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Neurodevelopment of Amazonian children exposed to ethylmercury (from Thimerosal in vaccines) and methylmercury (from fish)

Rejane C. Marques [a,*], Luciana Abreu [b], José V.E. Bernardi [c], José G. Dórea [c]

[a] Universidade Federal do Rio de Janeiro, Rio de Janeiro CEP 27930-560, RJ, Brazil
[b] Statistika Consultoria, Campinas, SP, Brazil
[c] Universidade de Brasília, Brasília, 7 0919-970, DF, Brazil

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ABSTRACT

Few studies have addressed co-occurring methylmercury (MeHg) from maternal origin and ethylmercury (EtHg) from Thimerosal-containing vaccines (TCVs) during infant's neurodevelopment. We studied children ($n=1139$) from the Western Amazon based on combined (low, intermediate, and high) exposure to chronic MeHg from fish consumption and acute TCV- EtHg. Neurodevelopment outcomes were age of walking and age of talking, and the Bayley Scale of Infant Development (BSID). The Mental Developmental Index (MDI) and Psychomotor Developmental Index (PDI) were measured at six and 24 months of age. Median hair-Hg (HHg) at birth was 6.4 $\mu g\, g^{-1}$ in mothers, and 1.94 $\mu g\, g^{-1}$ in newborns; total (pregnancy and infancy) EtHg exposure ranged from 0 to 187.5 μg. The combined (MeHg + EtHg) exposure showed significant differences for MDI but not for PDI; however, there was a significant decrease in both MDI and PDI scores at 24 months. The increase in BSID delays (scores < 80) between six and 24 months was not discernible with regards to EtHg or MeHg exposure. We found a statistically significant increase in neurodevelopmental (BSID) delays related to the combined exposure to Hg (MeHg > EtHg). Neurodevelopment delays due to low-doses of organic mercury (albeit undiscernible) are not predictable but can be avoided by choosing low-Hg fish and providing Thimerosal-free vaccines.

1. Introduction

During developmental periods extending from prenatal stages, the brain is more vulnerable to the adverse effects of toxic insults than at more mature stages; however, experience-guided development drives neuro-cognition achievements. Early brain susceptibility to mercury toxicity (Clarkson et al., 2003) and adverse neurological outcomes have been reported in animal experiments (*in vivo* and *in vitro*) as well as in human epidemiological studies (Dórea, 2013; Grandjean et al., 2014). In fish-eating populations of the Amazon, during prenatal and postnatal life, not only methylmercury (MeHg) but also ethylmercury (EtHg) in Thimerosal-containing vaccines (TCV) are the main forms of organic mercury exposure to the developing human brain (Marques et al., 2013a).

The first indication of EtHg neurotoxic effects on development resulted from high doses during the accidental mass poisoning in Iraq (Clarkson et al., 1976). Children born to women exposed to food contaminated with organic Hg (both MeHg and EtHg) showed impairment derived from neurological examination scores and milestone (age of first walking and first talking) delays (Marsh et al., 1987). Despite strong evidence of potential effects of low-doses of Thimerosal/EtHg (Geier et al., 2015), studies addressing only TCV-EtHg exposures and association with neurodevelopmental effects are conflicting in population studies conducted in developed countries (Dórea, 2010).

Despite a shorter residence time in the blood, compared with MeHg, Thimerosal-EtHg stays longer in the brain of monkeys (Burbacher et al., 2005). Thimerosal/EtHg toxicity tests conducted *in vitro* (molecular and cellular level) have shown perturbations of toxicity pathways of equal magnitude to that found for MeHg (Dórea, 2013). Experimental studies with Thimerosal/EtHg doses (simulating TCV) on tissue structure, function, and animal behavior have demonstrated neurotoxic effects in different species such as monkeys, hamsters, mice, and rats (Dórea, 2013); untoward effects of Thimerosal-EtHg on neurodevelopment have also been found in population studies (Dórea, 2013; Geier et al., 2015).

During pregnancy and lactation, subsistence fish-eating Amazonian mothers can pass relatively large amounts of Hg to fetuses and to breastfed infants (Marques et al., 2013a; Vieira et al., 2013).

* Corresponding author at: Universidade Federal do Rio de Janeiro, Rio de Janeiro, CEP 27930-560, RJ, Brazil.
E-mail addresses: rejanecmarques@globo.com (R.C. Marques), vicente.bernardi@yahoo.com.br (J.V.E. Bernardi), jg.dorea@gmail.com (J.G. Dórea).

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In some circumstances, the current TCV schedule can expose a six-month-old infant to 125 μg of EtHg which may correspond to half of that taken by a breastfed infant from a high fish-eating mother (Dórea, 2007); differently from milk-Hg, the injected TCV-EtHg is readily available to the suckling infant. Additionally, TCVs are adjuvanted with aluminum in a high (50:1) Al:EtHHg ratio (Marques et al., 2014a); the ratios of Al:Hg in cord blood (Rahbar et al., 2015) and milk (da Costa et al., 2005; Chao et al., 2014) occur at lower levels (circa 2:1 to 5:1). Thus, combined chronic (fish-MeHg) and acute EtHg (from TCV) exposures in fetuses and infants are an additional concern for those (vulnerable organisms) immunized with TCV in less economically developed countries.

The occurrence of multiple exposures to different chemical forms of mercury (MeHg and EtHg) is common in populations of high (Dórea, 2007; van Wijngaarden et al., 2013; Grandjean et al., 2014) and low (Lee and Ha, 2012; Jedrychowski, 2012) fish consumption habits that have used TCV to immunize nursing infants. Despite that, studies have focused mainly on chronic fish-MeHg, while for multiple doses of acute TCV-EtHg, research is relatively recent (Dórea, 2010, 2015). Nevertheless, studies have consistently showed that EtHg from Thimerosal co-occurs with other neurotoxic agents; when properly analyzed these combinations are frequently associated with negative responses in neurodevelopment (Dórea 2013; Dórea, 2015). In the Brazilian Amazon, neurodevelopmental delays have been reported in breastfed infants who were exposed to both EtHg-Al from TCV and MeHg from maternal fish consumption (Marques et al., 2014a; Dórea et al., 2014).

Hair is a choice matrix to monitor Hg exposure because it is non-invasive, can be easily collected, stored for long time at room temperature, and easily conditioned for shipment without biohazardous precautions. Because MeHg exposure is exclusively from fish and seafood consumption, HHg can also estimate fish consumption better than food frequency questionnaires (Dórea, 2009). These are prized features for assessing both maternal fish consumption and MeHg exposure in remote resource-poor settings. Although we have detected EtHg in post-vaccinated infant hair, its contribution to total HHg is negligible (Dórea et al., 2011a, 2011b). Maternal hair samples reflect MeHg exposure during pregnancy and lactation (Barbosa et al., 1998)

In isolated and remote parts of the Amazon region lacking roads and infrastructure, it is challenging to adhere to the vaccination schedule recommended for both pregnant mothers and infants; this naturally occurring situation created an opportunity to study multiple burdens of organic Hg. Therefore, our main objectives were a) to study co-occurring prenatal and postnatal exposure to organic-Hg forms resulting from maternal fish consumption (MeHg) and maternal and infant immunizations with TCVs (EtHg); and b) to address the association of Hg exposures with neurodevelopment (BSID and milestone achievements) in children at six and 24 months of age.

2. Materials and methods

Specific populations, such as riverines, are less likely than 'typical' counterparts to be studied in relation to environmental Hg exposure and effects. These Amazonian subsistence populations are disproportionately exposed to fish-MeHg and as infants they receive an additional load of EtHg in Thimerosal-containing vaccines.

The Madeira River Basin is the study area where most of our studies have been done. The study area covers several municipalities along the Madeira River in the state of Rondonia (Western Brazilian Amazon). A detailed map appeared in a parent publication (Marques et al., 2013a). Our research team is familiar with the geography, means of transportation, and has working experience with community leaders and families.

The study area was initially surveyed and publications dealing with children growth and neurodevelopment appeared elsewhere (Marques et al., 2011, 2012; Dórea et al., 2014) and served to identify expecting mothers that formed this cohort in January 2006. This cohort was formed to study growth and neurodevelopment of young children and it was approved by the Institutional Review Board of the Universidade Federal de Rondonia (Of. 001-07/CEP/NUSAU). The inclusion criteria were (1) residence of at least five years in the study area, (2) a healthy pregnancy (3) and absence of congenital malformations.

The studied families are dispersed in area running more than 733 km along the Madeira River Basin. Of the invited 1668 pregnant mothers, 215 declined participation in the study, and 20 (11 miscarried, and nine gave birth babies with congenital anomalies). In the present study we surveyed 1139 mother-newborn pairs from eight communities along the banks of the Jamari, Madeira and Mamoré Rivers, and former riverines now living on the outskirts of Ariquemes, Itapuã, Candeias, Porto Velho, and Guajará-Mirim, and rural communities; in regards to living conditions these communities were homogeneous. They were identified earlier as traditional or former riverines sharing socioeconomic characteristics that include fish-eating habits. These riverines are locally called "*ribeirinhos*". They live in small communities and rely on local food resources, especially fish and manioc, for their subsistence; they are racially a mixture of Amerindians, Caucasians (Portuguese descent), and Africans (Boischio and Cernichiari, 1998).

After signed informed consent, we conducted a comprehensive evaluation of health, growth and development of these children, including neuro-behavioral evaluation. The field researchers were health professionals with field experience; they were comprised of medical students, nurses and psychologists. They were trained to administer questionnaires, interviews, and collection of bio-data. Health information data were obtained from hospital records or from midwives.

Families were visited at six and 24 months; during visits we applied questionnaires (to obtain information on breastfeeding practices, age of walking and age of talking, as well as socioeconomic data), assessed neurodevelopment, and collected hair samples mothers and children. A bundle of hair strands were cut close to the scalp with scissors from the occipital area; the hair strands were tied with a cotton thread, properly identified, and stored in a plastic bag. The sampled hair was taken to the Radioisotopes Laboratory of the Federal University of Rio de Janeiro for analysis. At these visits we collected and updated immunization information from vaccination cards. Immunization scheme in Brazil recommends the first vaccine (Hepatitis B vaccine-HBV) to be taken as soon as possible after birth. Both HBV and DTP (diphtheria, tetanus and pertussis) vaccines contain Thimerosal (as preservative) and are subsequently recommended at 30, 60, 120 and 180 days. As stated by manufacturers (Korea Green Cross Corporation, Kiheung-Eup Yougin-Goon Kiyunggi-Do, Korea; Euvax B injectable, LG Life Sciences, Jeonbuk-Do, Korea) and diphtheria, tetanus and pertussis-DTP (Triple Antigen, Serum Institute of India Ltd., India;Vacina Tríplice, Instituto Butanta, São Paulo, Brazil), TCVs contained 0.01% thimerosal; these vaccines also contain Aluminum as adjuvants in Al:Hg ratio near 50-fold depending on the TCV (Dórea, 2015)

Hair preparation and analytical procedures to determine total Hg concentrations followed our standardized laboratory protocol (Fonseca et al., 2008). Briefly, the hair samples are comminuted with stainless steel scissors, washed with EDTA 01% (w/v), then dried in an oven at 50 °C. After weighing they are digested before analysis. The hair sample digestion is done at 80 °C for 40 min with concentrated HNO_3 (3 ml) and $KMnO_4$ (5% (w/v) in a

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