

Hair mercury in breast-fed infants exposed to thimerosal-preserved vaccines

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Received: 10 July 2006 / Revised: 9 October 2006 / Accepted: 28 October 2006
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Abstract Because of uncertainties associated with a possible rise in neuro-developmental deficits among vaccinated children, thimerosal-preserved vaccines have not been used since 2004 in the USA (with the exception of thimerosal-containing influenza vaccines which are routinely recommended for administration to pregnant women and children), and the EU but are widely produced and used in other countries. We investigated the impact of thimerosal on the total Hg in hair of 82 breast-fed infants during the first 6 months of life. The infants received three doses of the hepatitis-B vaccine (at birth, 1 and 6 months) and three DTP (diphtheria, tetanus, and pertussis) doses at 2, 4 and 6 months, according to the immunization schedule recommended by the Ministry of Health of Brazil. The thimerosal in vaccines provided an ethylmercury (EtHg) exposure of 25 µgHg at birth, 30, 60 and 120 days, and 50 µgHg at 180 days. The exposure to vaccine-EtHg represents 80% of that expected from total breast milk-Hg in the first month but only 40% of the expected exposure integrated in the 6 months of breastfeeding. However, the Hg exposure corrected for body weight at the day of immunization was much higher from thimerosal- EtHg (5.7 to 11.3 µgHg/kg b.w.) than from breastfeeding (0.266 µgHg/kg b.w.). While

mothers showed a relative decrease (−57%) in total hair-Hg during the 6 months lactation there was substantial increase in the infant's hair-Hg (446%). We speculate that dose and parenteral mode of thimerosal-EtHg exposure modulated the relative increase in hair-Hg of breast-fed infants at 6 months of age.

Keywords Thiomersal · Ethyl-mercury · Methyl-mercury · Breastfeeding · Immunization

Abbreviations

EtHg	ethyl-mercury
MeHg	methyl-mercury
DTP	difteria tetanus and pertussis
PUFA	polyunsaturated fatty acids
DHA	docosahexaenoic acid
EPA	eicosopentanoic acid
GSH	glutathione

Introduction

Great strides in the control of epidemics have been made possible by the effective production and safe use of vaccines. The elimination of polio, measles and other scourges are the hallmark of successful immunization programs. However, in the earlier days of vaccine production there were several tragic incidents that caused death in vaccinated children [1]. To reduce the risk of bacterial contamination, many countries require a preservative in vaccines. The antiseptic agent of choice to prevent bacterial growth is sodium ethylmercurithiosalicylate (thiomersal), also known as thimerosal (USA). Thimerosal contains 49.6% Hg by weight and has been used since the 1930s at the preservative level

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in vaccines. Some vaccines contain thimerosal at a concentration of 0.01%; thus a vaccine dose of 0.5 ml contains 50 µg of thimerosal or approximately 25 µg of Hg [1].

The amount of EtHg received from vaccinations by infants up to 6 months old is an additional exposure to Hg frequently found in human milk and formulas [10]. However, because the vaccine-Hg dose (extrinsic EtHg), unlike intrinsic milk-Hg, is not part of a food, it bypasses the barrier and detoxifying entero-hepatic system, and not being physiologically processed, it may instead migrate to the brain. The uncertainties about the safety of thimerosal-preserved vaccines and controversy about whether EtHg might be associated with neurodevelopmental disorders (including autistic spectrum disorder) are debatable issues that have involved both scientists and society [32]. Because Hg toxic effects are manifested long after the exposure, it is necessary to have reliable markers of Hg exposure. However, depending on the elapsed time, one of the difficulties in studying neurotoxic effect of vaccine-thimerosal is that post-exposure to vaccine-EtHg may not leave Hg traces to be measured.

The organic Hg form of thimerosal is ethyl-Hg which, like methyl-Hg (MeHg), can also bind to protein matrices. In vitro studies showed that the neuronal and glial cell toxicity of MeHg and EtHg are both mediated by glutathione depletion [15]. Although chemically and toxicologically related, ethyl-Hg and methyl-Hg have differences in metabolism. Compared to EtHg, MeHg binds more avidly to cysteine-groups and is more slowly metabolized. Thus, thimerosal-derived EtHg half-life in the blood of infant monkeys was 2.1 and 8.6 days after exposure (i.m. injection), significantly shorter than the 21.5 days elimination half-life of MeHg exposure by gavage [6]. Additionally, it was observed that the concentration of inorganic mercury in the brains of thimerosal-EtHg exposed monkeys was more than double that found in the MeHg exposed monkeys, and it was observed that the inorganic mercury in the brains of the thimerosal-EtHg exposed monkeys showed no significant decline 120 days after exposure. Pichichero et al. [26] estimated that half-life of EtHg in human infants is 6 days compared with 40 to 50 days for MeHg. Furthermore, concentrations of Hg were low in urine after vaccination, but were high in stools of thimerosal-exposed infants. Conversion to inorganic Hg is expected to yield high urine-Hg; thus, stool Hg elimination is a feature also found in MeHg metabolism. In such a scenario, the neurotoxic effects of thimerosal-EtHg evaluated by post-exposure markers such as hair-Hg can be compromised.

Stajich et al. [31] studied thimerosal-EtHg metabolism in preterm human infants by measuring total Hg levels in blood before and after the hepatitis-B vaccination. A significant increase in Hg was seen in both preterm and term infants. Additionally, preterm infants had greater than

tenfold higher mean Hg levels at the baseline reading compared with term infants. Although this difference was not statistically significant, Stajich et al. [31] speculated that it might indicate that preterm infants may not be able to metabolize Hg efficiently. Indeed we have shown that larger babies, as measured by liver weight, had significantly higher mean liver Cu concentrations, an indicator of metallothionein [16], than smaller babies [9]. Either immaturity of the liver could be responsible for less metallothionein to bind inorganic Hg or a difference in body composition of infants with less body mass could metabolize EtHg at a slower rate [31].

There are no studies that measured hair-Hg in infants at the time of vaccination, but Redwood et al. [29] developed models that predicted increases in hair-Hg concentrations after a full immunization schedule within the first 6 months of life. They assumed that neonates up to 6 months of age have low Hg excretion due to immature hepatic function, low bile production, and insufficient glutathione which binds the Hg.

Substances that accumulate in the body should be looked at specifically. In the case of Hg, not only the chemical form but also the exposure route, as well as the modifying effect of changing metabolism (body weight with accompanying blood volume and organ maturity) are critical for newborns. Therefore, we compared the exposure to injected EtHg in vaccines with the expected exposure to breast-milk Hg and examined the hair-Hg of breastfeeding mother-infant pairs after the recommended immunization schedule of the first 6 months.

Materials and methods

The research protocol to study the effects of Hg contamination of urban (Porto Velho, Brazil) mothers on neurodevelopment of pre-school children was approved by the Ethics Committee of Studies for Humans of the Universidade Federal de Rondonia and details appeared elsewhere [21]. In this study we used data from our previous study which was not collected with the purpose of evaluating the impact of immunization on hair-Hg, since when the research project started we were not aware of the EtHg issue in pediatric vaccines. Briefly, pregnant mothers were introduced to the study and invited to participate during their routine visits to the pre-natal clinics of three hospitals in Porto Velho (Hospital de Base, Hospital Panamericano and Hospital Regina Pacis). Only Hospital de Base is a state-run facility that receives mostly poor mothers.

Plain-language information about the study was presented and a written consent form signed by the volunteering mother. The written consent stated that participation was voluntary with assured confidentiality and the right to withdraw from the study at any time. Potential participants

were selected among mothers in good health, reporting no illness or complaints at the time of the study and who were willing to breast feed and adhere to the post-natal attendance of the pediatric clinic for the regular immunization program. One hundred mothers between the ages of 15 and 45 years were recruited.

At birth the infants were clinically examined with special attention to vitality, perinatal reflexes, maturity, and congenital malformations; weight, length, head circumference, and Apgar scores were recorded. Anthropometric data (weight, length, and head circumference) were recorded and samples of hair from the mothers and infants (fetal hair) were collected. Mothers followed the immunization schedule recommended by the Ministry of Health of Brazil and returned at 30, 60, 120 and 180 days. Only 86 mother-infant pairs reported for the programmed clinical and immunization at 6 months of age when infants were weighed and measured for length, and hair-samples were again collected from mothers and infants. Because Hospital de Base is a public health facility, only the babies born at this hospital (66%) received the hepatitis-B vaccine within the first day postpartum. Babies born at the Hospital Panamericano and the Hospital Regina Pacis received the hepatitis-B vaccine immediately after the mother's discharge (2–4 days postpartum). At this time the mothers were taken under our supervision to a state-run clinic where vaccines are distributed free.

Estimated exposure to Hg from vaccines (injected thimerosal) and breastfeeding

Differences in infants' weight at birth and at 6 months were used to estimate daily weight gain and integrated gain at 30, 60, 120 and 180 days. As stated by manufacturers, vaccines contained 0.01% Thimerosal; the Hg concentration of the doses delivered through vaccines was 25 µgHg/0.5 mL for hepatitis-B (Korea Green Cross Corporation, Kiheung-Eup Yougin-Goon Kiyunggi-Do, Korea; Euvax B injectable, LG Life Sciences, Jeonbuk-Do, Korea) and difteria, tuberculosis and pertussis-DTP (Triple Antigen, Serum Institute of India Ltd., India; Vacina Tríplice, Instituto Butanta, São Paulo, Brazil).

We used the data of breast milk-Hg concentrations (adapted from Dorea [8]) with the same approach as Bigham and Copes [4] to estimate Hg exposure during breastfeeding: infant mean weight × mean daily breast milk consumption (140 mL/kg) × number of days × mean total Hg concentration in breast milk (1.9 µg/L).

Hair Hg determinations

Hair sample preparation and Hg determination were done according to routine procedures previously established at

the Universidade Federal do Rio de Janeiro [3]. We followed routine laboratory procedures after adapting the analytical protocol used for Hg determination in previous studies analyzing blood, hair and fish-flesh matrices [3, 19, 20]. Briefly, the hair samples were comminuted with stainless steel scissors, weighed and digested before analysis. Blood samples were digested with concentrated HNO₃ (3 mL) and KMnO₄ (5%, 6 mL) using a microwave oven system for 35 min (CEM-Corporation, MDS 2000, Matthews, North Carolina, USA). Placenta and umbilical cord samples were weighed and digested with HNO₃:H₂SO₄ (1:1, 5 mL) and KMnO₄ (5%, 4 mL) using a digestion block at 80°C for 1 h (Tecnal Ltd., Piracicaba, São Paulo, Brazil). Human hair samples were washed with EDTA 0.01%, dried in an oven at 50°C, weighed and digested with 5 mL of HNO₃:H₂SO₄ (1:1) and 4 mL of 5% KMnO₄ using a digestion block at 80°C for 40 min. The determination of total Hg in the digested samples was done by cold vapor atomic absorption spectrometry with a flow injection system-FIMS (CV-AAS, Perkin-Elmer-FIMS 400, Ueberlingen, Germany). Glassware used in the analytical protocol was washed clean, rinsed with 5% EDTA and double distilled, and left to rest in 5% HNO₃ overnight. Then it was rinsed again in double distilled water, and dried at 100°C for 12 h. Precision and accuracy of Hg determinations were assured by the use of internal standards, use of triplicate analyses of samples and certified reference materials (IAEA-085 and 086, Vienna-Austria) with recoveries of 92%.

Statistical analysis and data summarization (mean, SD) were performed using SAS software (SAS Institute, Cary, NC, USA). For the statistical test, $p < 0.05$ was considered significant.

Results

The anthropometry and the hair-Hg concentrations of infants and mothers are shown in Table 1, while the immunization schedule and exposure to the injected thimerosal as well as the expected exposure to total Hg in breast milk are shown in Table 2.

The mean birth weight was slightly higher for girls, but there was a relatively higher weight gain for boys at 6 months. Every child received a cumulative dose of 150 µg/Hg (as Thimerosal-EtHg) distributed in five boluses during the first 6 months of life. An adaptation of world data on human milk-Hg concentrations [8] showed that the median mean Hg concentration is 1.9 ngHg/mL (Fig. 1). The estimated total Hg intake integrated over the first 180 days of breastfeeding showed a cumulative exposure of 290 µg of total-Hg (Fig. 2). Comparatively, the integrated EtHg exposure in the first month was 80% of that from

Table 1 Mean (and SD) of anthropometry, hair-Hg concentrations and Gesell scores of infants

	Girls	Boys
N	38	44
Birth weight, g	3,177.50 (450.8)	3,281.25 (393.4)
Weight at 180 d	7,012.63 (508.7)	7,010.23 (417.3)
Weight gain, g	3,835.13 (434.2)	3,728.98 (382.8)
Weight gain/d	21.31 (2.4)	20.72 (2.1)
Hair [Hg], µg/g		
Birth	2.58 (3.7)	2.32 (2.4)
180 days	4.14 (5.9)	3.9 (5.3)
Mean increase at 6 m	1.55 (5.8)	1.27 (5.3)
Relative increase, %	398.28 (541.9)	487.08 (1,515.0)
Maternal hair [Hg] µg/g		
Delivery, 0 days	7.14 (6.8)	7.55 (10.2)
180 days	2.97 (3.1)	3.38 (4.4)
Mean decrease at 6 m	4.17 (4.0)	4.16 (6.2)
Relative decrease, %	39.94 (16.2)	45.50 (23.1)

breast milk but declined to 40% at 180 days (Fig. 2). At two months the infants increased body weight by 60% but received an integrated EtHg challenge threefold higher than in the perinatal days (Table 2).

Per unit of body weight the amount of EtHg/kg b.w. given to newborn babies (before hospital discharge) was higher than at any other time (Fig. 3), even at 6 months when infants had increased body weight by more than 100%. The corrected exposure to thimerosal (EtHg) and breast-milk Hg per unit of body weight is shown in Fig. 3. Considering a milk volume transfer of 140 mL/kg b.w., and considering the median breast-milk Hg concentration of 1.9 ngHg/mL (Fig. 1), the exposure to breast-milk Hg was

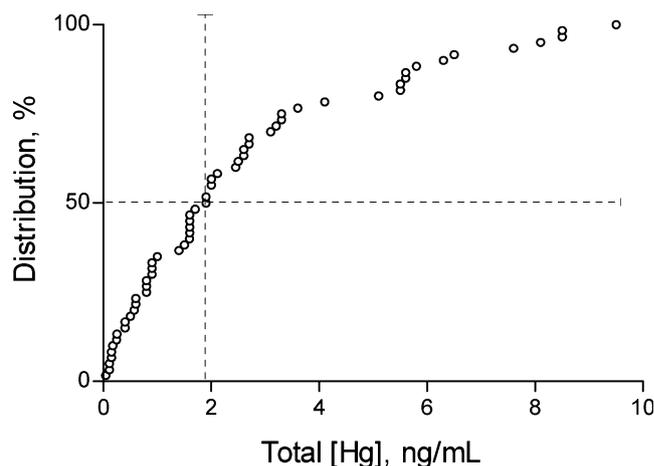
Table 2 Infant immunization schedule, type of vaccine, and Hg intake during the first 6 months

Age, days	Vaccine		Body weight, g	Breast milk ^b Hg intake, µg
	Type ^a	µgHg/dose		
0	Hp-B	25.0	3,233.17	0
30	Hp-B	25.0	3,862.87	30.83
60	DTP	25.0	4,492.56	44.65
90	–		5,067	
120	DTP	25.0	5,751.95	91.80
150	–		6,327	
180	DTP+ Hp-B	50.0	7,011.34	111.90
Total ^c		150.0		279.18

^a Hp-B: Hepatitis B (assumed 0.01%Thimerosal/dose, Korea Green Cross Corporation, Kiheung-Eup Yougin-Goon Kiyunggi-Do, Korea; Euvax B injectable, 0.01%Thimerosal, LG Life Sciences, Jeonbuk-do, Korea); DTP (Serum Institute of India Ltd; Vacina Triplíce, 0.01%Thimerosal/dose; Instituto Butanta, São Paulo, Brazil)

^b Integrated total Hg intake (estimated from Dorea [8])

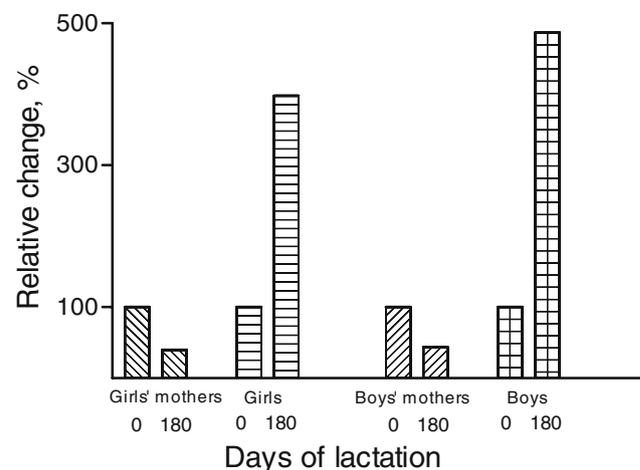
^c Total exposure of the respective Hg chemical forms

**Fig. 1** Distribution of mean total mercury concentrations in studies of different parts of the world (adapted from Dorea [8])

0.266 µgHg/kg b.w. at any time after the first immunization at 0 days. Excluding the small volumes of milk intake at birth (day 0 of first vaccine), this corresponds to a small percentage (4.7 to 8.1%) of the EtHg doses injected between 30 and 180 days.

Because of the variability in hair-Hg concentrations, the relative changes from 0 days (birth) to 180 days were calculated and shown in Fig. 4. While mothers had a mean decrease of 40% and 45%, their girls and boys had, respectively, increased hair-Hg concentrations by as much as 398.3% and 487.1%.

Another notable feature is the time of exposure to EtHg shown in Fig. 5. A distinct pattern is shown as a function of the immunization policy regarding the dispensation of vaccines. The infants that received the first vaccine within the first 24 hours after birth were all from the only public hospital in Porto Velho (Hospital de Base). Because the other two hospitals (Panamericano and Regina Pacis) are privately owned and operated, the infants are vaccinated in

**Fig. 2** Estimation of the integrated total-Hg intake during the first 180 days of breastfeeding

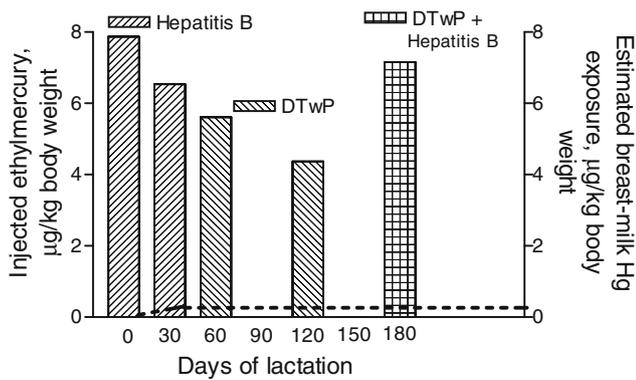


Fig. 3 Infants' vaccine schedule and injected ethyl-Hg (as Hg), and estimated total Hg in breast milk (– adapted from Dorea [8]) during the first 180 days of life

immunization clinics run by the Ministry of Health after the hospital discharge.

Discussion

Unlike breast-milk intake, there are no body-weight dosing adjustments for vaccines; therefore, neonates and young infants (up to 60 days) with less blood volume are exposed to more injected EtHg than older infants from 120 to 180 days (Table 2). Over the first 6 months, the estimated accrual of total Hg exposure from breast milk was higher than the five doses of injected EtHg (Table 2). However, differing from parenteral EtHg in vaccines, breast-fed infants were exposed to enteral Hg (intrinsic to the breast milk matrix) amortized over 6 months. Because the mean frequency of feeds/day is 5.5 [30], the milk-Hg exposure in the breastfed infant occurs in several small daily suckling episodes. This type of exposure amounts to only 4.7%–8.1% of the vaccine-Hg bolus (per kg/b.w.) taken on the day of the immunization. Furthermore, the ingested milk-Hg is hindered by all gastrointestinal barriers, metabolic and detoxifying mechanisms (albeit immature) attendant to enteral feeding. Coupled with that, another attenuating factor is the tendency of human milk Hg to decrease during initial lactation [8], i.e., from day four to 6 weeks after delivery [5]. Additionally, central to the toxic effects of Hg

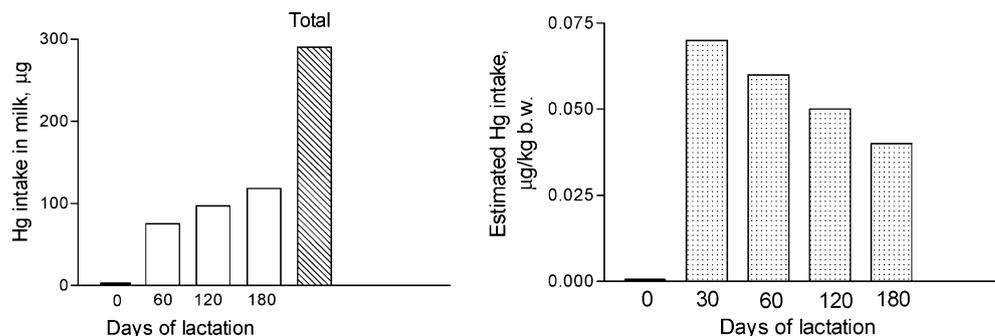
during early life is the unique chemical composition of human milk with its specific neurotoxic attenuating factors.

Neurodevelopment is rapid in the postnatal period and substantial amounts of long-chain polyunsaturated fatty acids (PUFAs) are critical to neurite growth and proper brain development. The beneficial PUFAs are docosahexaenoic acid (DHA=22:6n-3) and eicosopentanoic acid (EPA=20:5n-3). DHA is an essential component of nervous-system cell-membranes and is transported across the placenta and across the mammary gland into milk. During the critical window of early life, significant amounts of PUFAs are provided via breast milk and then avidly incorporated into membranes throughout the infant's nervous system [22]. Indeed, post-natal DHA is positively correlated with visual and language development in breast-fed infants [14].

Another mitigating factor against Hg-induced neurotoxicity is the intracellular defense provided by glutathione (GSH). Mercury has a high affinity for thiol (sulfhydryl groups), the thiol-containing antioxidant of GSH [15]. According to Cai and Sauve [7], thimerosal is a poorly membrane-permeable hydrophilic-molecule that oxidizes SH groups with a high affinity. It reduces S–S bonds of molecules such as GSH. Therefore, the potential protective effect of GSH against Hg toxicity can be compromised in situations limiting cysteine nutrition. Indeed, James et al. [15] showed that thimerosal-induced cytotoxicity was associated with depletion of intracellular GSH. Because brain cells cannot synthesize cysteine, (the rate limiting amino acid for GSH synthesis) it is dependent on the liver to synthesize and export cysteine to the brain for intracellular glutathione synthesis [15]. Human milk contains a high amount of bioavailable cysteine and differences between breast-fed and formula-fed infants have been reported with respect to cysteine concentrations and its metabolites [23].

The safety of thimerosal vaccines is assumed on the basis of the faster metabolism of EtHg compared to MeHg [6]. While we do not know if the infants' hair-Hg in this study was derived from the vaccine's thimerosal, it can be stated that there was an asymmetric change in infants' hair-Hg compared to the mothers' hair in the same period. We

Fig. 4 Relative hair-Hg changes (%) from birth (0 days) to 180 days in mothers according to infant gender



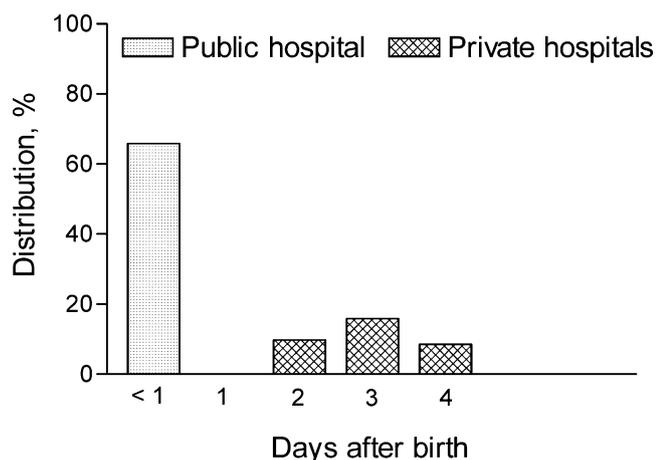


Fig. 5 Percent distribution of the time-after-birth interval of the first vaccine (hepatitis B)

have reported that fish-eating riverine mothers showed higher hair-Hg concentrations than their respective breast-fed infants [2]. In line with these observations it has been shown that maternal hair-Hg concentration was higher than fetal hair-Hg [28]. Recently, Lindow et al. [17] reported higher fetal hair-Hg concentrations in babies of mothers exposed to dental amalgam restoration either before or during pregnancy; they showed maternal/fetal Hg ratios > 1 in most samples (66%). However, contrary to these studies, Mohan et al. [24] reported that 80% of newborns had higher hair-Hg than their mothers. Nevertheless, the mean increase in hair-Hg concentrations we found at 6 months is compatible with the levels predicted by Redwood et al. [29].

Holmes et al. [12] describe that mothers from autistic children received more mercury during pregnancy than mothers from healthy controls and also hair-Hg differences between autistic and control infants at the time of their first haircut (11–24 months). They speculated that hair excretion patterns among autistic infants were significantly reduced relative to controls. Furthermore, they claimed that hair-Hg in control infants were significantly correlated to Hg exposure through prenatal maternal dental amalgam fillings, but that correlations were absent in the autistic group. Contrary to that, older (4–7 years old) autistic children showed higher hair-Hg concentrations than age-matched controls [11]. Hair-Hg association with behavioral disorders has also been extended to interactions with zinc deficiency [13]. However, the assessment of hair-Hg concentrations after immunizations containing thimerosal (EtHg) is incomplete without speciation of the Hg forms. Nevertheless, our total hair-Hg results are in line with previous work. Barbosa and Dorea [2] estimated that mean concentration of Hg in breast milk of “riverine” women (living near rivers, high fish eaters) with higher hair-Hg

(14.3 ppm) would give an exposure to breast-fed infants (mean hair Hg, 9.8 ppm) of 0.64 µg/kg body weight. In the present study the urban mothers are low fish eaters and show much lower mean hair-Hg concentration (7.5 ppm).

The neurotoxicity of administered MeHg is well known and seems to be higher than EtHg, the Hg metabolite of thimerosal [18]. There is limited information on human metabolism of EtHg and even less information is available for infant metabolism of thimerosal. Because there are no known studies of intrinsically incorporated EtHg in food matrices, comparing parenteral vaccine-EtHg to the breast milk-Hg (intrinsic-Hg) metabolism is challenging. The decomposition rate of EtHg is higher than MeHg because of the molecule size. Qvarnstrom et al. [27] reported that the carbon-Hg bond in $C_2H_5Hg^+$ is less stable than that in CH_2Hg^+ . In addition, the efficiency of the blood-brain barrier is directly proportional to the size of the organic radical [18].

Infant Hg exposure in early life can only happen through milk diets [10]. However, given the variation (24 to 67 h postpartum) of the onset of lactation [25], for many infants a high exposure to parenteral EtHg (25 µg) preceded the much lower (Fig. 2) enteral total-Hg (intrinsic to the human milk matrix). A second point is the impact caused by injected EtHg in an immature organism. It has been speculated that the removal of thimerosal from vaccines would produce no more than a 50% reduction of Hg exposure in infancy [4]. This reduction could only happen with human milk Hg concentrations greater than the median value (Fig. 1) and more frequently with formulas, which usually contain higher Hg concentrations [10]. Furthermore, this scenario assumes a debatable equivalency of a bolus (injected Hg) *versus* the integration (over 6 months) of ingested milk-Hg; it does not contemplate the tenets of Hg neurotoxicity: chemical form, dose, route of administration, attenuating neurotoxic factors of breast feeding, and enhancing neurotoxic factors of the perinatal period (birth weight/prematurity).

The expected metabolic consequences of such extremely different exposure routes and Hg chemical forms (injected EtHg *versus* ingested milk-Hg) as well as Hg dose are difficult to disentangle; we have the changing physiological interactions of a self-adjusted daily dose of intrinsic Hg (inorganic and MeHg) in breastfeeding against the full impact of an extrinsic dose of EtHg. With regard to breastfeeding, milk-Hg occurs in a context of proven benefits in a broad array of neurotoxic mitigating factors. In this study, we observed a relative increase in hair-Hg. This finding reinforces a mechanistic connection between thimerosal-EtHg and hair-Hg increase, supporting the use of hair-Hg in vaccinated children as a confounder of neurobehavioral maternal-Hg contamination.

Acknowledgements We are greatly in debt to the mothers for their participation in the study, to the staff and directors (Marinês R. dos Santos Cezar, Tereza Cristina Ramos, Daniele Brasil, Katia Wendt, Katiane G. Brandão, Laura Jane Marques) of the Hospitals (Hospital de Base Ary Pinheiro, Hospital Panamericano and Hospital Regina Pacis), Dr. Cezar Augusto Bezerra B. de Araújo (State Coordinator of the PNI-MS), the staff of the Fundação Universidade Federal de Rondônia and the Universidade Federal do Rio de Janeiro.

This work was supported by United Nations Educational, Scientific and Cultural Organization - UNESCO, Ministério da Saúde do Brasil (SC27824/2005/914BRA2000 Decit PRODOC) and The National Research Council of Brazil-CNPq (PNOPG project-55.0882/01-4 PPG7, project-556985/2005-2).

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