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Invited critical review

Thimerosal: Clinical, epidemiologic and biochemical studies ☆

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ABSTRACT

Introduction: Thimerosal (or Thiomersal) is a trade name for an organomercurial compound (sodium ethylmercury (Hg) thiosalicylate) that is 49.55% Hg by weight, which rapidly decomposes in aqueous saline solutions into ethyl-Hg hydroxide and ethyl-Hg chloride. Developed in 1927, it has been and is still being used as a preservative in some cosmetics, topical pharmaceuticals, and biological drug products, including vaccines. Concerns have been voiced about its use because it is toxic to human cells. Although it is banned in several countries, it continues to be added to some vaccines in the United States and many vaccines in the developing world.

Discussion: This critical review focuses on the clinical, epidemiological, and biochemical studies of adverse effects from Thimerosal in developing humans. This review will include research that examines fetal, infant, and childhood death; birth defects; neurodevelopmental testing deficits in children; and neurodevelopmental disorders (attention deficit/hyperactivity disorder, autism spectrum disorder, tic disorder, and specific developmental delays). The review will also look at the research that examined the outcomes of acute accidental ethyl-Hg poisoning in humans. The studies that examine the underlying biochemical insights into the neuronal cellular damage will also be explored.

Conclusion: The culmination of the research that examines the effects of Thimerosal in humans indicates that it is a poison at minute levels with a plethora of deleterious consequences, even at the levels currently administered in vaccines.

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Contents

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1. Introduction

Thimerosal (or Thiomersal) is a trade name for an organomercurial compound (sodium ethyl-mercury (Hg) thiosalicylate, $C_9H_9HgNaO_2S$) that is 49.55% Hg by weight. Thimerosal quickly decomposes in aqueous saline solutions into ethyl-Hg hydroxide and ethyl-Hg chloride [1]. Developed in 1927, it has been used as a preservative in cosmetics, pharmaceutical preparations, and biological products such as eye shadows, make-up removers, mascaras, and soap-free cleansers (cosmetic products); ear, eye and nose drops and ointments, antiseptic sprays, topical medications and tincture of Merthiolate (pharmaceutical preparations); and antitoxins, immune globulin preparations, skin-prick test antigens, and vaccines (biological products) [2].

Hg compounds have been used as disinfectants since bacteriology began [3]. For a long period of time, Hg compounds, such as mercury chloride ($HgCl_2$), were thought to be useful in the killing of bacteria and other microorganisms [3]. Despite this fact, as early as 1943, it was reported that plasma preserved with 1:10,000 Thimerosal was contaminated with viable micro-organisms, and it was concluded that Thimerosal cannot be considered the ideal preservative [4]. Subsequently, Morton et al. [3] reported that the label for Thimerosal (solution of 1:1000) stated that Thimerosal is a stainless and stable organic mercury compound of high germicidal value, especially in serum and other protein media. However, Morton et al. [3], based upon their experiments, found that Thimerosal is not highly germicidal and does not possess high germicidal value in the presence of serum and other protein mediums particularly. They further stated that the loss of antibacterial activity of mercurials in the presence of serum proves their incompatibility with serum. Furthermore, these investigators described that Thimerosal was 35-times more toxic to embryonic tissue cells than it was to bacteria, as well as more toxic to leukocytes than bacteria [3].

In more recent research, the effectiveness of Thimerosal as a preservative in Diphtheria–Tetanus–Pertussis (DTP) vaccine was evaluated by the United States (US) Centers for Disease Control and Prevention (CDC) [5]. The CDC reported that the choice and level of the preservative for inclusion in DTP vaccine were limited because of possible harmful effects on the vaccine's antigenicity, plus the need to ensure safety of the preservative. These investigators reported that Thimerosal, the preservative used in the production of DTP as an organic-Hg bacteriostatic agent, was only weakly bactericidal. The laboratory experiments revealed up to 2-week survival of bacterial cells in multi-dose DTP vaccine vials using Thimerosal as a preservative. These investigators concluded that at currently used concentrations, Thimerosal is not an ideal preservative. Higher concentrations were not recommended because it might reduce vaccine potency or pose a danger to individuals receiving the vaccine. As a result, the investigators suggested that those administering Thimerosal preserved vaccines should not rely on its effectiveness, but instead should apply particular attention to sterile technique when using multi-dose vials. Other investigators observed that Thimerosal failed to meet European Pharmacopoeia (EP) antimicrobial effectiveness acceptance criteria as a preservative due to lack of growth inhibition of Thimerosal on *Staphylococcus aureus* in both single and multi-challenge evaluations [6]. Finally, other investigators described the toxicity levels of commonly used preservatives in vaccines and biologics [7]. When comparing the relative cytotoxicity levels of the preservatives in US licensed vaccines, the observed relative toxicity of the compounds tested was phenol < 2-phenoxyethanol < benzethonium chloride < Thimerosal, and the relative toxicity indices (human neuroblastoma cells/bacterial cells) were 2-phenoxyethanol (4.6-fold) < phenol (12.2-fold) < Thimerosal (>330-fold). For the products tested, except for 2-phenoxethanol, the amounts needed to cause significant killing of bacteria were much higher than those routinely used in US licensed vaccine/biological preparations.

Despite all of the aforementioned concerns and the fact that there are other approved and effective preservatives available [6,7], Thimerosal continues to be used as a preservative in several vaccines to date and is a considerable source of Hg exposure for children [8,9]. About 50% of the Hg exposure in infants comes from the recurring bolus doses of Thimerosal from Thimerosal-containing vaccines administered in the first 2 years of life (cumulative doses of Hg exposure from Thimerosal-containing vaccines can be as high as 187.5 μg Hg in the first six months of life) [9]. Although this degree of exposure in the first six months of life has been reduced in the US in recent years, it remains unchanged in developing countries. There is considerable body of scientific and medical evidence supporting a role from Hg exposure causing harmful consequences [10]. To date, there are at least 180 studies that show harm from Thimerosal [11]. The purpose of this review is to specifically examine human clinical, epidemiological, and biochemical studies demonstrating the developmental adverse affects from human exposure to Thimerosal and its ethyl-Hg breakdown products.

2. Thimerosal exposure from vaccines

Until the beginning of this century, every tetanus-containing vaccine in the US (e.g., the DTP, tetanus toxoid (TT), diphtheria–tetanus (DT), and diphtheria–tetanus–acellular-pertussis (DTaP)), Haemophilus influenza type b (Hib), hepatitis B (HepB), and a polysaccharide meningococcal meningitis A, C, Y, and W-135 vaccine contained Thimerosal, many at a concentration of 0.01% Thimerosal. However, on July 7, 1999, the US Public Health Service (USPHS) and American Academy of Pediatrics (AAP) called for the elimination of Thimerosal from all vaccines in the US as soon as possible [12]. Then, as the vaccines were approved by the US Food and Drug Administration (FDA), reduced-Thimerosal vaccines began to displace the previous Thimerosal-preserved vaccines in the early 2000s. Finally, beginning in the late 2000s, no-Thimerosal vaccines began to replace the reduced-Thimerosal vaccines in the US. However, to date, the US FDA has not canceled the licenses for the Thimerosal-preserved vaccines or kept them from being produced and marketed [13].

As more of the reduced-Thimerosal and no-Thimerosal vaccines became available in the early 2000s in the US, the assumption was that the exposure to Thimerosal would sharply decrease. However, this expectation proved to not be accurate because of recommendation changes in the vaccination schedule. Starting in April of 2002, the US CDC began to recommend that influenza vaccines be given to infants and children, who were 6-to-23 months of age, when the only approved influenza vaccine for that age group was preserved with Thimerosal (Sanofi Pasteur's Fluzone®). In addition, the US CDC recommended influenza vaccines be given to women who were pregnant in their second and third trimesters, when the available influenza vaccines were also Thimerosal preserved [14]. In addition, through 2010, the US CDC progressively widened the age range for annual influenza vaccine such that very young children were supposed to get two doses of influenza vaccine initially (at 6 and 7 months of age) and then receive an additional dose every year. By this time, the US CDC had also discontinued the "second-and-third-trimester" constraint on giving influenza vaccines to pregnant women [15–17].

Thus, even though the US FDA eventually approved the reduced-Thimerosal and no-Thimerosal formulations of the tetanus-containing vaccines and some other vaccines, exposure to Thimerosal through vaccination has remained common in the US. As recently as 2013, more than half of all the influenza vaccines were still preserved with Thimerosal. Therefore, the approximate maximum lifetime exposure to Hg from Thimerosal-preserved vaccines has increased compared to the lifetime exposure under the US CDC's pre-2000 recommended vaccination schedule. It is estimated that it is now more than double what it would have been had the pre-2000 vaccination schedule been maintained. To date, in the US, Thimerosal is still a preservative in some of the other US FDA-approved vaccines including a multi-dose tetanus toxoid (TT) vaccine, and one multi-dose meningococcal meningitis vaccine [18]. Estimations suggest that there has not been a major decrease in Hg exposure from Thimerosal-preserved vaccines in vaccine-schedule-compliant children in the US.

Similarly, exposure to Hg from Thimerosal-preserved vaccines in pregnant women continues to occur through the use of inactivated-influenza vaccine still being given to pregnant women (that was first recommended by the US CDC in 1997) [19], since many of the inactivated-influenza vaccines and most of the available doses of those flu shots still contain Thimerosal [20,21]. To date, the amount of Hg still present in Thimerosal-preserved vaccines nominally ranges from 12.5 μg Hg to 25 μg Hg per dose (with some vaccines containing >25 μg Hg per dose) [18].

Globally, and especially in the developing world, Thimerosal is still used in many of the childhood vaccines such as tetanus toxoid, Hib, HepB, DTwP–HepB–Hib, DTP, and assorted influenza and meningococcal vaccines [22–24]. In addition, the tetanus toxoid vaccine (25 μg Hg per dose) is also recommended for pregnant women in some countries [25].

In estimating Hg exposure from Thimerosal-containing childhood vaccines, it was previously determined by Bigham and Copes [9] that some infants were exposed to cumulative doses of Hg from vaccines and environment that were over the Hg safety limits established by the US FDA, US CDC, US EPA, the World Health Organization (WHO) and Health Canada. In addition, it was observed that two-month-old US infants exposed to an average of 45.6 μg Hg (range 37.5 μg Hg–62.5 μg Hg) from Thimerosal-containing childhood vaccines by Pichichero et al. [26] had circulating blood Hg levels between 3.75 and 20.55 nM. Other researchers reported that Thimerosal-preserved vaccines given to human infants significantly increased the infants' blood Hg levels. Some of the infants had a total blood mercury level that was over the safety limit set by the US Environmental Protection Agency (US EPA) [27–29]. It was also observed that Thimerosal-containing childhood vaccines given to infants significantly increased the hair ethyl-Hg and total Hg levels. Importantly, some of infants had total hair Hg levels that were over the safety limit set by the US EPA [30].

3. Thimerosal exposure and fetal/infant/childhood death

Ethically and legally, the direct study of the effects of Thimerosal or ethyl-Hg compound exposure on fetal/infant/childhood death in humans is proscribed. On a theoretical basis, a number of previous researchers have investigated the potential toxicokinetics of Hg exposure from Thimerosal in pregnant women [20]. For example as Goldman [20] showed in his analysis of exposure to Hg from Thimerosal during pregnancy (assuming 50% of the total dose would accumulate in the fetus) that administration of a single Thimerosal-preserved influenza vaccine (25 μg Hg per dose) in comparison to the US EPA Hg safety limit would result in a fetus of average weight receiving a Hg dose that could be ≥125,000 times the EPA Hg safety limit if it was administered at ≤8 weeks of gestation and, by 42 weeks of gestation, result in a fetus of average weight receiving a Hg dose 34 times the EPA Hg safety limit. It was also determined by Goldman [20], given the aforementioned assumption that 50% of the total dose would accumulate in the fetus, that administration of a single Thimerosal-containing influenza vaccine with 1 μg Hg per dose in comparison to the US EPA Hg safety limit would result in a fetus of average weight receiving a Hg dose ≥5000 times the EPA Hg safety limit if it was administered at ≤8 week gestation and, by 42 week gestation, result in a fetus of average weight receiving a Hg dose 1.4 times the EPA Hg safety limit. Similarly, Brown and Austin [21] evaluated fetal exposure to Hg from one Thimerosal-preserved influenza shot during pregnancy (25 μg Hg per dose). Those investigators modeled exposure assuming hypothetical placental elimination of Hg at 0% (25 μg Hg exposure), 90% (2.5 μg Hg exposure), and 99% (0.25 μg Hg exposure). Overall, these investigators observed that doses of Hg exposure from administration of a single Thimerosal-preserved influenza vaccine during pregnancy resulted in a developing fetus receiving a dose of Hg in excess of the US EPA Hg safety limit from between 1,000,000 times to 10,000 times that safety limit at 1 week of development to 7.6 times to 0.1 times that limit at 38 weeks of development. It is interesting to note, from the Brown and Austin [21] modeling data, that, even assuming 99% elimination of the Hg dose by the placenta, a developing fetus even at 16 weeks-old would still receive a dose of Hg greater than 2.5 times the EPA Hg limit for safety. Overall, both Brown and Austin [21] and Goldman [20] concluded their toxicokinetic studies by suggesting that, given the magnitude in excess of the EPA Hg safety limits presented by exposure to a dose of Thimerosal-preserved vaccine during pregnancy, it is biologically plausible for such exposures to result in fetal/infant death and developmental disability.

In addition to toxicokinetic modeling studies of exposure to Thimerosal, many observational studies have examined the relationship between Hg exposure from Thimerosal or its ethyl-Hgcompounds decomposition products. For example, in the 2009–2010 influenza season, the US CDC recommended that women who are pregnant be administered a seasonal inactivated-influenza vaccine dose and a pandemic (A-H1N1-2009) inactivated-influenza vaccine dose, encouraged all pregnant women to get both vaccines, and allowed both vaccines to be administered at the same time [20]. The inactivated-influenza vaccine doses available for administration included Thimerosal-preserved vaccines, where about 50% of the available doses of the seasonal inactivated-influenza vaccine were Thimerosal-preserved doses; more than 50% of the pandemic inactivated-influenza vaccines were Thimerosal-preserved doses; and the CDC did not recommend that the pregnant women only be given the Thimerosal-free [20].

During the Fall of 2009, against a background of no more than five fetal-loss reports (spontaneous abortions and stillbirths) linked to the administration of an influenza vaccine in the Vaccine Adverse-Events Reporting System (VAERS), the number of fetal-loss reports to VAERS quickly exceeded 50 reports. By the end of 2009, the reports exceeded 100 fetal-loss instances before ending up at 178 fetal-loss reports in VAERS for the 2009–2010 flu season. An epidemiological study of the VAERS data by Goldman [20] that included correction for increased uptake and increased reporting, observed that there was more than an 8-fold fetal-loss reports increase in the 2009–2010 flu season over the average reporting rates for the prior (2008–2009) and subsequent (2010–2011) influenza seasons (roughly August through April of each year). Goldman [20] concluded from his analysis that a single Thimerosal-preserved influenza vaccine nominally containing 25 μg of Hg per dose was not sufficient to cause enough fetal-loss reports to VAERS to be of concern, but that two doses (one from a Thimerosal-preserved seasonal influenza vaccine and one from a Thimerosal-preserved pandemic influenza shot) were sufficient to generate a significant Thimerosal-exposure-related, fetal-loss-report signal.

As another example, Axton [31] reported about two adults and four children who were injected (accidently) with abnormally large quantities of Thimerosal from inappropriately prepared chloramphenicol-containing preparations. The children (aged between 6 weeks-old and 7 years-old) received between about 35 milligrams (mg) Hg per kilogram (kg) bodyweight and 162 mg Hg per kg bodyweight. All but one of them died within about 1 month (mortality rate = 75%).

As yet another example, topical application of Thimerosal to infants was shown to induce fatalities. Fagan et al. [32] reported on a case-series of 13 infants with omphaloceles (protrusions of the intestine and omentum through a hernia in the abdominal wall near the navel) treated with topical 0.1% Thimerosal solutions. It was reported that 10 of the 13 infants treated in this manner subsequently died (mortality rate = 77%), and analysis of Hg levels in their organs showed that the Hg ranged from 65–2700 times more than normal levels of Hg in the organs tested. Furthermore, Rohyans et al. [33] reported on an 18-month-old female who was administered Thimerosal ear irrigations on a daily basis for a month, and subsequently died of Hg poisoning.

In addition to fetal/infant/childhood fatalities observed following Thimerosal exposure, similar consequences were observed following exposure to its ethyl-Hg decomposition products. Cinca et al. [34]

described a case-series of four patients (3 children and their mother) poisoned by eating hog meat from hogs that were inadvertently fed seed treated with ethyl-Hg chloride fungicides. The symptoms of Hg intoxication began 10 days after exposure, and, overall, it was observed that 2 of the 3 children (aged between 10 and 15), both boys, died from Hg poisoning (mortality rate = 67%).

4. Thimerosal exposure and birth defects

Under the authority of the US National Institute of Neurological and Communicative Disorders and Stroke, the largest study to examine the relationship between Thimerosal exposure in pregnancy and birth defects was conducted between 1958 and 1965. The study was conducted on a prospective sample of 50,000+ women who were pregnant and their children [35]. This study is important because among 2277 children with malformations showing uniform rates by hospital in relation to exposure to topical antimicrobial drugs during lunar months 1–4 in 50,282 mother and child pairs, the survival and race standardized relative risk for Thimerosal was statistically significantly increased (2.69-fold). In addition, it was observed that influenza vaccine (at a time when it was preserved with Thimerosal) exposure was a risk factor for cleft palate (hospital standardized relative risk = 7.1), microcephaly (hospital standardized relative risk = 2.6), and pyloric stenosis (hospital standardized relative risk = 2.0) [35].

In addition, large-scale accidental poisonings of human populations with ethyl-Hg-based fungicides have provided information on the ability of in utero ethyl-Hg exposure to induce birth defects [36,37]. These changes (noted in the infants) included: (1) disorders of the central nervous system (CNS), hydrocephalus, cerebral paralysis, and spasms; (2) toxic encephalomyeloradiculoneuritis with prevalence of the syndromes of lesions of the cerebellum, brain stem, cerebral cortex, myelitis, and peripheral neurites, plus lesions of the motor centers and the pyramidal tracts, and encephalitis with irregular alpha-rhythm; (3) epilepsy (lasting up to 2 years) was observed in 10% of all the cases; (4) vegetoneurotic syndromes, bradycardia, tachycardia, arrhythmia, acrocyanosis, labile arterial pressure, and reduced blood cholinesterase activity; and (5) lesions of the heart, liver, kidney, and gastrointestinal tract.

5. Thimerosal and neurodevelopmental testing deficits in children

Recent studies comparing a cohort of infants at six months of age from communities with different fish-eating habits (rural communities in comparison to urban infants) who were simultaneously exposed to methyl-Hg and ethyl-Hg found that urban infants, who had the highest ethyl-Hg exposure from Thimerosal-containing vaccines and relatively lower methyl-Hg in comparison to rural infants, also had the highest risk of developmental delays (Gesell schedules below the median) [38]. Interestingly, examining this same cohort at 60 months of age failed to identify difference in development based upon Gesell schedules [39]. Finally, among a cohort of infants with multiple exposures to neurotoxic substances, those showing the most severe neurodevelopmental delays in psychomotor developmental index scores between six and 24 months of age were the ones with exposure to higher levels of ethyl-Hg from Thimerosal-containing vaccines [40].

Other investigators conducted a longitudinal cohort study to examine the relationship between infant exposure to Thimerosal from vaccines and child development in the first 3 years of life among 196 infants (born 2001–2003) in Krakow, Poland [41]. These investigators observed a significant adverse effect of neonatal exposure to Hg from Thimerosal on the children's psychomotor development index scores between the 12th and 24 months of life, and these deficits were observed to persist during the study's three-year follow-up period.

6. Thimerosal and neurodevelopmental disorders (ASD, ADHD, and TD)

Autism spectrum disorder (ASD), attention deficit/hyperactivity disorder (ADD/ADHD), and tic disorder (TD) are neurodevelopmental disorders that typically begin in childhood and are considered chronic conditions that last a lifetime. In all three disorders, males are affected more often than females. Shared symptomatology is also reported, such as problems with attention, social difficulties, obsessive compulsive behaviors, depression, anxiety, and ritualistic behaviors such as counting, repeating, or ordering and arranging. There has been a rise in these neurodevelopmental disorders in the last two decades [42]. However, to date, there is a debate as to the causes or contributing factors related to the increase. There are several studies that suggest that Thimerosal exposure in infancy increases the risk of a diagnosis of ASD, ADHD, and TD. This section will discuss the studies that show a relationship between Thimerosal exposure and ASD, ADHD, and TD.

6.1. Autism spectrum disorder (ASD)

For example, Geier et al. [43] recently undertook a two-phase study to examine the possible association between Hg exposure from Thimerosal in vaccines and the risk for an ASD diagnosis in the US. In the first phase of the study, a hypothesis generating cohort study was conducted to examine the possible relationship between exposure to Hg from a Thimerosal-containing DTaP vaccine in comparison to Thimerosal-free DTaP vaccines (from 1998 through 2000) and the risk of an ASD diagnosis in the VAERS database. In the second phase of the study, a hypothesis testing case–control study in the accessible Vaccine Safety Datalink (VSD) database was conducted to examine the relationship between Hg exposure from Thimerosal-containing hepatitis B vaccines given during specific time periods in the first six months of life among cases diagnosed with an ASD and controls without such exposures (born from 1991 through 1999). The results of the first phase of the study revealed a significantly increased risk ratio for the incidence of an ASD diagnosis following the receipt of Thimerosal-containing DTaP vaccine compared to the receipt of Thimerosal-free DTaP vaccine. In second phase of the study, cases diagnosed with an ASD were significantly more likely to have received increased Hg from Thimerosal-containing hepatitis B vaccine doses given within the first, second, and sixth month of life than controls.

In another example, Young et al. [44], examined the relationship between the birth cohort's prevalence of an ASD diagnosis and the birth cohort's cumulative exposures to Hg from Thimerosal in childhood vaccines given from birth to 7 months of age and from birth to 13 months of age among 278,624 subjects (born from 1990–1996) in the VSD. They used an ecological study design. Young et al. [44], found a dose–response relationship between increasing Hg exposure from Thimerosal in childhood vaccines given between birth and 7 months of age and between birth and 13 months of age for the risk of diagnosed autistic disorder and diagnosed ASD. Infants receiving an additional 100 μg Hg from Thimerosal in childhood vaccines between birth and 7 months of age showed a statistically significant increased rate ratio of 2.87 for an autistic disorder diagnosis and a statistically significant increased rate ratio of 2.44 for an ASD diagnosis.

Similarly, the relationship between administration of Thimerosal-containing hepatitis B vaccine within the first months of life and the subsequent risk of an individual being diagnosed with an autistic disorder was examined by Gallagher and Goodman [45]. They used the National Health Interview Survey (NHIS) 1997–2002 data sets. They reported that boys vaccinated with Thimerosal-containing hepatitis B vaccine during the first month of life were three times more likely to be diagnosed with autism than boys who were never vaccinated with that vaccine or boys who were vaccinated after the first month of life.

Geier and Geier [46] conducted a meta-analysis using statistical modeling to examine the relationship between exposure to Hg from

various Thimerosal-containing childhood vaccines and the risk of autism in the VAERS database. In that study, Geier and Geier [46] found a significant (1.6-fold) increased risk of autism adverse-event report in VAERS following additional doses of Hg from Thimerosal-containing vaccines.

In addition to postnatal exposure to Hg from Thimerosal in vaccines, researchers have also examined the relationship between prenatal exposure to Hg from Thimerosal-containing Rh_oD immune globulin preparations and the risk of a child being diagnosed with an ASD. The hypothesis was that if prenatal Rh_oD immune globulin preparation exposure was a risk factor for an ASD diagnosis then more children with an ASD diagnosis would have Rh-negative mothers as compared to children with Rh-positive mothers [47]. A previous study reported that children with an ASD diagnosis were significantly (28.30%) more likely (odds ratio = 2.35, $p < 0.01$) to have Rh-negative mothers (14.36%) [48]. In a second study, there was a comparable and significant increase in Rh-negative moms among children with a diagnosed ASD at two separate clinics (clinic A = 28.3%, B = 25.3%) compared to two separate control groups (clinic A = 12.1%, B = 13.9%) [47]. Supporting these findings, Holmes et al. [49] had previously reported that mothers of children with an autistic disorder were significantly more likely to have been given an increased number of $Rh_o(D)$ immune globulin preparation doses than the mothers of unaffected controls.

Finally, investigators have reported on a clinical case-series of eight children with a diagnosed Hg toxic encephalopathy showing regressive ASD symptoms following significant Hg exposure from Thimerosal-containing vaccines and $Rh_o(D)$ immune globulin preparation exposures [50]. The case-series revealed: (1) a consistent pattern of regressive ASD; (2) excretion of significant amounts of Hg post-chelation challenge test; (3) biochemical tests that showed that their glutathione pathways were compromised (decreased function in their Hg-related excretion pathways); and (4) no other known significant Hg exposures except from Thimerosal-containing vaccine and $Rh_o(D)$ immune globulin preparation doses. Moreover, any other cause of their regression had been ruled-out. In addition, there was a significant dose–response relationship between the total Hg dose received from Thimerosal-containing vaccine and $Rh_o(D)$-immune globulin-preparation exposures and ASD symptom severity. Based upon differential diagnoses, it was concluded that the eight patients examined had significant Hg exposure from $Rh_o(D)$ immune globulin preparations and Thimerosal-containing vaccines during their fetal and infant periods. Subsequently, these previously normally developing children suffered Hg toxic encephalopathy (between 12 and 24 months of age) that manifested with clinical symptoms consistent with a regressive ASD diagnosis.

It is noteworthy that the 2004 Consensus Report issued by the Institute of Medicine (IOM) failed to find an association between vaccines and autism [51]. The foundation of their conclusion was based upon a set of statistical population studies that were completed, funded and/or cosponsored by the United States Centers for Disease Control (US CDC). These studies include: (1) the Madsen et al. [52] ecological study of autism incidence in relation to Thimerosal exposure in Denmark, (2) the Stehr-Green et al. [53] ecological study of autism incidence in relation to Thimerosal exposure in Denmark, Sweden and California, (3) the Hviid et al. [54] study of autism incidence in relation to Thimerosal exposure in Denmark (also ecological), (4) the Andrews et al. [55] cohort study of autism incidence in relation to Thimerosal exposure in the United Kingdom, and (5) the published Verstraeten et al. [56] CDC cohort study of autism incidence in relation to Thimerosal exposure in the US. Later, the CDC published the Price et al. [57] study of autism incidence in relation to Thimerosal exposure in the US.

However, a recent review which critically examined these studies found several methodological issues of concern and, as such, concluded that their results are uninterpretable [58]. One methodological issue of concern was a statistical phenomenon called “overmatching” which was found in the Verstraeten et al. [56] and Price et al. [57] studies. Overmatching occurs when a matching variable is a significant predictor of exposure. This can artificially increase the chance that within-strata exposure is the same [59]. In addition, some of these studies examined cohorts with significantly different childhood vaccination schedules and with different diagnostic criteria for outcomes than those used in the US. Moreover, these studies used an insufficient length of time for the follow-up period. Follow-up period is a critical issue in all studies examining the relationship between exposures and the subsequent risk of a diagnosis of a neurodevelopmental disorder. Any follow-up period that fails to take into account the lag-time between birth and the subject's age of an initial diagnosis will not be able to observe the true relationship between exposure and outcome. Many other methodological issues of concern in these studies have been delineated. For a complete review of the methodological issues of concern in these aforementioned studies, please see Hooker et al. [58].

6.2. Attention deficit/hyperactivity disorder (ADD/ADHD)

Several studies, using several epidemiological methods in various databases, have also shown that Hg exposure from Thimerosal is a risk factor for ADD/ADHD diagnosis. For example, the aforementioned Young et al. [44] study observed that Hg exposure from Thimerosal in childhood vaccines given between birth and 7 months of age and between birth and 13 months of age was a significant dose-dependent risk factor for an ADD/ADHD diagnosis. Infants who received another 100 μg Hg from Thimerosal in childhood vaccines administered between birth and 7 months of age were found to have a significantly increased risk ratio (3.15) for diagnosed ADD/ADHD, and infants receiving another 100 μg Hg from Thimerosal in childhood vaccines administered between birth and 13 months of age, showed a significantly increased risk ratio (4.51) for diagnosed ADD/ADHD. Previously, Geier and Geier [60] conducted a cohort study in the VSD database to evaluate the relationship between increasing Hg exposure from Thimerosal in vaccines given within the first, second, third, and six months of life and the risk of an ADD diagnosis. That study reported a dose–response relationship between increasing cumulative Hg exposures from Thimerosal-containing vaccines administered within the first six months of life and the eventual risk of child receiving an ADD diagnosis.

Researchers have also examined the relationship between prenatal exposure to Hg from Thimerosal-containing Rh_oD immune globulin preparations and the risk of an ADD/ADHD diagnosis. The hypothesis was that if prenatal exposure to Rh_oD immune globulin preparation was a risk factor for an ADD/ADHD diagnosis then more children with an ASD diagnosis would have Rh-negative mothers compared to Rh-positive mothers [47]. Here, the epidemiological study found that maternal Rh-negativity in children diagnosed with ADD/ADHD in clinic A (26.3%) was significantly increased in comparison to the controls in both clinics (clinic A = 12.1%, B = 13.9%).

6.3. Tic disorder (TD)

A significant association between Hg exposure from Thimerosal-containing childhood vaccines and diagnosed TD has been found in six epidemiological studies [44,55,56,60–62]. These studies employed various epidemiological methods such as case–control or cohort designs, and were conducted on cohorts of children from several different countries. Some were US CDC-sponsored studies and others were studies conducted by independent investigators. In addition, several of these studies observed significant dose-dependent relationships between Hg exposure from Thimerosal in vaccines and the risk of diagnosed TD. A study by Young et al. [44], found a dose-dependent relationship between increasing Hg exposure from Thimerosal in vaccines given between birth and 7 months and also between birth and 13 months of age and the risk of a diagnosed TD. It was observed that, for a 100 μg Hg difference in exposure between birth and 7 months of age, the risk for diagnosed TD was significantly increased (3.39-fold). For the same

100 μg Hg difference in exposure between birth and 13 months of age, the risk for diagnosed tics was also found to be significantly increased (4.11-fold). In addition, Geier and Geier [60] conducted a cohort study in the VSD database to evaluate the relationship between increasing Hg exposure from Thimerosal in vaccines given within the first, second, third, and six months of life and the risk of TD diagnosis. That study found a dose–response relationship between increasing cumulative Hg exposures from Thimerosal in vaccines administered within the first three months of life and the risk of a TD diagnosis.

7. Specific developmental delays

A number of previous studies have observed that Hg administration from Thimerosal-containing childhood vaccines was a significant risk factor for specific delays in development [44–46,56,60]. For example Young et al. [44], observed a significant dose-dependent relationship between increasing Hg exposure from Thimerosal in childhood vaccines given from birth–7 months and birth–13 months and the risk of a diagnosis of specific delay in development. Specifically, these investigators observed a significantly increased rate ratio of 2.27 for diagnosed developmental disorder/learning disorder in infants who received another 100 μg Hg from Thimerosal in childhood vaccines from birth to 7 months of age. Furthermore, a significantly increased rate ratio of 2.91 for diagnosed developmental disorder/learning disorder was found in infants who received an additional 100 μg Hg from Thimerosal in childhood vaccines from birth to 13 months of age. Other investigators, using a cohort study design, examined the relationship between increasing Hg exposure from Thimerosal in childhood vaccines at 1-, 2-, 3-, and 6-months of age and the eventual risk of being diagnosed with specific delays in development [60]. A dose-dependent relationship between increasing cumulative Hg exposures from Thimerosal in vaccines was found to be associated with an increased risk of diagnosed unspecified developmental delay, language delay, and speech delay. As another example, investigators examined the association between being given three doses of Thimerosal in hepatitis B vaccine prior to 2000 and the risk of a child being diagnosed with a developmental disability from age 1–9 years, using the National Health Interview Survey (NHIS) 1999–2000 dataset [45]. Importantly, boys diagnosed with a development disability had a significantly greater odds ratio (9-fold) for receiving three doses of Thimerosal-containing hepatitis B vaccine in comparison to receiving no doses of Thimerosal-containing hepatitis B vaccine.

Previously, those investigators conducted a meta-analysis using statistical modeling to examine the association between additional doses of Hg from Thimerosal in childhood vaccines and neurodevelopmental disorder adverse event reports in the VAERS database [46]. This study found a statistically significant increased risk of speech disorder, mental retardation, personality disorder, thinking abnormality, and ataxia adverse event reports submitted to VAERS after the administration of additional doses of Hg from Thimerosal in vaccines.

Researchers have also examined the relationship between prenatal exposure to Hg from Thimerosal-containing Rh_oD immune globulin preparations and the risk of a neurodevelopmental disorder diagnosis (mentioned earlier) [47]. The researchers hypothesized that if prenatal exposure to Rh_oD immune globulin preparation was a risk factor for a neurodevelopmental disorder then more children with a neurodevelopmental disorder diagnosis would have Rh-negative mothers in comparison to children with Rh-positive mothers; and (2) if Thimerosal in the Rh_oD immune globulin preparations was the component associated with a neurodevelopmental disorder diagnosis, then the frequency of maternal Rh-negativity in children with a diagnosed neurodevelopmental disorder should be similar to control populations following the removal of Thimerosal from all manufactured Rh_oD immune globulin preparations from 2002 in the US. The researchers reported that children diagnosed with a neurodevelopmental disorder (clinic A = 24.2%) had a significant increase in maternal Rh-negativity in comparison to controls (clinic A = 12.1%, B = 13.9%). In addition, among children with a neurodevelopmental disorder diagnosis, born post-2001, a maternal Rh-negativity frequency (13.6%) comparable to that reported for the controls was observed [47].

8. Outcomes of acute ethylmercury poisoning in children

As discussed previously there have been numerous acute ethyl-Hg accidental poisonings of children around the world [63,64]. It was observed from these ethyl-Hg poisonings that adults have a higher survival rate than children [64] because children are more vulnerable to toxic metal poisoning [65]. For example, it was described in the 1960 Iraq ethyl-Hg poisoning episode that initially in February 1960, six members of a single family from Mhanawiya, Middle Euphrates district, were admitted to the Teaching Hospital, Baghdad, all with generalized weakness, tiredness, tremor, unsteadiness, dysarthria, and gastrointestinal symptoms. The family reported that 3 children, aged 6, 8, and 11 years-old, members of the same family, were stricken with the same symptoms and died at home before the rest of the family were admitted to the hospital [64]. Subsequently, investigators reported that, between February and April 1960, an estimated 1000 patients with ethyl-Hg poisoning were admitted to hospitals all over Iraq, and the predominant symptoms were those of the nervous system, although psychiatric disturbances and renal, cardiovascular, respiratory, and cutaneous manifestations were also present. Reportedly, about 200 patients died from the effects of poison [64]. As described by other investigators in more detail [66], numerous children between the ages of 7–18 were poisoned by ethyl-Hg. The symptoms of intoxication included gastrointestinal symptoms, ataxia, vision abnormalities, tremors, reflex abnormalities, and psychiatric symptoms. In an ethyl-Hg poisoning episode in Romania [34], the clinical outcomes showed that it was exceptionally toxic for the brain, spinal motoneurons, peripheral nerves, myocardium, and skeletal muscles. In China, forty-one people were poisoned by the ingestion of rice that had been treated with ethyl-Hg chloride [67]. It was reported that a significant number of children were impacted, and the consequences of exposure were dose-dependent (the lowest doses of ethyl-Hg exposure with adverse consequences were within about 10-fold of those administered to infants within the first 6 months of life as part of the routine childhood vaccine schedule in the US in the 1990s). In rural Ghana, 144 cases of ethyl-Hg poisoning were reported in 1974 [68]. The cases had eaten maize that was intended for sowing which was dressed with ethyl-Hg chloride. All showed clinical features typical of alkyl-Hg poisoning, and 20 individuals died. Children were more affected than adults in the poisoning, and symptoms in the children included speech disturbances, including complete lack of speech. Paralysis was seen, as well as mental issues and behavioral outbursts.

9. Biochemical insights into neuronal cellular damage

With a wide-range of human clinical and epidemiological studies revealing harmful consequences, especially neurological damage, in fetuses/infants/children from exposure to Thimerosal and its ethyl-Hg decomposition products, it is crucial to understand the biochemical basis for these compounds to induce cellular toxicity. As a result, human tissue culture studies are important to elucidate the biochemical mechanisms involved in cellular dysfunction and damage caused by Thimerosal and its ethyl-Hg decomposition product in humans, and also provide further insight into the specific uptake of these compounds within human cells following exposure.

Several studies have collectively shown that Thimerosal concentrations at nanomolar (nM) to low micromolar (μM) levels are acutely toxic to human neuronal cells in vitro [69–74]. For example, human neuroblastoma apoptosis has been reported to occur at levels as low as 25 nM [69]. Parran et al. [71] reported Thimerosal concentrations

for 50% cell death of SY5Y human neuroblastoma cells at 38.7 nM and 4.35 nM for 24- and 48-hour incubation periods, respectively.

Other investigators revealed that Thimerosal exposure significantly impacted the neuronal developmental process in vitro. For example, in a functional test based on the migration of human neural crest cells, investigators described an examination of developmental toxicants and signaling pathways [75]. These investigators described that impaired neural crest function is a known cause for teratologic effects and that testing a toxicant's effects should include testing the toxicant's effect on neural crest cells. After generating neural crest cells from human embryonic stem cells, and after establishing a migration assay of neural crest cells, the investigators tested environmental toxicants and inhibitors of physiological signal transduction pathways. These investigators observed that 1 nM concentrations of Thimerosal had a significant impact on their human neurodevelopmental model system, and when this value was compared to the lowest concentrations of other well-established neurodevelopmental toxins to induce a significant impact on their human neurodevelopmental model system, Thimerosal was more toxic than Pb acetate (about 1000-fold), methyl-Hg chloride (about 5-fold), $HgCl_2$ (about 50,000-fold), or valproic acid (about 1000-fold).

As another example, investigators evaluated the activation of methionine synthase by insulin-like growth factor-1 (IGF-1) as well as dopamine as a target for neurodevelopmental toxins [76]. These investigators described that methylation activity is important for the ability of growth factors to encourage normal development, and since neurodevelopmental toxins interrupt growth factor signaling, they may exert adverse effects on methylation. These investigators reported that in human neuroblastoma cells, IGF-1 and dopamine stimulated methionine synthase activity and folate-dependent methylation of phospholipids via a PI3-kinase- and MAP-kinase-dependent mechanism. Again, these investigators observed that a 10 nM concentration of Thimerosal completely eliminated IGF-1 and dopamine stimulated human neuroblastoma methionine synthase activity, and when this value was compared to the concentrations of other well-established neurodevelopmental toxins, Thimerosal was more toxic than $PbNO_3$, $HgCl_2$, ethanol, or CuCl.

Other investigators examined the ability of Thimerosal (at concentrations comparable to the levels in vaccines to infants) to induce an in vitro neurodevelopmental model system of the brain's pathological features observed in neurodevelopmental disorders such as ASD [70]. Overall, it was concluded that the effects caused by Thimerosal effects were consistent with the brain pathology found in ASD. For example, it was observed that the brain sample images from individuals with an ASD were virtually identical to the pathology found in the co-cultures of neuronal cells that were exposed to Hg. As another example, it was observed that the underlying neuronal cell damage caused by Thimerosal involved mitochondrial dysfunction and impaired oxidative-reduction. Importantly, studies have identified evidence for mitochondrial dysfunction and impaired oxidative-reduction in individuals with an ASD diagnosis [10].

A series of recent studies have also evaluated the vulnerability of individuals with an ASD diagnosis to the toxic adverse effects of Thimerosal. For example, investigators examined the cellular and mitochondrial glutathione redox imbalance in lymphoblastoid cells (LCLs) obtained from children with an ASD diagnosis [77]. These investigators reported that the redox ratio of reduced glutathione to oxidized glutathione was decreased. In addition, in the ASD individuals, the percentage of oxidized glutathione was increased in both cytosol and mitochondria. Moreover, Thimerosal exposure resulted in a greater decrease in the reduced glutathione to oxidized glutathione ratio and an increase in free radical generation, in the cells from the individuals with an ASD as compared to the controls. These investigators reported that LCLs from individuals with an ASD have a reduced glutathione reserve capacity in both cytosol and mitochondria that can limit antioxidant defense and detoxification capacity under pro-oxidant conditions.

In addition to this previous work, researchers recently examined the action of low levels, $\leq$1000 nM, of Thimerosal on immortalized B-cells taken from: (1) subjects diagnosed with an ASD, (2) their fraternal twins, a sibling, and (3) controls who were age/sex matched to the cases [78]. It involved eleven families and their matched controls. The effects of Thimerosal on cell proliferation and mitochondrial function from the B-lymphocytes were examined. In a subpopulation of eight individuals (4 diagnosed with an ASD, 2 twins, and 2 siblings) from four of the families, Thimerosal hypersensitivity was found; however, none of the matched control individuals showed this response. The cells that were hypersensitive to Thimerosal also had higher levels of oxidative stress markers, oxidant generation, and protein carbonyls. The study also found that mitochondria are the target organelle conferring Thimerosal sensitivity in the hypersensitive cells. Remarkably, the quantity of Thimerosal necessary to hinder cell proliferation in the cases was only 40% of that necessary to hinder cell proliferation in the matched controls.

In studies that have examined the distribution of Hg species within the body following Thimerosal-containing vaccine exposure, researchers have shown that ethyl-Hg species are transported across neuronal cellular membranes to the same degree as methyl-Hg species [79,80]. Moreover, both ethyl-Hg and methyl-Hg species were shown to be actively transported by the L-type neutral amino acid carrier transport (LAT) system into neuronal cells at the same rate [79]. Overall, the reported results were that ethyl-Hg partitions at a 5.6-fold greater concentration within a neuronal cell than outside, and within the cell, ethyl-Hg will partition by a factor of 1000-fold within the mitochondria [78].

10. Conclusion

Even though the research evidence regarding the toxicity of Thimerosal is, as seen in this review of the scientific literature from over eight decades of study, substantial, the issue on Thimerosal safety remains under debate. For example, the WHO's official statement [81] on the Thimerosal use in vaccines is “It is important to note that concerns about the toxicity of Thiomersal are theoretical and that there is no compelling scientific evidence of a safety problem related to its use in vaccines, although public perception of risk has been reported in some countries.” However, the culmination of the research that examines the effects of Thimerosal in humans indicates that it is a poison at minute levels with a plethora of deleterious consequences, and there is a clear cause for concern.

Conflict of interest

Six of the seven authors have been involved in vaccine/biologic litigation. One author has no conflict of interest.

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