdown in cultured cerebellar granule neurons exposed to methylmercury," *Journal of Neuroscience Research*, vol. 59, no. 6, pp. 775–787, 2000.

[84] N. K. Mottet, M. E. Vahter, J. S. Charleston, and L. T. Friberg, "Metabolism of methylmercury in the brain and its toxicological significance," *Metal Ions in Biological Systems*, vol. 34, pp. 371–403, 1997.

[85] S. Yee and B. H. Choi, "Methylmercury poisoning induces oxidative stress in the mouse brain," *Experimental and Molecular Pathology*, vol. 60, no. 3, pp. 188–196, 1994.

[86] F. Weinsberg, U. Bickmeyer, and H. Wiegand, "Effects of inorganic mercury (Hg^{2+}) on calcium channel currents and catecholamine release from bovine chromaffin cells," *Archives of Toxicology*, vol. 69, no. 3, pp. 191–196, 1995.

[87] C. S. Huang and T. Narahashi, "Mercury chloride modulation of the GABA(A) receptor-channel complex in rat dorsal root ganglion neurons," *Toxicology and Applied Pharmacology*, vol. 140, no. 2, pp. 508–520, 1996.

[88] W. D. Atchison and T. Narahashi, "Methylmercury-induced depression of neuromuscular transmission in the rat," *Neuro Toxicology*, vol. 3, no. 3, pp. 37–50, 1982.

[89] W. D. Atchison, "Extracellular calcium-dependent and -independent effects of methylmercury on spontaneous and potassium-evoked release of acetylcholine at the neuromuscular junction," *Journal of Pharmacology and Experimental Therapeutics*, vol. 237, no. 2, pp. 672–680, 1986.

[90] S. Halbach, S. Vogt, W. Köhler et al., "Blood and urine mercury levels in adult amalgam patients of a randomized controlled trial: interaction of Hg species in erythrocytes," *Environmental Research*, vol. 107, no. 1, pp. 69–78, 2008.

[91] G. Sandborgh-Englund, C. G. Elinder, S. Langworth, A. Schütz, and J. Ekstrand, "Mercury in biological fluids after amalgam removal," *Journal of Dental Research*, vol. 77, no. 4, pp. 615–624, 1998.

[92] D. A. Geier and M. R. Geier, "An assessment of the impact of thimerosal on childhood neurodevelopmental disorders," *Pediatric Rehabilitation*, vol. 6, no. 2, pp. 97–102, 2003.

[93] C. Cox, T. W. Clarkson, D. O. Marsh, L. Amin-Zaki, S. Tikriti, and G. G. Myers, "Dose-response analysis of infants prenatally exposed to methyl mercury: an application of a single compartment model to single-strand hair analysis," *Environmental Research*, vol. 49, no. 2, pp. 318–332, 1989.

[94] S. Hussain, D. A. Rodgers, H. M. Duhart, and S. F. Ali, "Mercuric chloride-induced reactive oxygen species and its effect on antioxidant enzymes in different regions of rat brain," *Journal of Environmental Science and Health—Part B*, vol. 32, no. 3, pp. 395–409, 1997.

[95] J. R. Brawer, G. F. McCarthy, M. Gornitsky, D. Frankel, K. Mehindate, and H. M. Schipper, "Mercuric chloride induces a stress response in cultured astrocytes characterized by mitochondrial uptake of iron," *NeuroToxicology*, vol. 19, no. 6, pp. 767–776, 1998.

[96] M. Königsberg, N. E. López-Díazguerrero, L. Bucio, and M. C. Gutiérrez-Ruiz, "Uncoupling effect of mercuric chloride on mitochondria isolated from an hepatic cell line," *Journal of Applied Toxicology*, vol. 21, no. 4, pp. 323–329, 2001.

[97] L. Massaroni, E. M. Oliveira, I. Stefanon, and D. V. Vassallo, "Effects of mercury on the mechanical and electrical activity of the Langendorff-perfused rat heart," *Brazilian Journal of Medical and Biological Research*, vol. 25, no. 8, pp. 861–864, 1992.

[98] E. M. Oliveira, D. V. Vassallo, J. J. F. Sarkis, and J. G. Mill, "Mercury effects on the contractile activity of isolated heart muscle," *Toxicology and Applied Pharmacology*, vol. 128, no. 1, pp. 86–91, 1994.

[99] J. Y. Su and W. J. Chen, "The effects of methylmercury on isolated cardiac tissues," *American Journal of Pathology*, vol. 95, no. 3, pp. 753–764, 1979.

[100] S. Halbach, G. Schonsteiner, and W. Vierling, "The action of organic mercury compounds on the function of isolated mammalian heart muscle," *European Journal of Pharmacology*, vol. 167, no. 2, pp. 255–264, 1989.

[101] S. Halbach, "Mercury compounds: lipophilicity and toxic effects on isolated myocardial tissue," *Archives of Toxicology*, vol. 64, no. 4, pp. 315–319, 1990.

[102] H. M. Rhee and B. H. Choi, "Hemodynamic and electrophysiological effects of mercury in intact anesthetized rabbits and in isolated perfused hearts," *Experimental and Molecular Pathology*, vol. 50, no. 3, pp. 281–290, 1989.

[103] W. R. Bastos, J. P. O. Gomes, R. C. Oliveira et al., "Mercury in the environment and riverside population in the Madeira River Basin, Amazon, Brazil," *Science of the Total Environment*, vol. 368, no. 1, pp. 344–351, 2006.

[104] M. Fillion, D. Mergler, C. J. Sousa Passos, F. Larribe, M. Lemire, and J. R. D. Guimarães, "A preliminary study of mercury exposure and blood pressure in the Brazilian Amazon," *Environmental Health*, vol. 5, article 29, 2006.

[105] E. Guallar, M. I. Sanz-Gallardo, P. Van'T Veer et al., "Mercury, fish oils, and the risk of myocardial infarction," *The New England Journal of Medicine*, vol. 347, no. 22, pp. 1747–1754, 2002.

[106] K. Yoshizawa, E. B. Rimm, J. S. Morris et al., "Mercury and the risk of coronary heart disease in men," *The New England Journal of Medicine*, vol. 347, no. 22, pp. 1755–1760, 2002.

[107] M. C. Houston, "The role of mercury and cadmium heavy metals in vascular disease, hypertension, coronary heart disease, and myocardial infarction," *Alternative Therapies in Health and Medicine*, vol. 13, no. 2, pp. S128–S133, 2007.

[108] J. T. Salonen, K. Seppanen, K. Nyyssonen et al., "Intake of mercury from fish, lipid peroxidation, and the risk of myocardial infarction and coronary, cardiovascular, and any death in Eastern Finnish men," *Circulation*, vol. 91, no. 3, pp. 645–655, 1995.

[109] J. K. Virtanen, S. Voutilainen, T. H. Rissanen et al., "Mercury, fish oils, and risk of acute coronary events and cardiovascular disease, coronary heart disease, and all-cause mortality in men in Eastern Finland," *Arteriosclerosis, Thrombosis, and Vascular Biology*, vol. 25, no. 1, pp. 228–233, 2005.

[110] J. Hulthe and B. Fagerberg, "Circulating oxidized LDL is associated with subclinical atherosclerosis development and inflammatory cytokines (AIR study)," *Arteriosclerosis, Thrombosis, and Vascular Biology*, vol. 22, no. 7, pp. 1162–1167, 2002.

[111] S. Ehara, M. Ueda, T. Naruko et al., "Elevated levels of oxidized low density lipoprotein show a positive relationship with the severity of acute coronary syndromes," *Circulation*, vol. 103, no. 15, pp. 1955–1960, 2001.

[112] M. C. Gonzalvo, F. Gil, A. F. Hernández, E. Villanueva, and A. Pla, "Inhibition of paraoxonase activity in human liver microsomes by exposure to EDTA, metals and mercurials," *Chemico-Biological Interactions*, vol. 105, no. 3, pp. 169–179, 1997.

[113] M. Mackness and B. Mackness, "Paraoxonase 1 and atherosclerosis: is the gene or the protein more important?" *Free Radical Biology and Medicine*, vol. 37, no. 9, pp. 1317–1323, 2004.

[114] M. Farina, M. Aschner, and J. B. T. Rocha, "Oxidative stress in MeHg-induced neurotoxicity," *Toxicology and Applied Pharmacology*, vol. 256, no. 1, pp. 405–417, 2011.

[115] L. Flohé, "Glutathione peroxidase," *Basic Life Sciences*, vol. 49, pp. 663–668, 1988.

[116] J. R. Arthur, "The glutathione peroxidases," *Cellular and Molecular Life Sciences*, vol. 57, no. 13-14, pp. 1825–1835, 2000.

[117] R. Brigelius-Flohé, "Glutathione peroxidases and redox-regulated transcription factors," *Biological Chemistry*, vol. 387, no. 10-11, pp. 1329–1335, 2006.

[118] L. Magos and T. W. Clarkson, "Overview of the clinical toxicity of mercury," *Annals of Clinical Biochemistry*, vol. 43, no. 4, pp. 257–268, 2006.

[119] T. W. Clarkson, "The toxicology of mercury," *Critical Reviews in Clinical Laboratory Sciences*, vol. 34, no. 4, pp. 369–403, 1997.

[120] M. Valko, C. J. Rhodes, J. Moncol, M. Izakovic, and M. Mazur, "Free radicals, metals and antioxidants in oxidative stress-induced cancer," *Chemico-Biological Interactions*, vol. 160, no. 1, pp. 1–40, 2006.

[121] H. E. Ganther, "Interactions of vitamin E and selenium with mercury and silver," *Annals of the New York Academy of Sciences*, vol. 355, pp. 212–226, 1980.

[122] S. I. Sherwani, S. Pabon, R. B. Patel et al., "Eicosanoid signaling and vascular dysfunction: methylmercury-induced phospholipase D activation in vascular endothelial cells," *Cell Biochemistry and Biophysics*. In press.
[123] L. J. Raymond and N. V. C. Ralston, "Mercury: selenuim interactions and health implications," *Seychelles Medical and Dental Journal*, vol. 7, no. 1, pp. 72–77, 2004.
[124] A. Naganuma, Y. Koyama, and N. Imura, "Behavior of methylmercury in mammalian erythrocytes," *Toxicology and Applied Pharmacology*, vol. 54, no. 3, pp. 405–410, 1980.
[125] J. Rungby and E. Ernst, "Experimentally induced lipid peroxidation after exposure to chromium, mercury or silver: interactions with carbon tetrachloride," *Pharmacology and Toxicology*, vol. 70, no. 3, pp. 205–207, 1992.
[126] L. V. Rossoni, S. M. C. Amaral, P. F. Vassallo et al., "Effects of mercury on the arterial blood pressure of anesthetized rats," *Brazilian Journal of Medical and Biological Research*, vol. 32, no. 8, pp. 989–997, 1999.
[127] A. C. Machado, A. S. Padilha, G. A. Wiggers, F. D. M. Siman, I. Stefanon, and D. V. Vassallo, "Small doses of mercury increase arterial pressure reactivity to phenylephrine in rats," *Environmental Toxicology and Pharmacology*, vol. 24, no. 2, pp. 92–97, 2007.
[128] L. Massaroni, E. M. Oliveira, I. Stefanon, and D. V. Vassallo, "Effects of mercury on the mechanical and electrical activity of the Langendorff-perfused rat heart," *Brazilian Journal of Medical and Biological Research*, vol. 25, no. 8, pp. 861–864, 1992.
[129] L. B. Furieri, M. Fioresi, R. F. R. Junior et al., "Exposure to low mercury concentration *in vivo* impairs myocardial contractile function," *Toxicology and Applied Pharmacology*, vol. 255, no. 2, pp. 193–199, 2011.
[130] L. Massaroni, L. V. Rossoni, S. M. C. Amaral, I. Stefanon, E. M. Oliveira, and D. V. Vassallo, "Haemodynamic and electrophysiological acute toxic effects of mercury in anaesthetized rats and in Langendorff perfused rat hearts," *Pharmacological Research*, vol. 32, no. 1-2, pp. 27–36, 1995.
[131] L. B. Furieri, M. Galán, M. S. Avendaño et al., "Endothelial dysfunction of rat coronary arteries after exposure to low concentrations of mercury is dependent on reactive oxygen species," *British Journal of Pharmacology*, vol. 162, no. 8, pp. 1819–1831, 2011.
[132] F. M. Pecanha, G. A. Wiggers, A. M. Briones et al., "The role of cyclooxygenase (COX)-2 derived prostanoids on vasoconstrictor responses to phenylephrine is increased by exposure to low mercury concentration," *Journal of Physiology and Pharmacology*, vol. 61, no. 1, pp. 29–36, 2010.
[133] G. A. Wiggers, F. M. Peçanha, A. M. Briones et al., "Low mercury concentrations cause oxidative stress and endothelial dysfunction in conductance and resistance arteries," *American Journal of Physiology*, vol. 295, no. 3, pp. H1033–H1043, 2008.
[134] J. Blanco-Rivero, L. B. Furieri, D. V. Vassallo, M. Salaices, and G. Balfagón, "Chronic $HgCl_2$ treatment increases vasoconstriction induced by electrical field stimulation: role of adrenergic and nitrergic innervation," *Clinical Science*, vol. 121, no. 8, pp. 331–341, 2011.

Hindawi Publishing Corporation
Journal of Biomedicine and Biotechnology
Volume 2012, Article ID 417652, 13 pages
doi:10.1155/2012/417652

Research Article

Effect of Marine Omega 3 Fatty Acids on Methylmercury-Induced Toxicity in Fish and Mammalian Cells *In Vitro*

O. J. Nøstbakken,[1,2] I. L. Bredal,[1,2] P. A. Olsvik,[1] T. S. Huang,[1] and B. E. Torstensen[1]

[1] *National Institute of Nutrition and Research (NIFES), P.O. Box 2029 Nordnes, 5817 Bergen, Norway*
[2] *Department of Molecular Biology, University of Bergen, 5020 Bergen, Norway*

Correspondence should be addressed to O. J. Nøstbakken, ono@nifes.no

Received 6 January 2012; Accepted 29 February 2012

Academic Editor: Marcelo Farina

Methylmercury (MeHg) is a ubiquitous environmental contaminant which bioaccumulates in marine biota. Fish constitute an important part of a balanced human diet contributing with health beneficial nutrients but may also contain contaminants such as MeHg. Interactions between the marine n-3 fatty acids eicosapentaenoic acid (20:5n-3, EPA) and docosahexaenoic acid (22:6n-3, DHA) with MeHg-induced toxicity were investigated. Different toxic and metabolic responses were studied in Atlantic salmon kidney (ASK) cell line and the mammalian kidney-derived HEK293 cell line. Both cell lines were preincubated with DHA or EPA prior to MeHg-exposure, and cell toxicity was assessed differently in the cell lines by MeHg-uptake in cells (ASK and HEK293), proliferation (HEK293 and ASK), apoptosis (ASK), oxidation of the red-ox probe roGFP (HEK293), and regulation of selected toxicological and metabolic transcriptional markers (ASK). DHA was observed to decrease the uptake of MeHg in HEK293, but not in ASK cells. DHA also increased, while EPA decreased, MeHg-induced apoptosis in ASK. MeHg exposure induced changes in selected metabolic and known MeHg biomarkers in ASK cells. Both DHA and MeHg, but not EPA, oxidized roGFP in HEK293 cells. In conclusion, marine n-3 fatty acids may ameliorate MeHg toxicity, either by decreasing apoptosis (EPA) or by reducing MeHg uptake (DHA). However, DHA can also augment MeHg toxicity by increasing oxidative stress and apoptosis when combined with MeHg.

1. Introduction

Methylmercury (MeHg) is an environmental contaminant produced from natural or anthropogenic sources of mercury by methylation in widespread sulphate reducing bacteria [1]. MeHg enters the aquatic food chain and accumulates to become a threat for higher-order aquatic mammals and fish, but also to human health through consumption of contaminated fish [2]. MeHg has been shown to be detrimental for human health [3], with many studies emphasizing its neurological toxicity [4, 5]. The molecular pathway by which MeHg exerts its toxicity has been the issue for extensive research. Although MeHg seems to induce specific cytotoxic symptoms, one main route for MeHg molecular toxicity has yet to be elucidated [6, 7]. However, MeHg has a strong affinity for thiol groups, making every cysteine-containing protein a potential target for MeHg-binding and disruption, meaning that there may not exist one specific route of toxicity [8]. In the search for a specific molecular mechanism of MeHg-cytotoxicity, several mechanisms have been suggested for example, oxidative stress [9, 10], excito-toxicological effects [7], microtubule and cell-structural damage [11], genotoxic effects [12], and elevated intracellular Ca^{2+} leading to apoptosis [11, 13].

The occurrence of MeHg in seafood has led to a debate regarding health promoting nutrients through fish consumption, versus the risk for contaminant exposure [14–16]. Fish serve as an important source of nutrients, vitamins, and minerals and constitute an important part of a balanced diet. Some of the beneficial nutrients in fish are the long chained marine n-3 fatty acids eicosapentaenoic acid (EPA, 20:5n-3) and docosahexaenoic acid (DHA, 22:6n-3), which has shown to be important for optimal cognitive health and neuronal development [17]. But in addition to its nutritional benefits, fish may also accumulate heavy metals and other environmental contaminants in edible parts, posing an exposure risk

for higher-order mammals. Many epidemiological studies have investigated the effects of chronic low-dose fetal exposure of MeHg in different geographical locations [6]. Some of these studies report no adverse effects [18, 19], while other studies have reported adverse effects [20]. Myers et al. [21] suggest that dietary effects may be responsible for the discrepancies in MeHg toxicity between different geographical localities. They argue that a study [18, 19], performed at the Seychelles which showed no adverse effects, is based on a mainly fish consuming population, while another, performed at the Pharoe island [20] which shows adverse effects, was based on populations consuming mainly whale meat. Following this argumentation, a fish-based diet may contain certain ameliorating nutrients that will reduce the toxicity of MeHg.

Recently there has been increasing focus on interactions between nutrients and toxicants and how nutrients and the nutrient composition of organisms may affect the toxicity of different environmental contaminants. Reviews have pointed to the lack of research on nutrient-MeHg interactions and suggest that an increased focus on nutrient-MeHg interaction may increase understanding of MeHg toxicological mechanisms [6]. Nutrients can affect MeHg toxicity and retention in fish, as shown by Bjerregaard et al. [22] who demonstrated that dietary selenite decreased MeHg retention in rainbow trout (*Oncorhynchus mykiss*). Marine n-3 fatty acids, in particular DHA, have also been shown to modulate MeHg-toxicity in rats [23], and to possibly modulate MeHg neurotoxicity, as demonstrated by *in vitro* studies [24].

The aim of this study was to elucidate possible intervening effects of n-3 marine PUFA (DHA and EPA) compared to the n-6 fatty acid arachidonic acid (ARA, 20:4n-6) on MeHg cytotoxicity in Atlantic salmon kidney (ASK) cells. Human embryonic kidney (HEK293) cells were included in certain aspects of the study, and MeHg-induced toxicity was compared between the two cell types by assessing effects on cell proliferation and death using the xCELLigence system. Interaction effects caused by fatty acids on MeHg toxicity were screened by investigating known mechanistic effects of MeHg, such as uptake of MeHg in both cell lines, apoptosis in ASK cells, and oxidation of roGFP in HEK293 cells. Additionally, we investigated the regulation of transcriptional markers for MeHg toxicity and fatty acids metabolism and how DHA, EPA, and MeHg affected these in ASK cells.

2. Materials and Methods

2.1. General Methodology

2.1.1. Cell Culture. ASK cells (CRL-2643) were purchased from ATCC (London, UK). The cells were grown in Leibowitz L15 media with 20% FBS and 1 × Penicillin/Streptavidin/Amphotericin (all from Sigma, St. Louis, MO, USA) at 20°C without adding CO_2. The cells were passaged when a confluence of almost 100% was reached.

HEK293 cells were a gift from Marc Niere (University of Bergen, Norway). The cells were grown in DMEM (Sigma-Aldrich, St. Louis, MO, USA) supplemented with 10% FBS, 1 × L-glutamine, and 1 × Penicillin/Streptavidin/Amphotericin at 37°C with 5% CO_2. The cells were passaged when reaching confluence of almost 100%.

2.1.2. Coupling of Fatty Acids (FAs) to Bovine Serum Albumin (BSA). Fatty acids were coupled to fatty-acid-free BSA (FAF-BSA) (PAA, Pasching, Austria) as described by [26]. Briefly, the fatty acids were weighed and 0.04 mL chloroform per mg FA was added. The chloroform was evaporated under N_2. Furthermore, Potassium hydroxide (KOH) was added in a 1 : 3 relationship and the vial shaken in a whirl mixer for ten minutes. FAF-BSA was added in a 2.5 : 1 ratio to FA. The solution was then shaken 45 minutes, sterile-filtered, and stored anoxic at −80°C until use.

2.1.3. Basic Experimental Design. ASK cells were seeded in concentrations of 24 000 cells per cm^2. After 24 hours the cells were preincubated with 300 μM DHA, 300 μM EPA, or 300 μM ARA, all purchased from Sigma, St. Louis, MO, USA. The controls were preincubated with equivalent amounts of BSA as would be found together with fatty acids at concentrations of 300 μM. The cells were preincubated with fatty acids or BSA for 72 hours, and the media were removed before MeHg, diluted in media, or control, just fresh media, was added. Several endpoints were subsequently measured within a time frame of 48 hours.

HEK293 cells were seeded, and similar to ASK they were preincubated with the same fatty acids; however, the incubation time was reduced to 24 hours due to a more rapid cell growth of HEK293 compared to ASK. MeHg, or control, was added and endpoint measurements were performed within a time frame of 48 hours.

2.2. Comparing ASK and HEK293

2.2.1. Uptake of MeHg in FA Preincubated Cells. ASK cells were seeded at a concentration of 24 000 cells per cm^2. Cells were treated as described in basic experimental design. 48 hours after MeHg addition, the cells were scraped off using a rubber policeman and washed 3 times in 1 × PBS. The cells were further divided into two aliquots, where one was added to a DMA 80 mercury analyzer. The other half of the cells were lyzed, and total protein was measured using the BCA protein assay (Thermo Scientific Pierce, Rockford, IL, USA) on a Labsystems iEMS Reader (Thermo Fisher Scientific Inc., Waltham, MA, USA).

HEK293 cells were seeded at a concentration of 15 000 cells per cm^2. Cells were treated as described in basic experimental design. 24 hours after MeHg addition, cells were harvested and analyzed as described previously for ASK cells.

2.2.2. Cell Toxicity/Impedance Assay. In order to investigate the effect of MeHg on cell viability and proliferation, we implemented the xCELLigence RTCA SP impedance assay from Roche Diagnostics (Mannerheim, Germany) where cell adherence is measured in real time. The instrument was used according to the manufacturer's instructions [27]. The base of the xCELLigence technology is the disposable E-plates,

which are similar to normal 96-well microtiter plates. These plates are connected to an RTCA analyzer and computer with RTCA-associated software. The E-plates contain gold-plated sensor electrodes in the bottom of each well, which enables impedance readings from a small current emitted into the system. The distance from one electrode to the next in the xCELLigence system is possibly obstructed by cell media and cells adhering to the electrodes. Cells will act as insulators at the electrodes and will alter impedance measurement based on cell density and cell adherence. Hence, this impedance measurement will correspond to cell growth, cell toxicity, and adherence qualities (morphology) of the cells. A broader introduction of the xCELLigence system and software has previously been presented in [27, 28].

ASK cells were seeded at a concentration of 24 000 cells per cm^2 in a 96-well E-plate. Fresh media containing BSA were added 24 hours after seeding, at a concentration of 150 μM (equivalent to the amount incorporated in FA). The cells were incubated another 72 hours, before they were washed twice in fresh media and MeHg was added in concentrations from 1 to 4 μM MeHg dispersed in fresh media. Impedance measurement was recorded every 30 minutes throughout the analysis and up to 48 hours after addition of MeHg.

HEK293 cells were seeded at a concentration of 15 000 cells per cm^2 in a 96-well E-plate. Fresh media containing BSA were added, similarly as in ASK cells. The cells were incubated for another 24 hours, before they were washed twice in media, and MeHg dispersed in media was added in concentrations of 1–7 μM. Impedance measurement was recorded every 30 minutes, with the exception of the first hour after any modification to the cells, where it was measured every 15 minutes. Impedance measurements were performed throughout the analysis and up to 48 hours after addition of MeHg.

After addition of MeHg to both ASK and HEK293, the xCELLigence plot showed a pronounced increase in cell adherence. Since this also occurred in the control, it was deemed likely that this specific effect was due to the washing of the cells with the resulting loss of cell adherence and increase in floating cells. When these cells adhered during the next couple of hours, a pseudoincrease in growth curve would be expected. Moreover, the renewal of the media with fresh nutrient was also likely to impose a sudden increase in growth of the cells. In order to focus on the more long-term effects of MeHg, it was chosen to normalize cells when the growth curve stabilized. In order to standardize this normalization, growth curve of control cells in both ASK and HEK cells was smoothed using the Prism 5.04 (Graphpad software Inc., San Diego, CA, USA), to get more continuous curves. After smoothing, the first derivate of the curve was calculated in order to investigate the slope of the curve. At the time point where the first-derivative first approached zero (where cell growth stabilized), the curve was normalized in all treatments for the respective cell type.

2.3. Analyses of ASK Cells

2.3.1. Apoptosis Assay. ASK cells were seeded at a concentration of 24 000 cells per cm^2 in 24-well culture plates and further treated as described in basic experimental design. After 48-hour exposure to MeHg, the cells were fixed with 4% Para formaldehyde (Chemi Teknik, Oslo, Norway) and stained with 4 μg mL^{-1} bisbenzimide H 33342 (Sigma, St. Louis, MO, USA). The percentage apoptosis was counted based on cell morphology in a Nikon fluorescence microscope. The fluorescent imaging was performed at the Molecular Imaging Center (FUGE, Norwegian Research Council), University of Bergen.

2.3.2. Real Time RT-PCR. ASK cells were preincubated with the different FA and exposed to MeHg in 6-well plates at a cell density of 24 000 cells/cm^2, as previously described. All treatments were performed in triplicates. After exposure to MeHg for 48 hours, the cells were harvested using a rubber policeman. Cells were lysed, and total RNA was extracted using RNeasy columns (Qiagen, Oslo, Norway). The quantity and quality of the RNA were assessed with the NanoDrop ND-1000 UV-Vis Spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). The concentration of RNA in one well of a 6-well plate containing 24 000 cells per cm^2 was approximately 50 ng μL^{-1}.

The expression of 11 target genes and two reference genes in ASK cells after FA and MeHg exposure (Table 1), was analyzed using a two-step real-time RT-PCR protocol [29]. The RT reactions were run in triplicates in a 96-well plate, with 250 ng RNA in each sample. For PCR efficiency calculations a 6-fold serial dilution (1000-31 ng) in triplicates was made from a pooled RNA sample. Template controls (ntc) and RT enzyme control (nac) were included in each 96-well plate. The RT reaction was performed according to manufacturer's instructions using TaqMan Reverse Transcription Reagents (Applied biosystems, Foster City, CA, USA) on a GeneAmp PCR 9700 machine (Applied Biosystems, Foster City, CA, USA). Reverse transcription was performed at 48°C for 60 min by using oligo dT primers (2.5 μM) for all genes in 30 μL total volume. The final concentration of the other chemicals in each RT reaction was $MgCl_2$ (5.5 mM), dNTP (500 mM of each), 10X TaqMan RT buffer (1X), RNase inhibitor (0.4 U/μL), and Multiscribe reverse transcriptase (1.67 U/μL) (Applied Biosystems). Diluted cDNA (1 : 2) (2.0 μL cDNA from each RT reaction) was transferred to a new 96-well reaction plate and the qPCR run in 10 μL reactions on the LightCycler 480 Real-Time PCR System (Roche Applied Sciences, Basel, Switzerland). Real-time PCR was performed by using SYBR Green Master Mix (LightCycler 480 SYBR Green master mix kit, Roche Applied Sciences,), which contains FastStart DNA polymerase and gene-specific primers (500 nM). PCR was achieved with a 5 min activation and denaturizing step at 95°C, followed by 45 cycles of a 15 s denaturing step at 95°C, a 60 s annealing step and a 30 s synthesis step, at 72°C. Target gene mean-normalized expression (MNE) was determined using a normalization factor calculated by the *geNorm* software [30] based on two selected reference genes, $ef1a_B$ and arp. *geNorm* determines the individual stability of a gene within a pool of genes and calculates the stability according to the similarity of their expression profile by pairwise comparison, using the geometric mean as a normalizing factor. The gene with the highest *M*,

Table 1: Primer data.

Gene	Accession number	Fwd primer (5′–3′)	Rev. primer (5′–3′)	Amplicon size	PCR efficiency
arp (ref. gen)	AY255630	GAAAATCATCCAATTGCTGGATG	CTTCCCACGCAAGGACAGA	101	1,813
ef1ab (ref. gene)	BG933853	TGCCCCTCCAGGATGTCTAC	CACGGCCCACAGGTACTG	59	2,013
fatp	CA373015/AF023258	TGGGAGCTTGTGGGTTCAA	ACTTTCATGAGGCGGATTGG	64	2,243
actb	BG933897	CCAAAGCCAACAGGGAGAAG	AGGGACAACACTGCCTGGAT	92	2,007
gst	BQ036247	ATTTTGGGACGGGCTGACA	CCTGGTGCTCTGCTCCAGTT	81	2,07
tuba	BT049768	GAGCCAGCCAATCAGATGGT	TGCGCTTGGTCTTGATTGTG	110	2,009
gpx	BQ036408	TTCTCCACCACACTGGGATCA	GGAAATGGCATCAAGTGGAATT	101	2,011
hsp-70	BG933934	CCCCTGTCCCTGGCTATTG	CACCAGGCTGGTTGTCTGAGT	121	1,895
cox2	AJ238307	CGTCCTGAGGCAGGAGCAT	TGAGGCGTGTGGTCTGGAA	62	2,18
cpt1	AM230810	CTTTGGGAAGGGCCTGATC	CATGGACGCCTCGTACGTTA	121	1,917
bcl-x	NM_001141086	GCCTGGACGCAGTGAAAGAG	GGACGGCGTGATGTGTAGCT	107	2,127

Full gene names: acidic ribosomal protein (arp), elongation factor 1 alpha b (ef1ab), fatty acid transport protein (fatp), β-actin (actb), glutathione-s-transferase (gst), tubulin α (tuba), glutathione peroxidase (gpx), heat shock protein 70 (hsp-70), cyclooxygenase 2 (cox2), carnitine palmitoyltransferase 1 (cpt1), and B-cell lymphoma x (bcl-x).

that is, the least stabile gene, is then excluded in a stepwise fashion until the most stabile genes are determined. Here, a normalizing factor based on two examined reference genes was used to calculate the MNE.

2.4. Analysis of HEK293 Cells

2.4.1. Red-ox Sensitive GFP (roGFP). In the fluorescent vector pEGFP-N, already containing a C48S mutation, serine and glutamine at positions 147 and 204 have been replaced by two cysteines to create the roGFP2 C48S/S147C/Q204C [25]. The thiol group in these cysteines reacts to changes in the red-ox environment of the cell, altering the conformation of the roGFP-protein, and subsequently its conformation and fluorescent properties. The roGFP has two emission peaks (Figure 1(a)), which vary in intensity according to red-ox status in the cell. By measuring the emission of the roGFP at these two different excitation wavelengths, a ratiometric value representing the red-ox status in the cell can be obtained [25].

The plasmid pEGFP-N1/roGFP was purchased from University of Oregon. A stable cell line expressing roGFP (Figure 1(b)) was made by transfecting HEK293 using Fugene HD transfection reagent (Roche diagnostics, Mannheim, Germany) according to manufacturer instructions, and then the cells were selected using 400 μg mL^{-1} Geneticin (G418) (Invitrogen, By land). After selection, the cells were grown in media containing 200 μg mL^{-1} Geneticin.

Cells containing roGFP were excited at 400 and 488 nm, and emission at the two different excitation points was measured at 510 nm using a Optima FLUOstar plate reader (BMG labtech, Offenburg, Germany). The emission-ratio between the two excitation points was calculated. A ratiometric value from the emission at the two excitation points was obtained by

$$\frac{\text{emission at excitation point 488 nm}}{\text{emission at excitation point 405 nm}} = \text{Red/ox ratio}. \quad (1)$$

When roGFP positive cells grow, the total signal will increase, and since the two peaks measured have different emission maxima, a general increase in signal leads to a small change in the ratio. In order to adjust for this every treated sample was normalized to control at each time point:

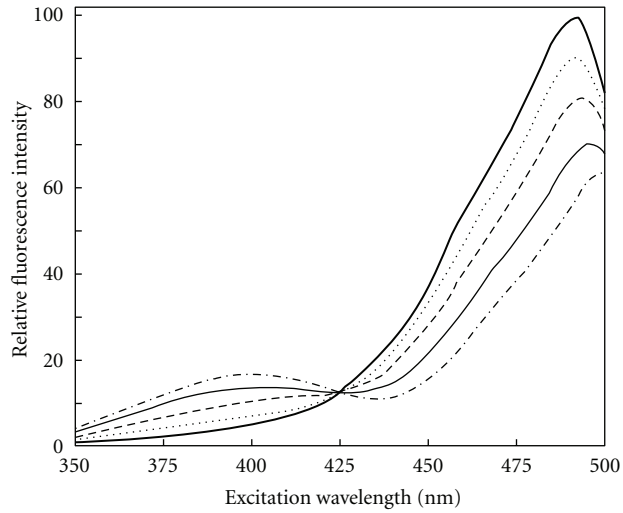
$$\frac{\text{red ox ratio}}{\text{average control red ox ratio}} = \text{relative red/ox ratio}. \quad (2)$$

In order to reduce systematic errors in the assay, the relative red-ox ratios were also normalized to their relative red-ox ratio at the start of the experiment (t_0):

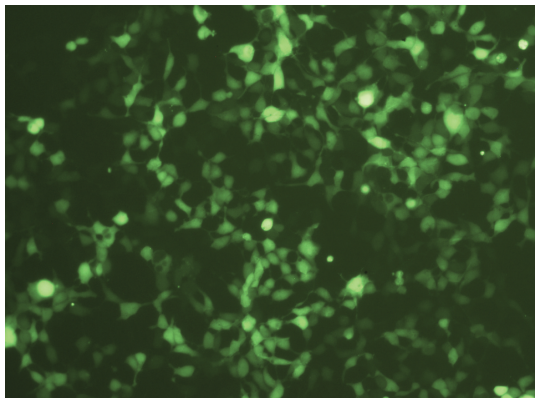
$$\frac{\text{relative red ox ratio}}{\text{red ox ratio at } t_0} = \text{normalized red ox ratio}. \quad (3)$$

To compare red-ox ratio of fatty acids and MeHg to a known oxidative inducer, a titration curve using H_2O_2 was run and a standard curve was calculated using linear curve fitting (Figure 1(c)) of the log-transformed H_2O_2 concentration. Oxidative effects of fatty acids and MeHg were then calculated into corresponding concentrations of H_2O_2, to visualize oxidative effect.

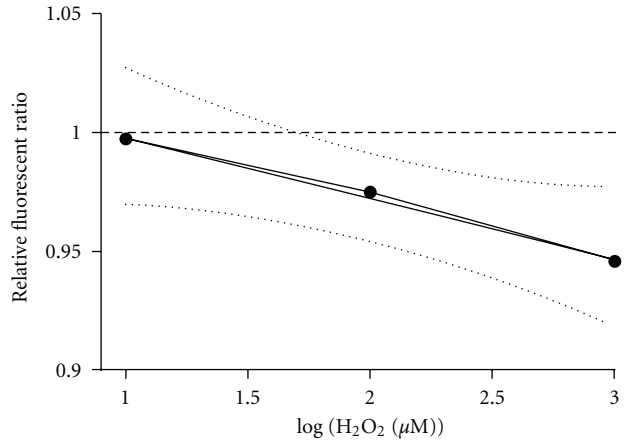
2.5. Statistical Analyses. All statistic analysis was performed using Statistica 9 (StatSoft Inc., Tulsa, USA) and Graphpad Prism 5.04 (Graphpad software Inc., San Diego, CA, USA). xCELLingence data were processed using regression analysis. Uptake data, apoptosis count, and red-ox measurement were processed using one-way ANOVA followed by post hoc Dunnett's test. Real-time RT-PCR data were processed using the nonparametric Kruskal-Willis with post hoc paired comparisons. Differences were regarded as significant when $P < 0.05$ with both statistical analysis.



(a)



(b)



(c)

Figure 1: Different aspects of roGFP as marker for oxidative changes in cells. (a) Emission intensity of roGFP measured at serial excitation points. The flat line represents reductive conditions while the semidotted line represents oxidative conditions. The lines in between are different levels of red-ox conditions (image modified from Hanson et al. [25]) (b) Image of HEK293 cells stably transfected with roGFP. (c) Linear regression of log-transformed concentration of H_2O_2 in roGFP cells. Slope is -0.02617 ± 0.004004, Y intercept when $X = 0$ is 1.027 ± 0.008649, and R^2 is 0.8104.

3. Results

3.1. Comparisons between ASK and HEK293

3.1.1. DHA Decrease Uptake of MeHg in HEK293 Cells. Uptake of MeHg was investigated in cells treated with the different fatty acids. Cells were preincubated with the marine fatty acids: DHA or EPA, the n-6 polyunsaturated fatty acid ARA, or BSA as control, before addition of MeHg. ASK cells preincubated with DHA, EPA, or ARA showed no difference in uptake of MeHg compared to MeHg-control (Figure 2(a)). In HEK293 cells, however, DHA significantly decreased the uptake of MeHg (Figure 2(b)).

3.1.2. MeHg Affects Cell Viability Similarly in ASK and HEK293 Cells. Cell toxicity of MeHg in both ASK and HEK293 cells was investigated using xCELLigence impedance measurement. Both cell lines showed a clear dose-response curve after titration versus MeHg (Figure 3). In HEK293, impedance measurements were stabilized at a concentration of 5 μM MeHg, where no further decrease was measured. Therefore, concentrations above 5 μM were considered as too high to be statistically relevant, and only data from 0–5 μM MeHg were used in regression analysis of dose response. Impedance measurement of ASK cells also seemed to reach a plateau as the MeHg concentration increased similar to HEK293, but more noticeable was the early onset of toxicity from control to 1 μM MeHg (Figure 3). Area under curve (AUC) was calculated in ASK cells from time point 102 h–144 h after seeding, as a measurement of cell toxicity, and used in regression analysis. This dose-response curve of ASK AUC followed a nonlinear regression curve with $r^2 = 0.9824$ (Figure 3(c)). AUC was also calculated from HEK cells from time point 53–96 h. The dose-response curve of HEK AUC followed a linear curve fitting with $r^2 = 0.9491$ (Figure 3(d)).

3.2. ASK Cells

3.2.1. Fatty Acids Affect MeHg-Induced Apoptosis. Apoptosis was measured using morphological analysis of ASK cells after fatty acid preincubation and MeHg exposure. A dose-response curve of MeHg was investigated by preincubating cells with BSA, instead of fatty acids, followed by a titration to different MeHg concentrations. Apoptosis count was plotted, and curve fitting was performed (Figure 4(a)). The dose response followed a nonlinear regression curve ($r^2 = 0.9564$). From the dose response curve we chose a concentration of 2.5 μM MeHg to test the effect of fatty acid preincubation on MeHg-induced apoptosis, since this particular concentration of MeHg induced a level of apoptosis which can be remedied by molecular intervention. Cells preincubated with different fatty acids were compared to control cells preincubated with BSA and exposed to MeHg using one-way ANOVA and post hoc Dunnett's test. Preincubating DHA significantly ($P = 0.000014$) increased while EPA significantly ($P = 0.043948$) decreased apoptosis induced by 2.5 μM MeHg. ARA showed no effects on apoptosis (Figure 4(b)).

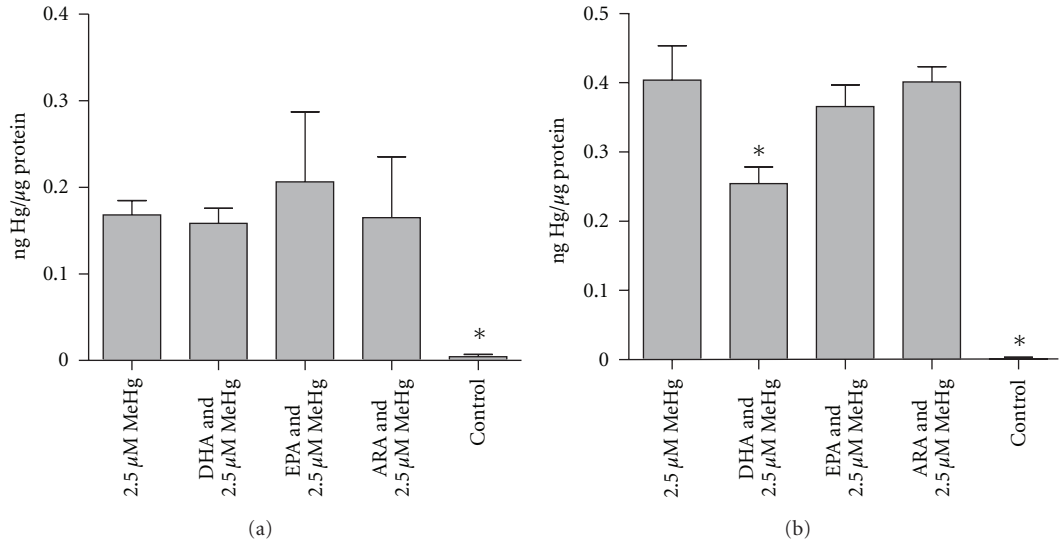


Figure 2: Uptake of MeHg after FA preincubation in ASK cells (a) and HEK cells (b). Results are presented as mean ± SD. Statistical differences are revealed by one-way ANOVA and post hoc Dunnett's test where $n = 4$, except for Hg- where $n = 3$. Significant differences compared to 2.5 μM MeHg ($P \leq 0.05$) are indicated by asterix.

3.2.2. MeHg Severely Affected, While Fatty Acids Only Induced, Minor Changes in Gene Expression. Total RNA from ASK cells, preincubated with the different fatty acids and exposed to MeHg, was extracted and the gene expression of selected markers was investigated. When comparing all groups exposed to MeHg versus control (MeHg main effect), the B-cell lymphoma 2 (bcl2) like protein (bclX), heat shock protein 70 (hsp70), and cyclooxygenase 2 (cox2) were all upregulated by MeHg (Figures 5 and 6). Fatty acid transport protein (fatp), carnitine-palmitoyl transferase (cpt1), and tubulin α (tuba) were downregulated by MeHg (Figures 5 and 6). When comparing the different fatty acids (fatty acid main effect) versus control, no effects were observed. When comparing all groups, hsp70 was significantly upregulated in ARA- and MeHg-treated cells compared to control. fatp was significantly downregulated in cells preincubated with DHA compared to the control, while tuba was significantly downregulated in cells preincubated with EPA and exposed to MeHg compared to the control. No significant effects of the treatments were observed in glutathione peroxidase 2 (gpx2) or glutathione-S-transferase (gst) (Figures 5 and 6).

3.3. HEK293 Cells

3.3.1. DHA and MeHg Alter Red-Ox Status in HEK293 Cells. HEK293 cells were stably transfected with roGFP plasmid and showed a clear fluorescence after G418-selection (Figure 1(b)). These cells were used to investigate effects of fatty acids and MeHg on oxidative status in cells. Oxidative stress caused by fatty acids and MeHg treatment was compared to equivalent signals generated by the known oxidative inducer H_2O_2 (Table 2).

Incubating HEK293 cells with EPA and ARA did not induce any change in oxidative status However, both DHA and MeHg significantly induced an oxidative state in the cells after 5 minutes of incubation (Figure 7(a)). DHA steadily kept cells in a state of oxidative stress during its preincubation, but after addition of fresh media a normal oxidative status was recovered (Figure 7(b)). MeHg also rapidly induced oxidation in the cells, but cells recovered their original oxidative status within approximately 1 hour (Figure 7(c)). Preincubating cells with fatty acids did not affect the change in the oxidative status caused by later MeHg exposure (Figure 7(a)).

4. Discussion

In this study, the mediating effects of the marine n-3 fatty acids DHA and EPA on MeHg toxicity were studied. EPA ameliorated the apoptotic response of ASK to MeHg, while DHA reduced the uptake of MeHg into HEK293 cells. These results indicate that the metabolism of marine n-3 fatty acids may ameliorate MeHg toxicity at the molecular level and that the nutrient content of a diet may affect the potential negative effect of MeHg-contaminated fish. However, DHA also augmented the MeHg-induced apoptosis in ASK cells, indicating potential potentiating effects of DHA on MeHg toxicity.

4.1. MeHg Affects FA Metabolism in ASK Cells. The metabolism of nutrients into applicable energy for the cell is a vital part in maintaining cell survival. In different exploratory studies performed on fish exposed to MeHg, changes in metabolic genes or proteins have been reported [8, 31]. In our study we found a significant decrease in cpt1 and fatp gene expression as a result of MeHg exposure. cpt1 is responsible for a rate limiting step in mitochondrial β-oxidation of fatty acids: the transport of fatty acids into mitochondria for catabolism [32]. The regulation of this protein is mainly based on its gene transcription [33]. Fatp is a membrane

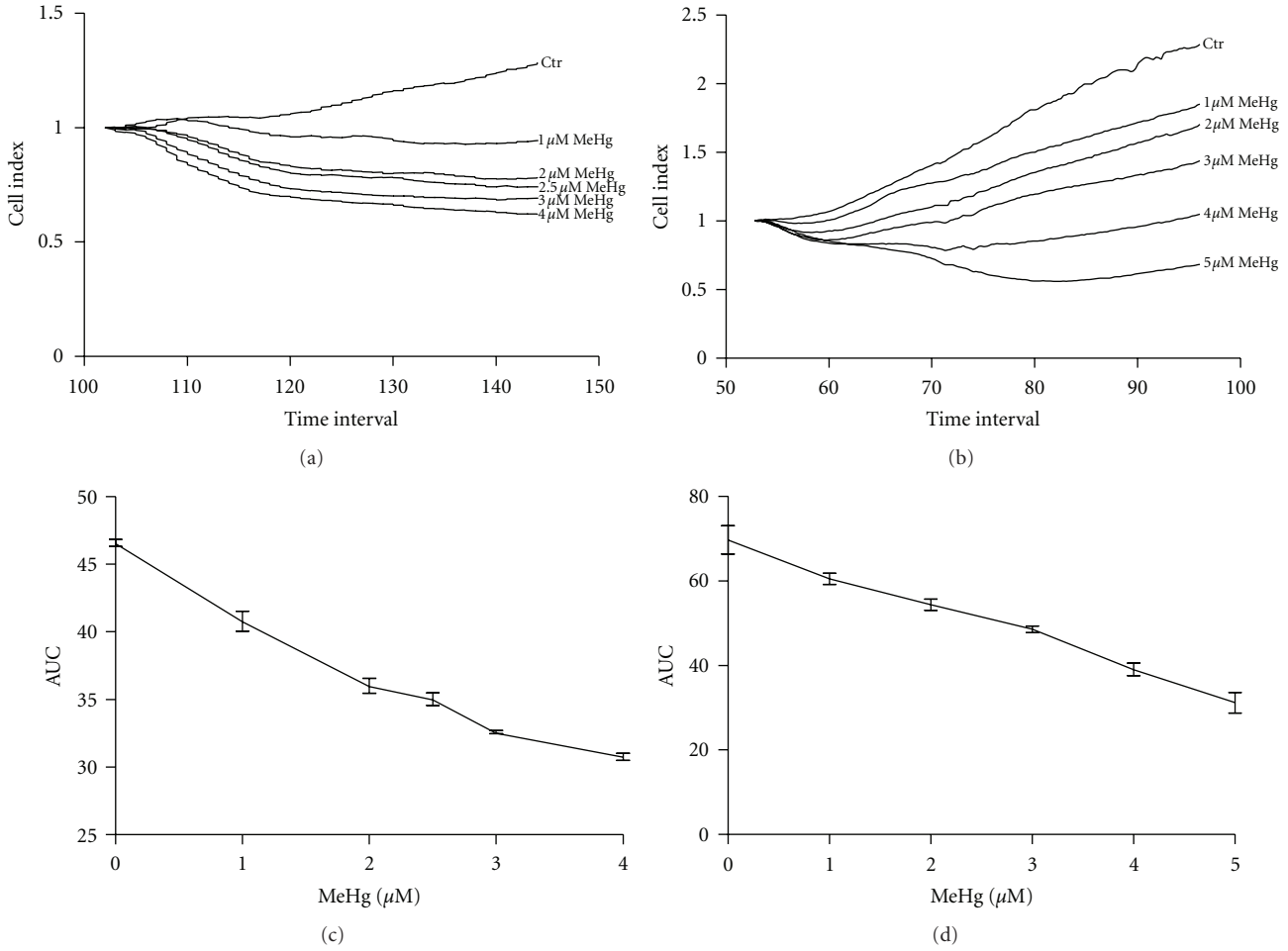


Figure 3: Titration of MeHg in ASK and HEK293 cell culture. (a) Growth pattern of ASK in xCELLigence after normalization. (b) Growth pattern of HEK293 in xCELLigence after normalization. (c) Area under curve from (timespan = 48 h after MeHg exposure), representing loss of adherent cells or decreased growth in ASK cells. The dose response showed a nonlinear curve ($r^2 = 0.9824$). (d) Area under curve (timespan = 48 h after MeHg exposure), representing loss of adherent cells or decreased growth in HEK 293 cells. The dose response showed a linear curve ($r^2 = 0.9491$).

Table 2: Comparison of relative red-ox ratio to the known oxidative inducer H_2O_2 in HEK293.

	DHA	EPA	ARA	MeHg	DHA and MeHg	EPA and MeHg	ARA and MeHg
Average of relative red-ox ratio	0.971	0.993	1.002	0.952	0.954	0.951	0.939
Equiv. [H_2O_2]	142	≈0	≈0	710	606	811	>1000

Values below 12 are not significantly different from control, and oxidative stress is assumed almost zero. Calculations are made based on exponential curve fitting and its formula.
">1000" represents a value that exceeds the standard made from H_2O.

bound protein involved in uptake of fatty acids into cells [34]. The regulation of fatp is thought to be governed through peroxisome proliferator receptors (PPARs) [35], hence being dependent on induction of PPAR through ligand-binding. fatp is also, in part, responsible for the import of fatty acids into mitochondria by interacting with cpt1 [36]. When transcription of both cpt1 and fatp is decreased in our cell system, this may signify a decrease in the availability of fatty acids as substrate for energy metabolism in the mitochondria and a subsequent reduced metabolic throughput. This could be a direct effect of MeHg on the proteins themselves or their regulatory mechanisms, or alternatively it could be that the transcriptional expression of these genes is downregulated as a secondary response to metabolic shutdown after MeHg-induced apoptosis is initiated. Disruption of mitochondrial metabolism by MeHg has previously been reported. Studies have shown that MeHg can disrupt the mitochondrial energy metabolism more directly, possibly through inhibiting phosphorylation of ADP to ATP [37], or depolarizing the mitochondria [10]. The detrimental effect of MeHg

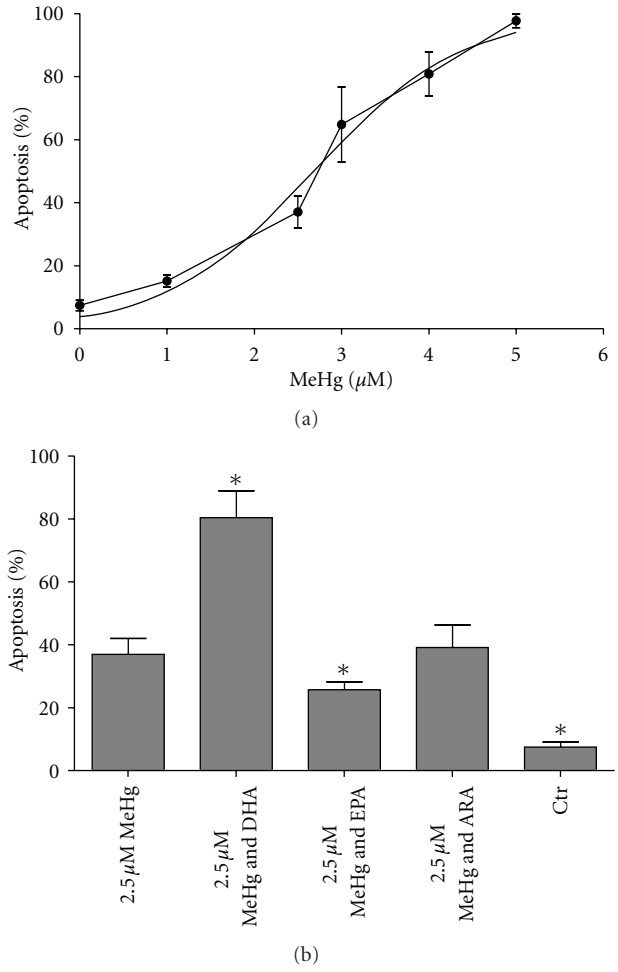


Figure 4: Apoptosis count of ASK cells after treatment with FA and MeHg. (a) MeHg dose-response with curve following a logarithmic nonlinear curve disposition ($r^2 = 0.9564$); from this curve a concentration which gave 40% apoptosis (approx. 2.5 μM MeHg) was used in assay including fatty acids. (b) Apoptosis count after preincubation of cells with fatty acids. Results are presented as mean ± SD. Statistical differences are revealed by one-way ANOVA and post hoc Dunnett's test where $n = 4$. Significant differences compared to 2.5 μM MeHg ($P \leq 0.05$) are indicated by asterix.

on mitochondrial metabolism in the cell may also increase leakage of reactive oxygen species from mitochondria and thereby increasing ROS [38].

An important metabolic pathway for 20 carbon polyunsaturated fatty acids (EPA and ARA) is the eicosanoid pathway, where activation of cytosolic phospholipase A_2 (cPLA2) catalyzes the breakdown of membrane phospholipids and releases ARA for subsequent metabolite formation. cPLA2 activity has been shown to be increased by MeHg [39]. An important enzyme in this pathway is cyclooxygenase 2 (cox2), which we showed to be upregulated in response to MeHg exposure. This may be a secondary response to MeHg-induced oxidative stress since cox2 expression has been shown to increase in response to oxidative stress [40, 41]. Cox2 is responsible for the conversion of ARA into prostanoids, such as the prostaglandins. An increase in production of prostaglandins may increase the inflammatory response in the cell, meaning that MeHg may induce proinflammatory metabolites of ARA. Metabolites derived from ARA such as the prostaglandin E_2 have also shown to increase intracellular Ca^{2+} in osteoblast-like cells [42]. Increase of intracellular Ca^{2+} is also a known toxic effect of MeHg [43], meaning that increased cox 2 leading to increased production of prostaglandins may be part of the underlying cause of MeHg-induced Ca^{2+} influx, with consequent increase in apoptosis.

4.2. EPA Reduces MeHg-Induced Apoptosis. The enzymes in the eicosanoid pathway have the ability to utilize both ARA and EPA as substrate for production of different metabolites [44]. However, ARA- and EPA-induced eicosanoids exhibit different molecular effects in the cell [45]. In our study, EPA ameliorated the apoptotic effect of MeHg in the ASK cells, while ARA did not. If excess EPA out competes ARA in the eicosanoid pathway, the resulting metabolites show more anti-inflammatory effects than the respective ARA metabolites [45]. EPA might even reduce the inflammatory effects of ARA-metabolites created in the same system [46]. The abandoning of ARAs proinflammatory and possible Ca^{2+} releasing effects could be part of the explanation why EPA reduces MeHg-induced apoptosis in ASK cells. ARA supplementation did not augment the toxicity of MeHg, which one might expect if the proinflammatory ARA-derived eicosanoids are damaging to the cell. However, the FBS added to the cell media in the MeHg-control probably already contains relatively high ARA-to-EPA ratio, making the production of ARA-derived metabolites saturated, and not dependent on cox2 or the availability of substrate.

Another side of the story is that by replacing ARA with EPA in the eicosanoid system, more EPA-derived eicosanoids will be produced, which could reduce inflammation and affect MeHg toxicity directly, or through apoptosis signaling pathways. The effect of EPA-derived eicosanoids on MeHg toxicity generally, and apoptosis specifically, is not yet known, and further investigations are necessary to elucidate this.

Oxidative effects of fatty acids with and without MeHg were investigated using roGFP-HEK293 cells, and as opposed to DHA, EPA and ARA did not affect the oxidation of roGFP in the cells. These results are consistent with the lack of antioxidant protection against MeHg of fish oil, shown in previous studies [47]. There are, however, studies showing that EPA can cause oxidative stress in muscle and liver cells of Atlantic salmon [48, 49]. However, since we did not see any indications of this, there could be other molecular mechanisms explaining the antioxidant effects noted elsewhere in the literature [50].

EPA, unlike DHA, did not affect the uptake of MeHg into HEK293 cells, suggesting that there must be intracellular molecular mechanisms responsible for EPA's ameliorating effects on MeHg-induced apoptosis.

4.3. DHA Modulates MeHg Uptake and Toxicity. DHA preincubation significantly decreased the uptake of MeHg in

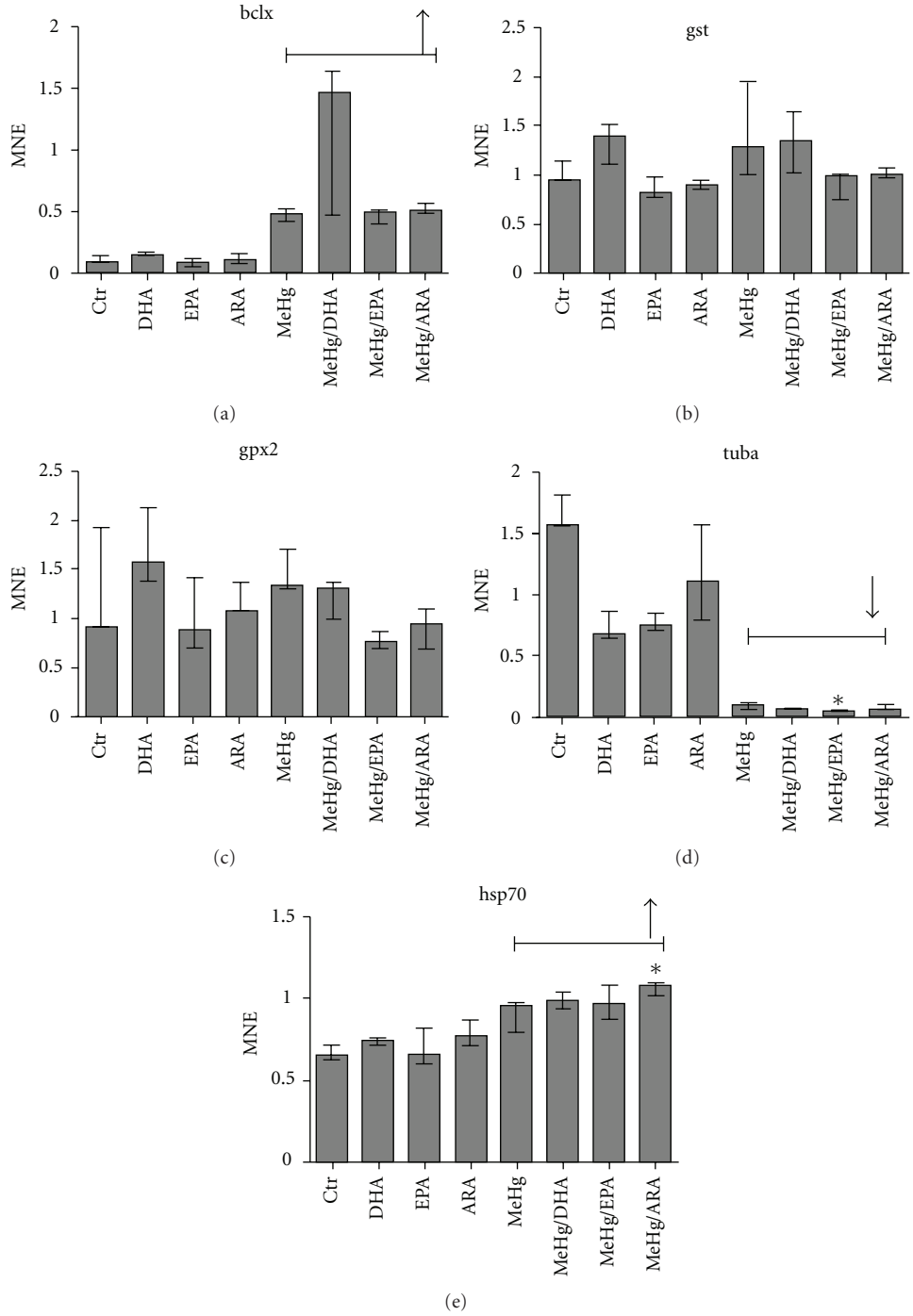


Figure 5: Mean-normalized expression (MNE) of (a) bclx, (b) gst, (c) gpx2, (d) tuba, and (e) hsp70 in ASK cells. Data are represented by median, with error bars representing interquartile range. Data are treated through factorial design, where $n = 3$ for all groups (columns shown), and significant differences ($P \leq 0.05$) are indicated by asterix. MeHg and FA effects were calculated across groups where $n = 12$ and $n = 6$, respectively. Significant effects ($P \leq 0.05$) of MeHg are shown using bracket lines. No fatty acid effect compared to control was observed. Statistical analysis was performed using nonparametric Kruskal Willis with post hoc paired comparisons.

HEK293 cells, which is consistent with other studies using human cerebellar astrocytes and cerebellar neurons [51]. No significant difference in uptake of MeHg due to DHA was observed in the ASK cells. However, ASK cells were grown at lower temperature and displayed much lower metabolic rate than HEK293 cells. Given that uptake of MeHg is an active metabolic process linked to carrier mediated processes [52], the effect of DHA on MeHg uptake may be disguised in our experimental settings. Unfortunately, this leaves our results in ASK cells indicative instead of conclusive, and further

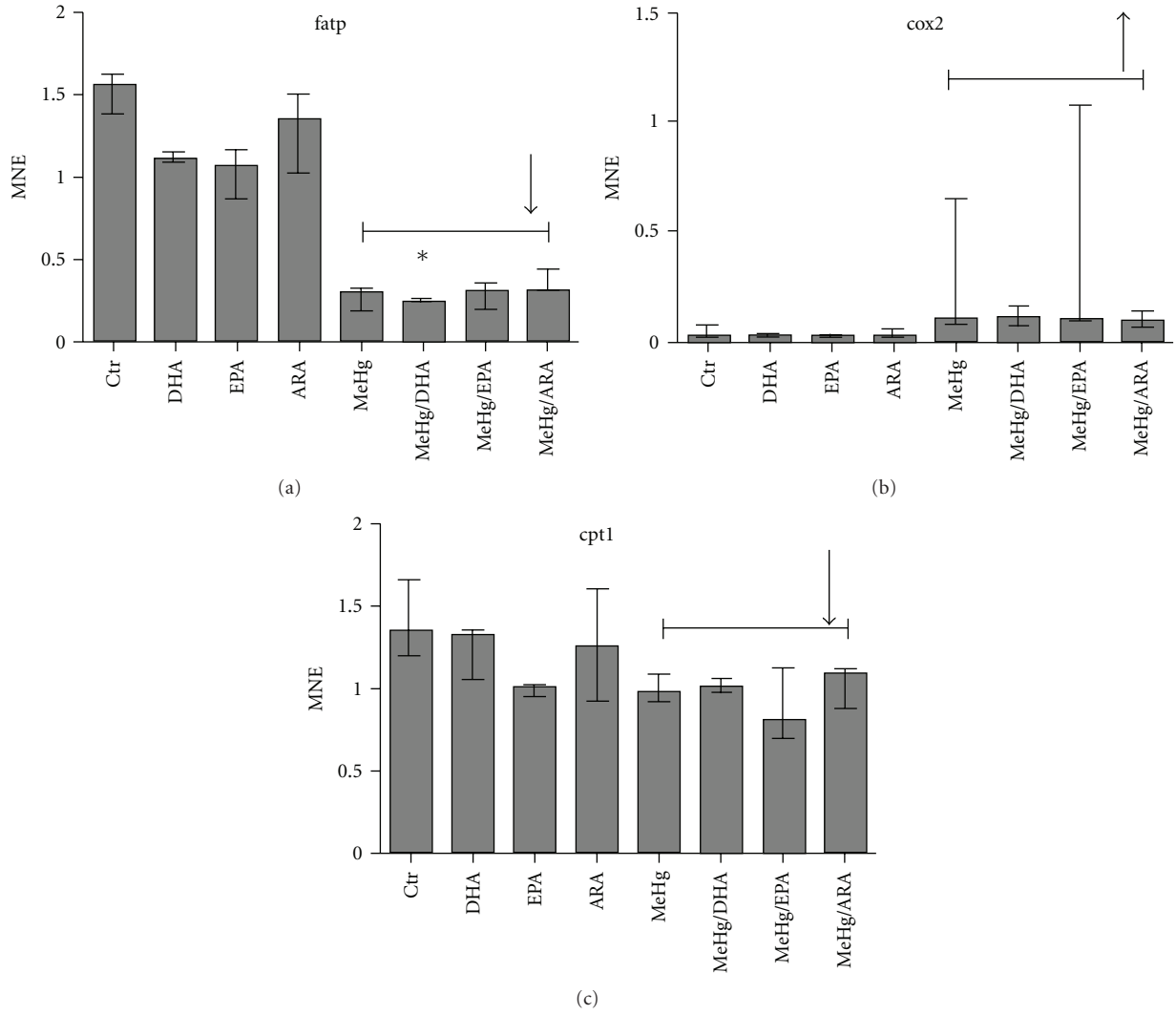


Figure 6: Mean-normalized expression (MNE) of (a) fatp, (b) cox2, and (c) cpt1 in ASK cells. Data are represented by median, with error bars representing interquartile range. Data are treated through factorial design, where $n = 3$ for all groups (columns shown), and significant differences ($P \leq 0.05$) are indicated by asterix. MeHg and FA effects were calculated across groups where $n = 12$ and $n = 6$, respectively. Significant effects ($P \leq 0.05$) of MeHg are shown using bracket lines. Fatty acid effects compared to control are shown by the letters FA. Statistical analysis was performed using nonparametric Kruskal Willis with post hoc paired comparisons.

research of uptake mechanisms affected by DHA, in different cell types, is needed.

The amended uptake of MeHg observed in the HEK293 cells may be due to altered membrane properties after DHA preincubation. PUFAs are important constituents in cell membranes, where they affect several functions such as membrane organization, elasticity, microdomain formation, and permeability of the lipid bilayer. However, due to the difference in chain length and the number of double bonds, DHA is thought to differ in flexibility and conformational freedom compared to EPA and hence will cause different structure disorganization [53]. By changing physical properties of cell membranes, DHA may affect uptake of extracellular compounds, such as MeHg, into cells.

Although DHA decreased the uptake of MeHg in the HEK293 cells, we observed an increase in apoptosis of ASK cells after DHA preincubation and MeHg exposure. This increase was profound, and real-time RT-PCR analysis of the apoptotic regulator bclx in ASK cells suggested a similar effect, though not significant compared to cells only exposed to MeHg. The apoptotic effect seems to be triggered by the addition of MeHg, since DHA treatment alone did not induce increase in apoptosis (results not shown). A possible explanation for the severe apoptotic effect of DHA together with MeHg may be due to changes in the red-ox status in the cells. Although some literature reports DHA as an inhibitor of oxidative stress in certain cells [54], DHA has also been reported to induce oxidative stress and production of ROS [48, 55]. MeHg is known to be a pro-oxidant [56], so the observation can be explained by a potential effect of the compounds. Another possible explanation could be that DHA has inhibited the cells innate antioxidant defense system over

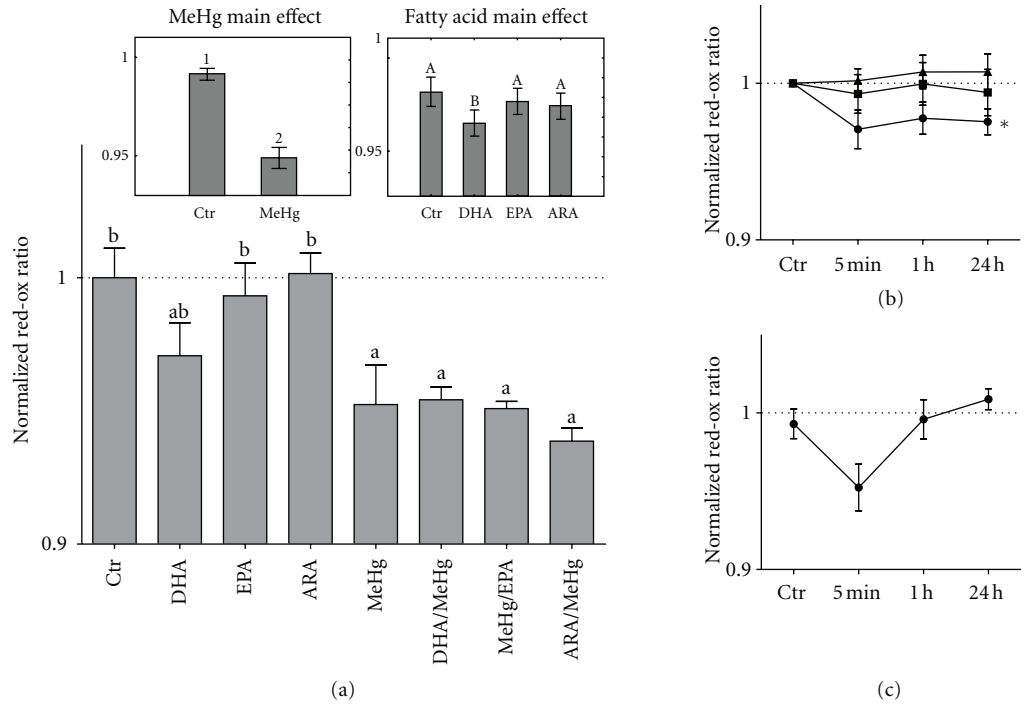


Figure 7: Fatty acid and MeHg effect on red/ox balance in HEK293 cells. (a) Relative fluorescent value of the cells 5 minutes after exposure to the different compounds. Data are treated by two-way ANOVA and main effects analyzed by post hoc Fischer's test. Significant differences ($P < 0.05$) due to MeHg exposure (MeHg main effect) are represented using lettering. Significant differences ($P < 0.05$) due to fatty acid treatment (fatty acid main effect) are represented using large letters. Interaction effects were analyzed using post hoc Dunnett's test where significant differences ($P < 0.05$) compared to control are visualized by small letter a, while significant differences ($P < 0.05$) compared to MeHg are visualized using small letter b. Dotted line represents normalized control values. (b) Relative fluorescent ratio of fatty acids compared to control over time. Circles represent DHA, squares represent EPA, and triangles represent ARA. The dotted line represents normalized control values. Data are treated with one-way ANOVA followed by a post hoc Dunnett's test; lines significantly different ($P < 0.05$) from control are marked with $*$. (c) Relative fluorescent ratio of MeHg compared to control over time. Black dots represent MeHg, while the dotted line represents normalized control values. Error margins are signified using standard deviation.

time [24]. When we investigated oxidation of roGFP in the HEK293 cells, both MeHg and DHA were observed to oxidize roGFP. DHA kept roGFP in a continuous oxidative state, whilst MeHg affected the oxidation of roGFP moretemporarily. EPA and ARA did not oxidize roGFP in the cells, indicating that the pro-oxidative effect of these fatty acids, compared to DHA, has been obliterated or kept under control in the cells. Conversely, no significant effects of DHA were noted on the transcriptional regulation of the two antioxidant enzymes gpx2 and gst in ASK cells. However, this may be due to normalization of these levels after replenishing control cells with fresh media after the fatty acid preincubation.

5. Conclusion

In this study we have shown that the marine n-3 fatty acid DHA can decrease MeHg uptake in HEK293 cells as well as increase MeHg-induced apoptosis in ASK cells. Furthermore, the proapoptotic effect of DHA may depend on its ability to induce changes in the red-ox environment in the cell. To our knowledge, this is the first time that ameliorating effects of the marine fatty acid EPA on MeHg-induced apoptosis are reported. Taken together, the effects shown by DHA and EPA on MeHg-induced toxicity, in this study, may help explain the differing toxicity observed in response to type of dietary MeHg source [21].

Acknowledgments

This work was funded by the National Research Council (NFR) of Norway, through the project: Seafood and Mental Health: Uptake and Effects of Marine Nutrients and Contaminants Alone or in Combination on Neurological Function (Project no. 186908). The authors would like to thank Ms Aina Cathrine Øvergård (IMR) for feedback on the paper.

References

[1] S. Jensen and A. Jernelöv, "Biological methylation of mercury in aquatic organisms," *Nature*, vol. 223, no. 5207, pp. 753–754, 1969.

[2] F. M. M. Morel, A. M. L. Kraepiel, and M. Amyot, "The chemical cycle and bioaccumulation of mercury," *Annual Review of Ecology and Systematics*, vol. 29, pp. 543–566, 1998.

[3] S. Diez, "Human health effects of methylmercury exposure," *Reviews of Environmental Contamination and Toxicology*, vol. 198, pp. 111–132, 1998.

[4] A. F. Castoldi, T. Coccini, S. Ceccatelli, and L. Manzo, "Neurotoxicity and molecular effects of methylmercury," *Brain Research Bulletin*, vol. 55, no. 2, pp. 197–203, 2001.

[5] A. F. Castoldi, T. Coccini, and L. Manzo, "Neurotoxic and molecular effects of methylmercury in humans," *Reviews on Environmental Health*, vol. 18, no. 1, pp. 19–31, 2003.

[6] L. Chapman and H. M. Chan, "The influence of nutrition on methyl mercury intoxication," *Environmental Health Perspectives*, vol. 108, supplement 1, pp. 29–56, 2000.

[7] M. Aschner, T. Syversen, D. O. Souza, J. B. T. Rocha, and M. Farina, "Involvement of glutamate and reactive oxygen species in methylmercury neurotoxicity," *Brazilian Journal of Medical and Biological Research*, vol. 40, no. 3, pp. 285–291, 2007.

[8] K. Berg, P. Puntervoll, S. Valdersnes, and A. Goksøyr, "Responses in the brain proteome of Atlantic cod (*Gadus morhua*) exposed to methylmercury," *Aquatic Toxicology*, vol. 100, no. 1, pp. 51–65, 2010.

[9] M. H. G. Berntssen, A. Aatland, and R. D. Handy, "Chronic dietary mercury exposure causes oxidative stress, brain lesions, and altered behaviour in Atlantic salmon (*Salmo salar*) parr," *Aquatic Toxicology*, vol. 65, no. 1, pp. 55–72, 2003.

[10] T. K. Garg and J. Y. Chang, "Methylmercury causes oxidative stress and cytotoxicity in microglia: attenuation by 15-deoxy-delta 12, 14-prostaglandin J2," *Journal of Neuroimmunology*, vol. 171, no. 1-2, pp. 17–28, 2006.

[11] C. Johansson, A. F. Castoldi, N. Onishchenko, L. Manzo, M. Vahter, and S. Ceccatelli, "Neurobehavioural and molecular changes induced by methylmercury exposure during development," *Neurotoxicity Research*, vol. 11, no. 3-4, pp. 241–260, 2007.

[12] M. E. Crespo-López, A. Lima de Sá, A. M. Herculano, R. Rodríguez Burbano, and J. L. Martins do Nascimento, "Methylmercury genotoxicity: a novel effect in human cell lines of the central nervous system," *Environment International*, vol. 33, no. 2, pp. 141–146, 2007.

[13] K. Miura and N. Imura, "Mechanism of methylmercury cytotoxicity," *Critical Reviews in Toxicology*, vol. 18, no. 3, pp. 161–188, 1987.

[14] C. Bouzan, J. T. Cohen, W. E. Connor et al., "A quantitative analysis of fish consumption and stroke risk," *American Journal of Preventive Medicine*, vol. 29, no. 4, pp. 347–352, 2005.

[15] J. T. Cohen, D. C. Bellinger, W. E. Connor et al., "A quantitative risk-benefit analysis of changes in population fish consumption," *American Journal of Preventive Medicine*, vol. 29, no. 4, pp. 325–334, 2005.

[16] G. L. Ginsberg and B. F. Toal, "Quantitative approach for incorporating methylmercury risks and omega-3 fatty acid benefits in developing species-specific fish consumption advice," *Environmental Health Perspectives*, vol. 117, no. 2, pp. 267–275, 2009.

[17] J. R. Hibbeln, T. A. Ferguson, and T. L. Blasbalg, "Omega-3 fatty acid deficiencies in neurodevelopment, aggression and autonomic dysregulation: opportunities for intervention," *International Review of Psychiatry*, vol. 18, no. 2, pp. 107–118, 2006.

[18] G. J. Myers, D. O. Marsh, C. Cox et al., "A pilot neurodevelopmental study of Seychellois children following in utero exposure to methylmercury from a maternal fish diet," *NeuroToxicology*, vol. 16, no. 4, pp. 629–638, 1995.

[19] G. J. Myers, D. O. Marsh, P. W. Davidson et al., "Main neurodevelopmental study of Seychellois children following in utero exposure to methylmercury from a maternal fish diet: outcome at six months," *NeuroToxicology*, vol. 16, no. 4, pp. 653–664, 1995.

[20] P. Grandjean, P. Weihe, R. F. White et al., "Cognitive deficit in 7-year-old children with prenatal exposure to methylmercury," *Neurotoxicology and Teratology*, vol. 19, no. 6, pp. 417–428, 1997.

[21] G. J. Myers and P. W. Davidson, "Prenatal methylmercury exposure and children: neurologic, developmental, and behavioral research," *Environmental Health Perspectives*, vol. 106, supplement 3, pp. 841–847, 1998.

[22] P. Bjerregaard, B. W. Andersen, and J. C. Rankin, "Retention of methyl mercury and inorganic mercury in rainbow trout Oncorhynchus mykiss (W): effect of dietary selenium," *Aquatic Toxicology*, vol. 45, no. 2-3, pp. 171–180, 1999.

[23] X. Jin, E. Lok, G. Bondy et al., "Modulating effects of dietary fats on methylmercury toxicity and distribution in rats," *Toxicology*, vol. 230, no. 1, pp. 22–44, 2007.

[24] P. Kaur, K. Schulz, M. Aschner, and T. Syversen, "Role of docosahexaenoic acid in modulating methylmercury-induced neurotoxicity," *Toxicological Sciences*, vol. 100, no. 2, pp. 423–432, 2007.

[25] G. T. Hanson, R. Aggeler, D. Oglesbee et al., "Investigating mitochondrial redox potential with redox-sensitive green fluorescent protein indicators," *The Journal of Biological Chemistry*, vol. 279, no. 13, pp. 13044–13053, 2004.

[26] C. Ghioni, D. R. Tocher, and J. R. Sargent, "The effect of culture on morphology, lipid and fatty acid composition, and polyunsaturated fatty acid metabolism of rainbow trout (Oncorhynchus mykiss) skin cells," *Fish Physiology and Biochemistry*, vol. 16, no. 6, pp. 499–513, 1997.

[27] Roche-Diagnostics-GmbH, *RTCA SP Instrument Operator's Manual*, ACEA Biosciences, 2009.

[28] E. Urcan, U. Haertel, M. Styllou, R. Hickel, H. Scherthan, and F. X. Reichl, "Real-time xCELLigence impedance analysis of the cytotoxicity of dental composite components on human gingival fibroblasts," *Dental Materials*, vol. 26, no. 1, pp. 51–58, 2010.

[29] P. A. Olsvik, F. Kroglund, B. Finstad, and T. Kristensen, "Effects of the fungicide azoxystrobin on Atlantic salmon (*Salmo salar* L.) smolt," *Ecotoxicology and Environmental Safety*, vol. 73, no. 8, pp. 1852–1861, 2010.

[30] J. Vandesompele, K. De Preter, F. Pattyn et al., "Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes," *Genome Biology*, vol. 3, no. 7, 2002.

[31] R. Klaper, B. J. Carter, C. A. Richter, P. E. Drevnick, M. B. Sandheinrich, and D. E. Tillitt, "Use of a 15 k gene microarray to determine gene expression changes in response to acute and chronic methylmercury exposure in the fathead minnow Pimephales promelas Rafinesque," *Journal of Fish Biology*, vol. 72, no. 9, pp. 2207–2280, 2008.

[32] G. Jogl, Y. S. Hsiao, and L. Tong, "Structure and function of carnitine acyltransferases," *Annals of the New York Academy of Sciences*, vol. 1033, pp. 17–29, 2004.

[33] J. P. Bonnefont, F. Djouadi, C. Prip-Buus, S. Gobin, A. Munnich, and J. Bastin, "Carnitine palmitoyltransferases 1 and 2: biochemical, molecular and medical aspects," *Molecular Aspects of Medicine*, vol. 25, no. 5-6, pp. 495–520, 2004.

[34] J. E. Schaffer and H. F. Lodish, "Expression cloning and characterization of a novel adipocyte long chain fatty acid transport protein," *Cell*, vol. 79, no. 3, pp. 427–436, 1994.

[35] B. I. Frohnert and D. A. Bernlohr, "Regulation of fatty acid transporters in mammalian cells," *Progress in Lipid Research*, vol. 39, no. 1, pp. 83–107, 2000.

[36] D. Sebastián, M. Guitart, C. García-Martínez et al., "Novel role of FATP1 in mitochondrial fatty acid oxidation in skeletal

muscle cells," *Journal of Lipid Research*, vol. 50, no. 9, pp. 1789–1799, 2009.

[37] J. P. Bourdineaud, S. Cambier, G. Bénard et al., "At environmental doses, dietary methylmercury inhibits mitochondrial energy metabolism in skeletal muscles of the zebra fish (*Danio rerio*)," *International Journal of Biochemistry and Cell Biology*, vol. 41, no. 4, pp. 791–799, 2009.

[38] S. Yee and B. H. Choi, "Oxidative stress in neurotoxic effects of methylmercury poisoning," *NeuroToxicology*, vol. 17, no. 1, pp. 17–26, 1996.

[39] G. Shanker, R. E. Hampson, and M. Aschner, "Methylmercury stimulates arachidonic acid release and cytosolic phospholipase A2 expression in primary neuronal cultures," *NeuroToxicology*, vol. 25, no. 3, pp. 399–406, 2004.

[40] P. Rockwell, J. Martinez, L. Papa, and E. Gomes, "Redox regulates COX-2 upregulation and cell death in the neuronal response to cadmium," *Cellular Signalling*, vol. 16, no. 3, pp. 343–353, 2004.

[41] Y. Sun, J. Chen, and B. Rigas, "Chemopreventive agents induce oxidative stress in cancer cells leading to COX-2 overexpression and COX-2-independent cell death," *Carcinogenesis*, vol. 30, no. 1, pp. 93–100, 2009.

[42] H. Tokuda, M. Miwa, Y. Oiso, and O. Kozawa, "Autoregulation of prostaglandin E2-induced Ca^{2+} influx in osteoblast-like cells: inhibition by self-induced activation of protein kinase C," *Cellular Signalling*, vol. 4, no. 3, pp. 261–266, 1992.

[43] M. Sue Marty and W. D. Atchison, "Pathways mediating Ca^{2+} entry in rat cerebellar granule cells following *in vitro* exposure to methyl mercury," *Toxicology and Applied Pharmacology*, vol. 147, no. 2, pp. 319–330, 1997.

[44] A. A. Spector, "Essentiality of fatty acids," *Lipids*, vol. 34, no. 6, supplement 3, pp. S1–S3, 1999.

[45] P. C. Calder, "n-3 Polyunsaturated fatty acids, inflammation, and inflammatory diseases," *American Journal of Clinical Nutrition*, vol. 83, no. 6, supplement, pp. 1505S–1519S, 2006.

[46] D. Bagga, L. Wang, R. Farias-Eisner, J. A. Glaspy, and S. T. Reddy, "Differential effects of prostaglandin derived from ω-6 and ω-3 polyunsaturated fatty acids on COX-2 expression and IL-6 secretion," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 100, no. 4, pp. 1751–1756, 2003.

[47] D. Grotto, J. Vicentini, J. P. Friedmann Angeli et al., "Evaluation of protective effects of fish oil against oxidative damage in rats exposed to methylmercury," *Ecotoxicology and Environmental Safety*, vol. 74, no. 3, pp. 487–493, 2011.

[48] M. A. Kjaer, M. Todorcević, B. E. Torstensen, A. Vegusdal, and B. Ruyter, "Dietary n-3 HUFA affects mitochondrial fatty acid beta-oxidation capacity and susceptibility to oxidative stress in Atlantic salmon," *Lipids*, vol. 43, no. 9, pp. 813–827, 2008.

[49] T. K. Østbye, M. A. Kjær, A. M. B. Rørå, B. Torstensen, and B. Ruyter, "High n-3 HUFA levels in the diet of Atlantic salmon affect muscle and mitochondrial membrane lipids and their susceptibility to oxidative stress," *Aquaculture Nutrition*, vol. 17, no. 2, pp. 177–190, 2011.

[50] N. Nakamura, R. Kumasaka, H. Osawa et al., "Effects of eicosapentaenoic acids on oxidative stress and plasma fatty acid composition in patients with lupus nephritis," *In Vivo*, vol. 19, no. 5, pp. 879–882, 2005.

[51] P. Kaur, I. Heggland, M. Aschner, and T. Syversen, "Docosahexaenoic acid may act as a neuroprotector for methylmercury-induced neurotoxicity in primary neural cell cultures," *NeuroToxicology*, vol. 29, no. 6, pp. 978–987, 2008.

[52] W. Wang, T. W. Clarkson, and N. Ballatori, "γ-Glutamyl transpeptidase and L-Cysteine regulate methylmercury uptake by HepG2 cells, a human hepatoma cell line," *Toxicology and Applied Pharmacology*, vol. 168, no. 1, pp. 72–78, 2000.

[53] R. Gorjão, A. K. Azevedo-Martins, H. G. Rodrigues et al., "Comparative effects of DHA and EPA on cell function," *Pharmacology and Therapeutics*, vol. 122, no. 1, pp. 56–64, 2009.

[54] M. Shimazawa, Y. Nakajima, Y. Mashima, and H. Hara, "Docosahexaenoic acid (DHA) has neuroprotective effects against oxidative stress in retinal ganglion cells," *Brain Research*, vol. 1251, pp. 269–275, 2009.

[55] K. S. Kang, P. Wang, N. Yamabe, M. Fukui, T. Jay, and B. T. Zhu, "Docosahexaenoic acid induces apoptosis in MCF-7 cells In Vitro and In Vivo via reactive oxygen species formation and caspase 8 activation," *PLoS One*, vol. 5, no. 4, Article ID e10296, 2010.

[56] C. P. Lebel, S. F. Ali, M. McKee, and S. C. Bondy, "Organometal-induced increases in oxygen reactive species: the potential of 2′,7′-dichlorofluorescin diacetate as an index of neurotoxic damage," *Toxicology and Applied Pharmacology*, vol. 104, no. 1, pp. 17–24, 1990.

Hindawi Publishing Corporation
Journal of Biomedicine and Biotechnology
Volume 2012, Article ID 132876, 9 pages
doi:10.1155/2012/132876

Research Article

Neurodevelopment of Amazonian Infants: Antenatal and Postnatal Exposure to Methyl- and Ethylmercury

José G. Dórea,[1] Rejane C. Marques,[2] and Cintya Isejima[3]

[1] *Department of Nutrition, University of Brasília, 70919-970 Brasília, DF, Brazil*
[2] *Federal University of Rio de Janeiro, Campus Macaé, 27971-550 Rio de Janeiro, RJ, Brazil*
[3] *Departament of Statistics, Federal University of São Carlos, 13566-590 São Carlos, SP, Brazil*

Correspondence should be addressed to José G. Dórea, dorea@rudah.com.br

Received 5 December 2011; Accepted 4 February 2012

Academic Editor: João B.T. Rocha

Neurodevelopment as Gesell development scores (GDSs) in relation to mercury exposure in infants (<6 months of age) of one urban center and two rural villages, respectively, of fisherman and cassiterite miners. Mean total hair-Hg (HHg) concentrations of infants from Itapuã (3.95 ± 1.8 ppm) were statistically ($P = 0.0001$) different from those of infants from Porto Velho (3.84 ± 5.5 ppm) and Bom Futuro (1.85 ± 0.9 ppm). Differences in vaccine coverage among these populations resulted in significantly higher ($P = 0.0001$) mean ethylmercury (EtHg) exposure in urban infants (150 μg) than in infants from either village (41.67 μg, Itapuã; 42.39 μg, Bom Futuro). There was an inverse significant (Spearman $r = -0.2300$; $P = 0.0376$) correlation between HHg and GDS for infants from Porto Velho, but not for the rural infants from Bom Futuro (Spearman $r = 0.1336$; $P = 0.0862$) and Itapuã (Spearman $r = 0.1666$; $P = 0.5182$). Logistic regression applied to variables above or below the median GDS showed that EtHg exposure (estimated probability = −0.0157; $P = 0.0070$) and breastfeeding score (estimated probability = −0.0066; $P = 0.0536$) score were significantly associated with GDS. *Conclusion*. In nurslings whose mothers are exposed to different levels of fish-MeHg (HHg), a higher score of neurological development at six months was negatively associated with exposure to additional TCV-EtHg. Results should be interpreted with caution because of unaccounted variables.

1. Introduction

The first years are critical for children's future health and development. A healthy infanthood can be monitored by physical growth and neurodevelopment (social, emotional, language, and cognitive domains). There are sufficient studies documenting the vulnerability of the developing central nervous system (CNS) to adverse effects of neurotoxicants [1], and mercury is the most prevalent of these among fish-eating populations. Among high consumers of seafood it has been shown that maternal exposure to methylmercury (MeHg) during pregnancy can affect neurodevelopment of children [2]. Indeed, Björnberg et al. [3] have shown that infant exposure to mercury is higher before birth than during the breastfeeding period.

Nutrition, toxic, and stimulatory events in this early phase can influence neurobehavioral scores in childhood [4, 5] by interfering with the neural programming of the CNS. During critical periods of early CNS development, depending on the neurotoxic insult, neurological delays can have an indelible effect. Indeed, the developmental origin of neurological diseases/dysfunction is a concept supported by current epidemiological studies [1]. However, it is recognized that, in most circumstances, exposure occurs to more than one neurotoxic substance. In this regard, Stewart et al. [6] have shown negative association between prenatal MeHg exposure and neurocognitive tests at 38 months to be higher in preschoolers with higher levels of other neurotoxic substances.

The traditional Amazon lifestyle has been characterized by consumption of abundant fish during pregnancy and breastfeeding [7]. However, the growing human occupation and development of the Western Amazon has brought deforestation by fire (for agriculture) and open-cast mining, both of which cause environmental degradation and pollution. Additionally hydroelectric reservoirs have displaced

traditional living [8]. It is also amply recognized that in mining areas there are elevated risks of coexposure to neurotoxic elements resulting from ore extraction and metal processing activities [9]. In new agricultural frontiers of the Amazon there is evidence that anthropogenic activities have contributed to the release of naturally accumulated Hg [10] and have also increased organochlorine residues in the aquatic ecosystem. Rabitto et al. [11] found that fish from the Samuel reservoir are contaminated with DDT residues and also with high Hg concentrations.

For mothers eating fish there is attendant exposure to MeHg [8, 9, 12]. However, not only do other neurotoxicants reach the Amazonian aquatic food web, but there is also iatrogenic exposure to ethylmercury (EtHg) in thimerosal-containing vaccines (TCVs) which are the norm in Brazil's successful immunization program [13]. Despite early-life exposures to preservative (thimerosal) and adjuvant (aluminum) in TCV given to pregnant mothers and babies [14, 15], studies assessing the neurological status of Amazonian children have only considered fish-MeHg [16], missing differences in TCV-EtHg exposure [17].

In this transitioning demography of the Western Amazon we have shown different levels of environmental Hg exposure attendant on variations in maternal fish consumption in children. In these studies we addressed preschool (under five years of age) children's growth and neurodevelopment for urban children [13] and for the families of fishermen [8] and tin miners [9]. In the present work we studied environmental differences (due to maternal fish-MeHg) coupled with variations in postnatal TCV coverage between urban and rural infants. In order to capture early effects of pre- and postnatal Hg exposure, we compared only six-month-old infants' neurodevelopment in three distinct sociocultural situations in urban (Porto Velho) and rural (fishing village and mining settlement) environments.

2. Methods

2.1. Background. We have been studying nutritional status, growth, and neurodevelopment, as well as health of Amazonian children since the late 1990s. In the state of Rondania we have been able to address growth and development in the city of Porto Velho and in impacted rural areas. Results of a longitudinal cohort of 5 years in urban children have appeared in several publications [13]. A second (cross-sectional) study started in 2007, aimed at all children under 5 years of age in rural communities formed after displacement of traditional families by the flooding of a hydroelectric reservoir (Itapuã) and new settlers (Bom Futuro) attracted by cassiterite mining [8, 9]. The protocol of these studies followed ethical recommendations and received approval from the Ethics Committee for Studies in Humans of the Universidade Federal de Rondania [8, 9, 12]; these publications carry maps showing the geographic location of the city of Porto Velho (capital of the state of Rondania) and the studied communities. Written consent was always obtained from the volunteering mother with assured confidentiality. Mothers could withdraw from the study at any time.

2.2. Exposure Assessment and Neurological Tests. The data presented in this paper comprise a specific subset of children 1 to 6 months of age (281) representing all infants surveyed in the two rural communities of Itapuã ($n = 33$; former subsistence fisherman families) and Bom Futuro ($n = 166$; families of cassiterite mining settlers) and the first part of the study with a sample of urban infants of Porto Velho ($n = 82$); respective publications on linear growth and neurodevelopment for preschool children (1 to 60 months of age) have appeared elsewhere [8, 9, 12]. We made anthropometric measurements and collected hair for all infants.

Briefly, during home visits we also applied a questionnaire to the mothers to collect information on breastfeeding duration and immunization records. Because of easy access to health facilities the children of Porto Velho received the full immunization scheme. As for the rural villagers, vaccination coverage experiences a number of difficulties and is not always followed as recommended; however, all vaccines applied had been recorded and this information was acquired from the vaccination cards. The total amount of EtHg was computed from information on the TCV (hepatitis B and DTP) given during the first six months; because all these TCVs are formulated to contain aluminum salts as adjuvant, total amount of adjuvant-Al was also estimated for the vaccinated infant.

During visits, trained professionals conducted interviews and applied the Gesell neurodevelopment tests. The neurodevelopment of infants was assessed by trained professionals using the Gesell Developmental Schedules (GDSs) [18, 19]. The GDSs include reflexes and reactions (voluntary, spontaneous, or learned) as well as postural reactions, hand pressure, locomotion and coordination, constructive ability (which is influenced by motor development), and visible and audible communication; individual reactions regarding people and stimulations (depending mainly on the temperament of the child and the surroundings) were also evaluated [12]. The results were expressed as scores for the assessed domains (motor skills, language development, adaptive behavior, and personal social behaviors).

Breastfeeding for the infants of Porto Velho lasted for six months or more [13], whereas for the rural infants we estimated a breastfeeding score based on the information given at the time of the GDS testing. A score was calculated in relation to the day of the visit and age of the infant (breastfeeding score = age of infant in days/breastfeeding days $\times$ 100). The published parent publications [8, 9, 12] describe the protocol and methods for anthropometry and hair-Hg determinations. Strands of hair were collected from the children and were stored, processed, and analyzed according to our routine laboratory protocol after washing with detergents and digesting with acid [8, 9, 12].

2.3. Statistical Analysis. Data summarization (means, standard deviation) and correlation analysis were done by the statistical packages; Prism software (Prism, version 10IC; GraphPad Software Inc., San Diego, CA, USA) was used to generate graphs and determine Spearman's (P) correlation

Table 1: General characteristics of families and respective infants in the study.

Communities	Mean (SD) Itapuã	Bom Futuro	Porto Velho	*P*
Family				
Fish meal, week[a]	2.63 (2.70)	2.4 (1.4)	1.97 (2.8)	<0.000
Family income US$	227.93 (193.9)	225.2 (146.1)	247.30 (301.8)	0.4771
Maternal education, y	6.56 (3.49)	6 (2.7)	8.7 (3.5)	<0.000
Children				
n	33	166	82	
Birth				
Weight (g)	3445.0 (510.9)	3278.5 (462.8)	3233.2 (421.6)	0.0787
Length (cm)	50.7 (2.7)	50.8 (2.6)	49.8 (2.4)	<0.000
Age at visit, months	3.7 (1.7)	2.8 (1.5)	6	N.T.[c]
Breastfeeding score %[b]	96.1 (16.9)	81.0 (32.6)	100	<0.000
W/H Z-scores	0.16 (1.3)	0.03 (1.4)	−1.22 (1.2)	<0.000
H/A Z-scores	−0.27 (1.5)	0.52 (0.8)	0.38 (1.3)	0.001
W/A Z-scores	−0.22 (1.2)	0.22 (0.9)	−0.77 (0.7)	<0.000
TCV-Hg, μg	41.7 (29.8)	42.4 (24.5)	150	<0.000
Infant's hair Hg, $\mu g \cdot g^{-1}$	3.95 (1.8)	1.85 (0.9)	3.84 (5.5)	<0.000

[a]Number of fish meals a week; [b]breastfeeding score = age of infant in days/breastfeeding days × 100; [c]N.T., not tested per se but as age-dependent variable for anthropometry and breastfeeding scores; W/H: weight for height; H/A: height for age, W/A: weight for age; TCV: uptake of mercury in thimerosal-containing vaccines.

between the variables of interest. A logistic regression analysis was used to test the effect of chosen variables on children's GDS by SAS (SAS Institute, Cary, NC, USA) statistical package.

2.4. Controlling for Confounders. Potential confounders known to interfere with neurodevelopment that were part of the study were considered in the logistic regression model. The rationale behind the choice of covariate variables was based on published literature that includes our own work. The frequency of family fish consumption is associated with total mercury concentrations in infants' hair [20, 21]. Breastfeeding is known to modulate neurobehavior [22] and also to counteract negative effects of maternal exposure to neurotoxic substances [23]. TCV is the only source of EtHg which is a relevant neurotoxic *per se* and, in the case of vaccines, because of its obligatory association with adjuvant-Al salts [15], it actually represents a combination of thimerosal and aluminum salts sufficiently defined (and constant) for it to be treated as a unit here.

3. Results

3.1. General Characteristics and Mercury Exposure. The general characteristics of mothers and infants in the three settings are summarized in Table 1; for all parameters, except family income, there were statistically significant differences. Only breastfeeding score and EtHg exposure showed a similar pattern distinguishing urban from rural settings. In the urban sample, the infants had a full TCV schedule and breastfed for at least six months. Infants' exposure to mercury from maternal fish consumption is represented as HHg while iatrogenic sources such as EtHg from TCV were estimated from vaccination cards and represent a combination of EtHg and attendant adjuvant-Al. As a result, a more precise (in time, quantity, and route) measure of exposure to EtHg was assessed from vaccine cards, showing a statistically significant difference between groups (Table 1). While infants from Porto Velho had received the full vaccine schedule (150 μg of EtHg), rural infants were less exposed (by approximately one-third) to this mercury source because of age variation, less effective vaccine coverage, and schedule adherence; the overall exposure to EtHg in the rural settings was 42.4 μg and 41.7 μg of EtHg for Bom Futuro and Itapuã villages, respectively. For statistical modeling and interpretation of the GDS responses, the EtHg exposure actually represents a combination of two neurotoxicants (thimerosal and adjuvant-Al salts); they are an integral part of the vaccine formulation.

Except for the children of Porto Velho who were exclusively breastfed for at least six months (breastfeeding score of 100%), breastfeeding for the other groups (Itapuã and Bom Futuro) showed lower scores (Table 1). In order to adjust for these differences at the time of the neurobehavioral testing, this variable is presented as "breastfeeding score" (estimated at the day of the visit). At six months, when the Porto Velho infants were tested, all of them (100%) were still breastfeeding, whereas the mean lactation score was relatively less (96.0, Itapuã; 81.0, Bom Futuro) for rural infants (Table 1).

In Figure 1 the cumulative distribution of HHg indicated different patterns of maternal fish consumption in the three settings. The HHg concentrations of the Porto Velho infants showed the highest proportion of low values in sharp contrast with fisherman infants (Itapuã) who showed

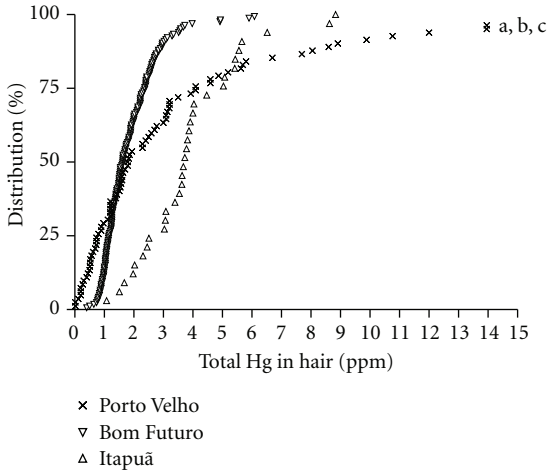


Figure 1: Cumulative percent distribution of total hair Hg concentrations in infants from different localities and maternal fish-eating habits; a, b, c are off-chart values of Porto Velho infants' HHg concentrations, respectively, of 18.3, 26.8, 32.9 ppm.

the highest proportion of high values. In fact, the median HHg of fisherman infants (3.7 ppm) was more than twice the median found in urban infants from Porto Velho (1.8 ppm) and that of the cassiterite mining settlements (1.6 ppm). At this early age, infants' HHg reflects *in utero* exposure as well as maternal milk (mostly breastfeeding) exposure; both types of exposure are influenced by maternal fish-eating habits. Indeed, correlation between infants' HHg and family fish consumption (Figure 2) was statistically significant for each of the three settings: Porto Velho (Spearman $r = 0.489$, $P = 0.0001$), Bom Futuro (Spearman $r = 0.1531$, $P = 0.0489$), and Itapuã (Spearman $r = 0.3763$, $P = 0.0309$).

3.2. Neurodevelopment Scores. The cumulative distribution of the neurodevelopment scores (GDS) is illustrated in Figure 3. A greater proportion of low GDS occurred in infants from Porto Velho, in contrast with a greater proportion of higher scores that occurred in infants from the fishing villages—Itapuã families (Figure 3). It is important to notice that proportionately more infants (95%) from the Itapuã families showed HHg above 1.5 ppm; in this regard there were far more infants from Bom Futuro (35%) and Porto Velho (40%) with HHg concentrations lower than 1.5 ppm (Figure 1). The box plot in Figure 4 illustrates the general GDS and specific domains tested; collectively, the infants of Itapuã seemed to have a more homogenous neurodevelopment than infants from the other study sites. Thus the pattern of distribution of GDS shows an opposite trend compared with the HHg distribution (Figures 1 and 3).

Because of the differences in mercury exposure (maternal fish consumption and TCV-EtHg) and GDS we tested for correlation between these variables in the three settings. The Spearman correlation analyses of HHg and GDS are illustrated in Figure 5. While correlation of HHg and GDS in infants from Porto Velho (Spearman $r = -0.2300$; $P = 0.0376$) was statistically significant, in infants from Bom Futuro (Spearman $r = 0.1336$; $P = 0.0862$) and in infants from Itapuã (Spearman $r = 0.1666$; $P = 0.5182$) these correlations did not reach statistical significance.

A multivariate model (logistic regression) tested the probability of a child showing a GDS above the median, and results are summarized in Table 2. Among the variables tested, exposure to EtHg and breastfeeding score showed negative associations that were statistically significant (EtHg exposure: estimate $= -0.0157$, $P = 0.0070$; "breastfeeding score": estimate $= -0.0065$, $P = 0.0536$).

4. Discussion

This paper shows differences in pre- and postnatal exposure to organic mercury, revealing negative associations with neurodevelopment in six-month-old infants. Overall, most infants had HHg above 1.5 ppm, but the group from Porto Velho with the lowest median HHg concentration had a higher frequency of low GDS. Curiously, these infants had the highest exposure to vaccine-EtHg. Although the measured delays were overcome by 36 months [13], these findings are nevertheless important to show that researchers should consider all sources of Hg in populations using TCVs. Indeed, when vaccine-EtHg exposures are included in statistical model measuring neurological outcome of children there are indications concurring with our results. In South Korea, Lee and Ha [24] observed a marginal level of significance in mental development index related to maternal smoking when considering vaccination history. Also in Poland, Jedrychowski [25] informs that TCV effects on psychomotor development appeared to be significant, although very weak.

Chronic neurotoxic sequelae attributed only to the high burden of MeHg resulting from subsistence fish eating in the Amazon were found by some but not by others. Neurobehavioral studies in Amazonian children of high fish consumers (mean HHg concentration >10 ppm) tended to show a significant decrease in tested neurobehavioral functions [26, 27], while in other studies (with mean HHg <5 ppm) children showed no such significant effects [28]. Although differences in Hg exposure may seem to explain differences in study results, the neurodevelopment status of rural Amazonian children is not an exclusive function of the assessed fish-Hg exposure. Fonseca et al. [29] showed that low results in neurofunctional testing among children from isolated communities are related to causes other than MeHg derived from fish consumption; subsistence riverine children with hair-Hg concentrations 66 times higher than those found in non-Amazonian agrarian communities had comparably poor neurofunctional outcomes.

Grandjean et al. [2] noticed that prenatal low levels of MeHg exposure that may affect brain function can only be detected after the child is old enough to be tested in cognitive domains. However, addressing neurological development as a function of breastfeeding, Grandjean et al. [30] reported that infants with higher HHg at 12 months of age reached the milestone criteria earlier and attributed this to breastfeeding

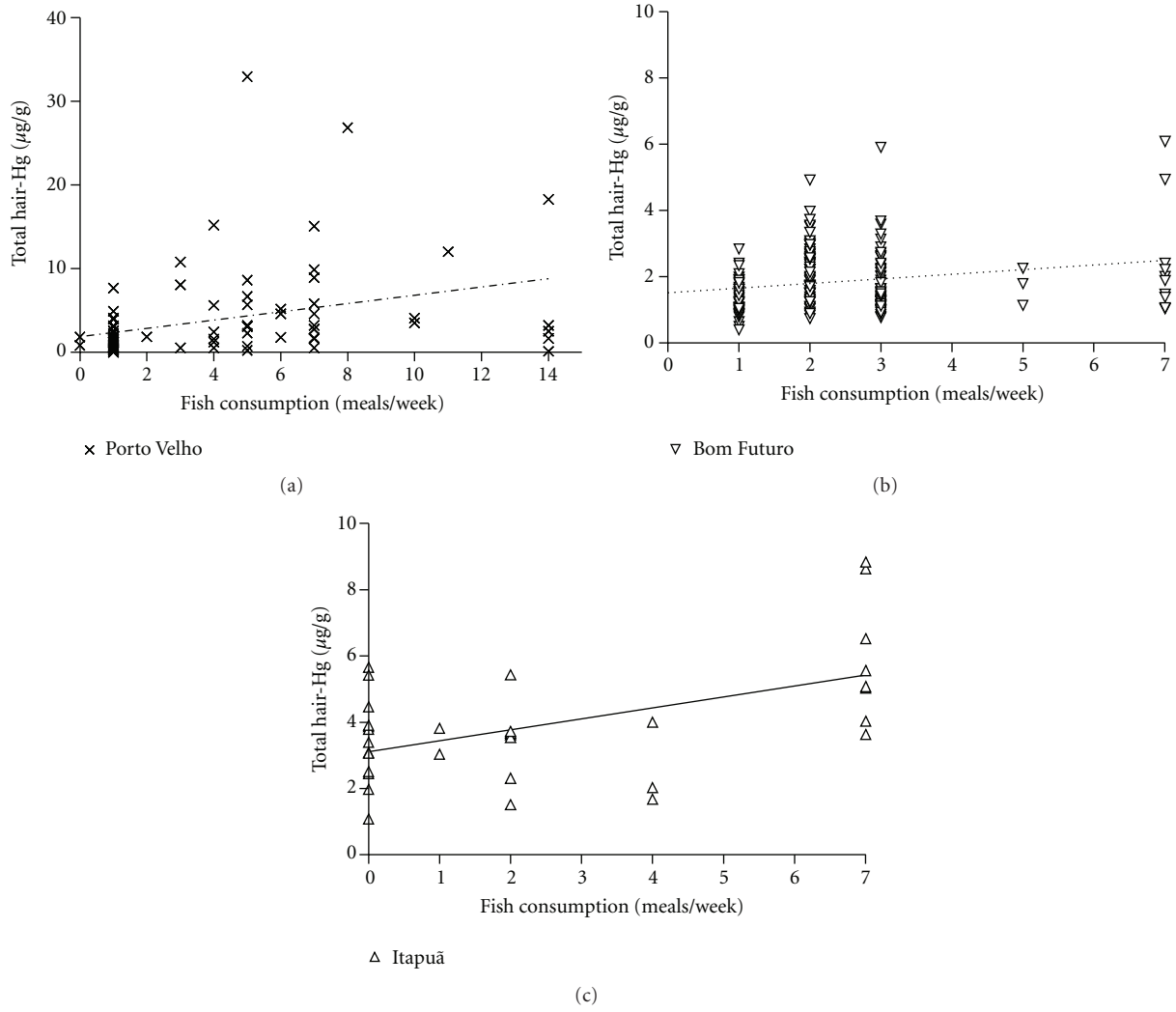


Figure 2: Scatter plots of infant's total hair-Hg concentrations as a function of maternal fish consumption; the Porto Velho data "high" refers to eating >2 fish meals a week.

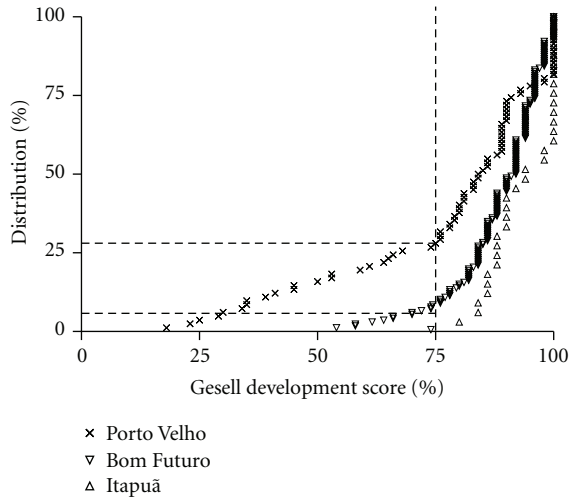


Figure 3: Cumulative percent distribution of infants' neurological test at 6 months; Gesell total development quotient.

duration. In the infants of Porto Velho neurodevelopmental delays (attributed to Hg that included TCV) were inversely correlated with length of breastfeeding and were overcome by age of 5 yrs [13]. At six months, however, comparing groups of infants with different Hg exposure (chemical forms and time) it was possible to reveal significant associations with neurobehavioral testing. In this logistic regression breastfeeding seemed less than that associated with TCV-EtHg (Table 2).

There are few publications reporting HHg concentrations in infants who are still under the influence of maternal Hg exposure. As an exposure marker, HHg has been reported for babies in Italy [31], in Surinam [32] and in the Faroe Islands [20]. In Italy mean HHg was 1.22 ppm, while in Surinam there was a median of 1.6 ppm, and in the Faroe Islands the interquartile range was from 0.46 to 2.04 ppm. Babies in the present study showed higher HHg concentrations than in other countries but lower than previously (>15 yrs ago) found in traditional riverine communities of the Rio Madeira

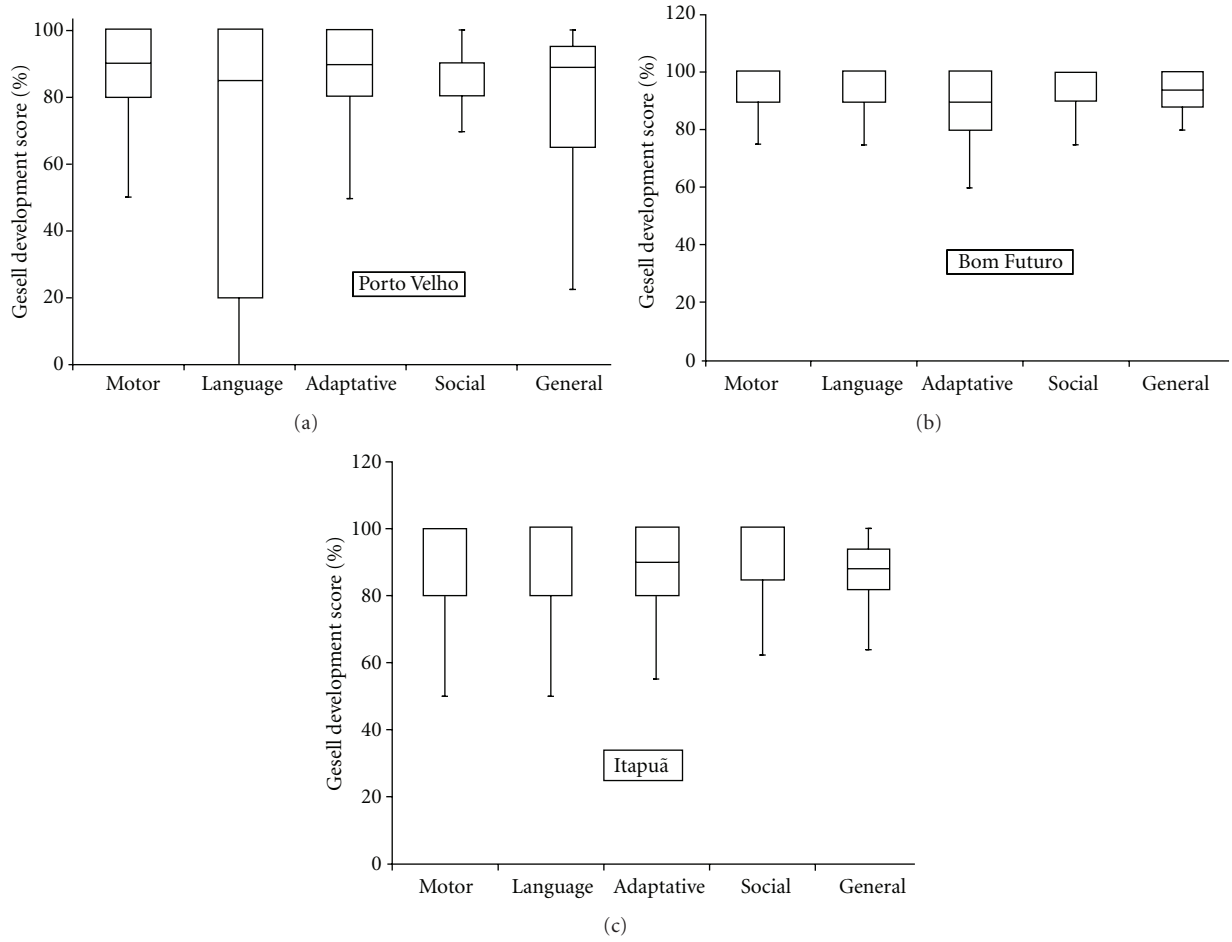


Figure 4: Box plot of Gesell development scores-% (Y axis) representing PV, BF, and Itapuã infants.

Table 2: Results of the logistic regression of a child showing a score above the median; a negative sign demonstrates a diminished probability.

Parameter	Coefficient (β)	Standard error	Wald χ^2	Pr > χ^2
Intercept	−1.5406	3.4667	0.1975	0.6568
Sex	0.1659	0.2940	0.3184	0.5726
Age	0.0639	0.2216	0.0832	0.7731
Birth weight	−0.00010	0.000286	0.1169	0.7325
W/H-Z score	0.2268	0.1457	2.4229	0.1196
W/A-Z score	−0.2695	0.2180	1.5295	0.2162
TCV-EtHg	−0.0157	0.00583	7.2714	0.0070
HHg	−0.0694	0.0537	1.6691	0.1964
Breastfeeding score	−0.00655	0.00339	3.7265	0.0536

W/H: weight for height; W/A: weight for age; VCT-EtHg: thimerosal-containing vaccine-ethylmercury; HHg: hair mercury; breastfeeding score = age of infant in days/breastfeeding days × 100.

(9.8 ppm—[33]). During breastfeeding, a slow elimination of body Hg has been linked to continuous exposure of the nursling to Hg in maternal milk [20]. Indeed, we have also observed that postnatal HHg increases in breastfed infants [34]. However, despite the increased exposure to EtHg derived from TCV, this source of organic Hg is captured by babies' hair in very small concentrations [35, 36]; due to the acute nature of TCV exposure, specific EtHg in baby's hair is only correlated with time of last TCV [36]. Therefore, at this early age infants' HHg showed to be significantly correlated to maternal fish consumption (Figure 2). This is in agreement with previous observations for Amazonian riverine populations [33].

The present study can only suggest association of Hg exposure from different sources (MeHg assessed from HHg and EtHg assessed from vaccination cards) and neurobehavioral outcomes. In such observational studies it is difficult to establish a reliable association between markers of exposure and corresponding neurologic effects. While HHg in infants (still breastfeeding and most certainly not eating fish) can be a good indicator of maternal Hg transferred from fish consumption [33] EtHg could be tracked from vaccination cards. Of the many types of exposure occurring before birth and continuing during the immediate perinatal period, we were able to account for postnatal TCV and breastfeeding. Other aspects related to nutrition and stimulatory factors are complex to tackle in such circumstances. However, the strength of the study is

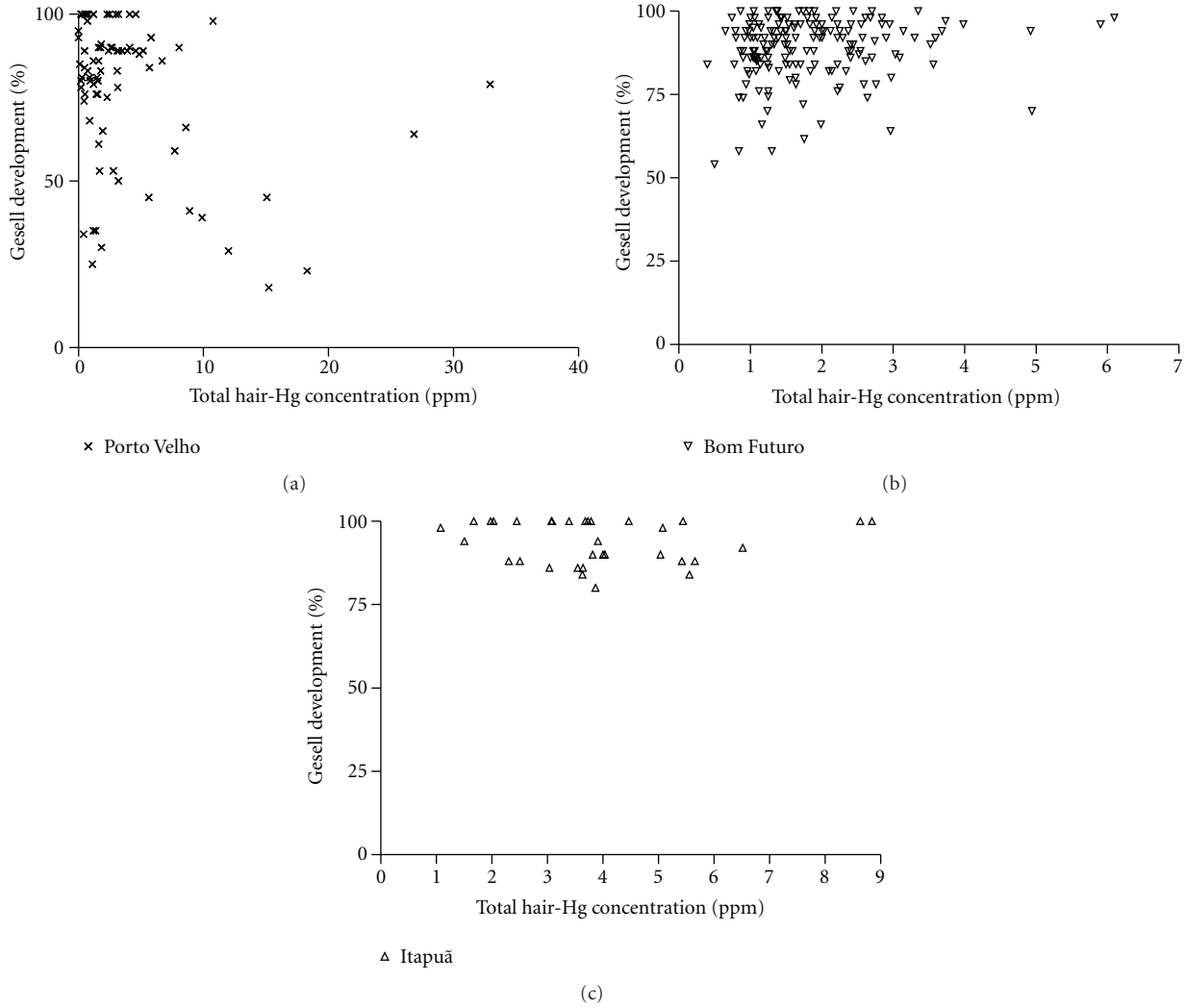


Figure 5: Scatter plots of total hair-Hg concentrations and Gesell development scores in the 3 settings of tested infants.

that we could compare urban and rural settings accounting indirectly for maternal fish consumption (HHg) and, directly, for exposure to TCV-EtHg. Because Amazonian children are exposed perinatally to TCV-EtHg, neurological assessment at such early ages has to be interpreted with caution. Another weakness relates to the limitation in capturing the many potential confounding variables that can influence experience-dependent synapse formation in such diverse environments (urban and rural settings).

Evidence from animal studies demonstrates the neurodevelopmental impact of prenatal [37, 38] and perinatal exposure to vaccine-EtHg [39, 40]; these studies included infants of several species (mice, rats, and macaques) and suggest that the cumulative effects of TCV-Hg could lead to cognitive and social deficits and delayed acquisition of reflexes. Because this literature is very recent, it has not yet modeled the wide spectrum of human infant diversity and the full interaction with other neurotoxicants [41]. Perinatal TCV-EtHg exposure occurs worldwide, with a wide variation in dose, interval, and postnatal time, in accordance with countries' immunization schedules. Furthermore, nutritional status, degree of poverty, and dispossession attendant on large massed populations in developing countries mean that millions of young children may experience coexposures with other toxic substances that could be more common and more severe.

5. Conclusions

For Amazonian infants, not only exposure to environmental fish-MeHg (in maternal diets) but also the additional burden of iatrogenic EtHg (in TCV) is relevant to neurobehavioral testing. In the present case, infants from Porto Velho are exposed to city pollution, Itapuã villagers to more fish-MeHg, and Bom Futuro to open-mining operations. However, the neurotoxic effects associated with the combined chemical forms of Hg seemed sensitive for infants exposed to additional TCV-Hg.

Conflict of Interests

The authors declare that they have no competing financial interests.

Acknowledgment

The present study was partly supported by the National Research Council of Brazil-CNPq (PPG7; CT-HIDRO, project-555516/2006-7; CT-AMAZONIA, Project 575573/2008-2).

References

[1] P. Grandjean and P. Landrigan, "Developmental neurotoxicity of industrial chemicals," *The Lancet*, vol. 368, no. 9553, pp. 2167–2178, 2006.

[2] P. Grandjean, P. Weihe, R. F. White, and F. Debes, "Cognitive performance of children prenatally exposed to "safe" levels of methylmercury," *Environmental Research*, vol. 77, no. 2, pp. 165–172, 1998.

[3] K. A. Björnberg, M. Vahter, B. Berglund, B. Niklasson, M. Blennow, and G. Sandborgh-Englund, "Transport of methylmercury and inorganic mercury to the fetus and breast-fed infant," *Environmental Health Perspectives*, vol. 113, no. 10, pp. 1381–1385, 2005.

[4] C. R. Gale, F. J. O'Callaghan, K. M. Godfrey, C. M. Law, and C. N. Martyn, "Critical periods of brain growth and cognitive function in children," *Brain*, vol. 127, no. 2, pp. 321–329, 2004.

[5] C. R. Gale, F. J. O'Callaghan, M. Bredow, and C. N. Martyn, "The influence of head growth in fetal life, infancy, and childhood on intelligence at the ages of 4 and 8 years," *Pediatrics*, vol. 118, no. 4, pp. 1486–1492, 2006.

[6] P. W. Stewart, J. Reihman, E. I. Lonky, T. J. Darvill, and J. Pagano, "Cognitive development in preschool children prenatally exposed to PCBs and MeHg," *Neurotoxicology and Teratology*, vol. 25, no. 1, pp. 11–22, 2003.

[7] A. C. Barbosa, S. R. Silva, and J. G. Dórea, "Concentration of mercury in hair of indigenous mothers and infants from the Amazon basin," *Archives of Environmental Contamination and Toxicology*, vol. 34, no. 1, pp. 100–105, 1998.

[8] R. C. Marques, J. G. Dórea, C. McManus et al., "Hydro-electric reservoir inundation (Rio Madeira Basin, Amazon) and changes in traditional lifestyle: impact on growth and neurodevelopment of pre-school children," *Public Health Nutrition*, vol. 14, no. 4, pp. 661–669, 2011.

[9] R. C. Marques, J. G. Dórea, R. S. Leão et al., "The role of methylmercury exposure (from fish consumption) on growth and neuro-development of children under 5 y of age living in a transitioning (tin-mining) area of the Western Amazon, Brazil," *Archives of Environmental Contamination and Toxicology*, vol. 62, no. 2, pp. 341–350, 2012.

[10] A. Béliveau, M. Lucotte, R. Davidson, L. O. Lopes, and S. Paquet, "Early Hg mobility in cultivated tropical soils one year after slash-and-burn of the primary forest, in the Brazilian Amazon," *Science of the Total Environment*, vol. 407, no. 15, pp. 4480–4489, 2009.

[11] I. S. Rabitto, W. R. Bastos, R. Almeida et al., "Mercury and DDT exposure risk to fish-eating human populations in Amazon," *Environment International*, vol. 37, no. 1, pp. 56–65, 2011.

[12] R. C. Marques, J. Garrofe Dórea, W. R. Bastos, M. de Freitas Rebelo, M. de Freitas Fonseca, and O. Malm, "Maternal mercury exposure and neuro-motor development in breastfed infants from Porto Velho (Amazon), Brazil," *International Journal of Hygiene and Environmental Health*, vol. 210, no. 1, pp. 51–60, 2007.

[13] R. C. Marques, J. G. Dórea, J. V. Bernardi, W. R. Bastos, and O. Malm, "Prenatal and postnatal mercury exposure, breastfeeding and neurodevelopment during the first 5 years," *Cognitive and Behavioral Neurology*, vol. 22, no. 2, pp. 134–141, 2009.

[14] R. C. Marques, J. G. Dórea, and J. V. E. Bernardi, "Thimerosal exposure (from tetanus-diphtheria vaccine) during pregnancy and neurodevelopment of breastfed infants at 6 months," *Acta Paediatrica*, vol. 99, no. 6, pp. 934–939, 2010.

[15] J. G. Dórea and R. C. Marques, "Infants' exposure to aluminum from vaccines and breast milk during the first 6 months," *Journal of Exposure Science and Environmental Epidemiology*, vol. 20, no. 7, pp. 598–601, 2010.

[16] C. J. Passos and D. Mergler, "Human mercury exposure and adverse health effects in the Amazon: a review," *Cadernos de Saude Publica*, vol. 24, supplement 4, no. 4, pp. S503–S520, 2008.

[17] J. G. Dórea, "Comparing fish-mercury exposed Amazonian children: should not we consider thimerosal-preserved vaccines?" *NeuroToxicology*, vol. 30, no. 3, pp. 485–486, 2009.

[18] A. Gesell, *A Criança de 0 a 5 Anos*, Martins Fontes, São Paulo, Brazil, 6th edition, 2003.

[19] A. Gesell and C.S. Amatruda, *Psicologia do Desenvolvimento-do Lactente a Criança Pequena, bases Neuropsicológicas e Comportamentais*, Atheneu, São Paulo, Brazil, 1st edition, 2000.

[20] P. Grandjean, P. J. Jørgensen, and P. Weihe, "Human milk as a source of methylmercury exposure in infants," *Environmental Health Perspectives*, vol. 102, no. 1, pp. 74–77, 1994.

[21] R. C. Marques, J. G. Dórea, J. V. Bernardi, W. R. Bastos, and O. Malm, "Maternal fish consumption in the nutrition transition of the Amazon basin: growth of exclusively breastfed infants during the first 5 years," *Annals of Human Biology*, vol. 35, no. 4, pp. 363–377, 2008.

[22] J. W. Anderson, B. M. Johnstone, and D. T. Remley, "Breast-feeding and cognitive development: a meta-analysis," *American Journal of Clinical Nutrition*, vol. 70, no. 4, pp. 525–535, 1999.

[23] J. G. Dórea, "Maternal smoking and infant feeding: breastfeeding is better and safer," *Maternal and Child Health Journal*, vol. 11, no. 3, pp. 287–291, 2007.

[24] B. E. Lee and E. H. Ha, "Response to commentary "Co-exposure and confounders during neurodevelopment: we need them in the bigger picture of secondhand smoke exposure during pregnancy"," *Environmental Research*, vol. 112, article 235, 2012.

[25] W. Jedrychowski, "Reply to the correspondence letter by M.D. Majewska: Krakow's children and cognitive function: can the study by Jedrychowski et al. show us the bigger picture?" *European Journal of Pediatrics*, vol. 171, no. 2, p. 407, 2011.

[26] P. Grandjean, R. F. White, A. Nielsen, D. Cleary, and E. O. Santos, "Methylmercury neurotoxicity in Amazonian children downstream from gold mining," *Environmental Health Perspectives*, vol. 107, no. 7, pp. 587–591, 1999.

[27] S. Cordier, M. Garel, L. Mandereau et al., "Neurodevelopmental investigations among methylmercury-exposed children in French Guiana," *Environmental Research*, vol. 89, no. 1, pp. 1–11, 2002.

[28] L. M. Tavares, V. M. Câmara, O. Malm, and E. C. Santos, "Performance on neurological development tests by riverine children with moderate mercury exposure in Amazonia, Brazil," *Cadernos de Saúde Pública*, vol. 21, no. 4, pp. 1160–1167, 2005.

[29] M. F. Fonseca, J. G. Dórea, W. R. Bastos, R. C. Marques, J. P. Torres, and O. Malm, "Poor psychometric scores of children living in isolated riverine and agrarian communities and fish-methylmercury exposure," *Neurotoxicology*, vol. 29, no. 6, pp. 1008–1015, 2008.

[30] P. Grandjean, P. Weihe, and R. F. White, "Milestone development in infants exposed to methylmercury from human milk," *Neurotoxicology*, vol. 16, no. 1, pp. 27–33, 1995.

[31] F. Valent, F. Pisa, M. Mariuz et al., "Fetal and perinatal exposure to mercury and selenium: baseline evaluation of a cohort of children in Friuli Venezia Giulia, Italy," *Epidemiologia e Prevenzione*, vol. 35, no. 1, pp. 33–42, 2011.

[32] S. Mohan, M. Tiller, G. van Der Voet, and H. Kanhai, "Mercury exposure of mothers and newborns in Surinam: a pilot study," *Clinical Toxicology*, vol. 43, no. 2, pp. 101–104, 2005.

[33] A. C. Barbosa and J. G. Dórea, "Indices of mercury contamination during breast feeding in the Amazon Basin," *Environmental Toxicology and Pharmacology*, vol. 6, no. 2, pp. 71–79, 1998.

[34] R. C. Marques, J. G. Dórea, W. R. Bastos, and O. Malm, "Changes in children hair-Hg concentrations during the first 5 years: maternal, environmental and iatrogenic modifying factors," *Regulatory Toxicology and Pharmacology*, vol. 49, no. 1, pp. 17–24, 2007.

[35] J. G. Dórea, W. Wimer, R. C. Marques, and C. Shade, "Automated speciation of mercury in the hair of breastfed infants exposed to ethylmercury from thimerosal-containing vaccines," *Biological Trace Element Research*, vol. 140, no. 3, pp. 262–271, 2011.

[36] J. G. Dórea, V. L. V. A. Bezerra, V. Fajon, and M. Horvat, "Speciation of methyl- and ethyl-mercury in hair of breastfed infants acutely exposed to thimerosal-containing vaccines," *Clinica Chimica Acta*, vol. 412, no. 17-18, pp. 1563–1566, 2011.

[37] M. Ida-Eto, A. Oyabu, T. Ohkawara, Y. Tashiro, N. Narita, and M. Narita, "Embryonic exposure to thimerosal, an organomercury compound, causes abnormal early development of serotonergic neurons," *Neuroscience Letters*, vol. 505, no. 2, pp. 61–64, 2011.

[38] Z. L. Sulkowski, T. Chen, S. Midha, A. M. Zavacki, and E. M. Sajdel-Sulkowska, "Maternal thimerosal exposure results in aberrant cerebellar oxidative stress, thyroid hormone metabolism, and motor behavior in rat pups; sex- and strain-dependent effects," *Cerebellum*. In press.

[39] J. G. Dórea, "Integrating experimental (in vitro and in vivo) neurotoxicity studies of low-dose thimerosal relevant to vaccines," *Neurochemical Research*, vol. 36, no. 6, pp. 927–938, 2011.

[40] M. Duszczyk-Budhathoki, M. Olczak, M. Lehner, and M. D. Majewska, "Administration of thimerosal to infant rats increases overflow of glutamate and aspartate in the prefrontal cortex: protective role of dehydroepiandrosterone sulfate," *Neurochemical Research*, vol. 37, no. 2, pp. 436–447, 2012.

[41] C. Heilmann, E. Budtz-Jørgensen, F. Nielsen, B. Heinzow, P. Weihe, and P. Grandjean, "Serum concentrations of antibodies against vaccine toxoids in children exposed perinatally to immunotoxicants," *Environmental Health Perspectives*, vol. 118, no. 10, pp. 1434–1438, 2010.