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OVERVIEW

Integrating Experimental (In Vitro and In Vivo) Neurotoxicity Studies of Low-dose Thimerosal Relevant to Vaccines

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Abstract There is a need to interpret neurotoxic studies to help deal with uncertainties surrounding pregnant mothers, newborns and young children who must receive repeated doses of Thimerosal-containing vaccines (TCVs). This review integrates information derived from emerging experimental studies (in vitro and in vivo) of low-dose Thimerosal (sodium ethyl mercury thiosalicylate). Major databases (PubMed and Web-of-science) were searched for in vitro and in vivo experimental studies that addressed the effects of low-dose Thimerosal (or ethylmercury) on neural tissues and animal behaviour. Information extracted from studies indicates that: (a) activity of low doses of Thimerosal against isolated human and animal brain cells was found in all studies and is consistent with Hg neurotoxicity; (b) the neurotoxic effect of ethylmercury has not been studied with co-occurring adjuvant-Al in TCVs; (c) animal studies have shown that exposure to Thimerosal-Hg can lead to accumulation of inorganic Hg in brain, and that (d) doses relevant to TCV exposure possess the potential to affect human neuro-development. Thimerosal at concentrations relevant for infants' exposure (in vaccines) is toxic to cultured human-brain cells and to laboratory animals. The persisting use of TCV (in developing countries) is counterintuitive to global efforts to lower Hg exposure and to ban Hg in medical products; its continued use in TCV requires evaluation of a sufficiently nontoxic level of ethylmercury compatible with repeated exposure (co-occurring with adjuvant-Al) during early life.

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Introduction

The prevalence of emerging neuro-developmental disabilities has been directly linked to environmental neurotoxic substances which are estimated to affect 3% of children [1]; environmental mercury exposure, mainly methylmercury from seafood [1] and elemental mercury from coal combustion (used in electrical utilities) as well as municipal and medical waste incinerators [2], is at the center of concerns. However, a considerable part of these disabilities (25%) may arise as a result of interaction with individual genetic susceptibilities [1]. Indeed it is known that Hg neurotoxicity involves long latencies and atypical responses between low and high doses [3]; additionally, it has now been shown that exposure to different forms of mercury (such as methylmercury and Hg vapor) can act synergistically in increasing neurotoxic risks [3].

Organic and inorganic forms of mercury have a long history of use in medicine and pediatrics. Until the 1950s mercury preparations were part of the therapeutic resources to deal with common childhood ailments [4]. Because of its role in pink disease and also with the advent of more specific therapeutic drugs, mercury formulations have been withdrawn from children's medication [4]. Nevertheless, Thimerosal (sodium ethyl mercury thiosalicylate) has remained in wide use as a preservative in pharmaceutical products. Thimerosal in topical formulations has been eliminated in many parts of the world but its use in vaccines for pregnant women, newborns and young children continues in developing countries [5]. Although breast-fed infants can be exposed to elemental Hg from maternal

dental amalgam [6], outside the most developed countries, ethylmercury (EtHg), the metabolite of Thimerosal, remains the first exposure a vaccinated infant has to a potentially neurotoxic substance.

Thimerosal (which is 49% EtHg) is used as a preservative (at 0.01% of the formulation) in multidose vials of some vaccines. Thimerosal has been in use since the 1930s and it only became a toxicological issue in the early 2000s when public health professionals in the USA raised concerns about possible untoward effects caused by EtHg on newborns and infants. Thimerosal is known as a contact allergen, and caution has been urged regarding significant side effects in therapeutic agents [7] and in vaccines [8] with specific issues related to infant-CNS (central nervous system); however, its effects have been focused only relatively recently [9]. Indeed, these issues remain outside the scope of surveillance of post-license Thimerosal-containing vaccine (TCV) safety [10]. Post-vaccine adverse-effects that receive attention are restricted to extreme cases of reactogenicity (from components other than preservatives and adjuvants). Although there are neurologic adverse reactions related to vaccines, they do not capture long latencies compatible with low-dose Hg toxicity. Rare adverse neurologic reactions following vaccination include clinical syndromes such as encephalopathy, Guillain–Barre syndrome, meningo-encephalitis, poly-neuropathy, peripheral neuritis, per se or in combination [11]; these clinical syndromes can occur in association with vaccines (rabies, diphtheria-tetanus-polio, smallpox, measles, mumps, rubella, Japanese B encephalitis, pertussis, hepatitis B, and influenza) that may or may not contain Thimerosal. Furthermore, these reactions occur hours or within few weeks after vaccination [11] and are not compatible with low-dose exposure to mercury. However, recent increase in neuro-developmental disorders has been thoroughly discussed in relation to vaccines, addressing both immunologic and neurotoxic issues related to Thimerosal [12].

Environmental safety managers and public health professionals have attributed neurologic risks to Hg contamination and have successfully educated the public about the undesirable effects of exposure to it through fish consumption and dental amalgam; these concerns are now extended to populations living in developing countries where TCVs are largely used [13]. Such efforts have led to a general awareness of mercury in pharmaceuticals and, as a result of withdrawing Thimerosal from medicines, a deep-rooted concern has emerged regarding the presence of Hg in vaccines still in use for pregnant mothers, newborns, and infants. The WHO convened a group of experts that examined the complexities surrounding production and use of TCV [14]. The Organization's decision to uphold TCV-Hg safety was based on expert opinions when scientific information on low-dose effects of Thimerosal was limited.

Vaccine-Thimerosal exposure is an important pre- and post-natal neurotoxic stressor. In this regard, in vitro tests are useful to unravel mechanisms of specific effects caused by toxic substances while animal controlled experiments can extract information on exposure, dose, and related toxic outcomes. We still do not have an integrated overview of current knowledge that could serve as a tool to guide the decisions of pediatric and health professionals and help them to debate effectively the uncertainties posited by conventional toxicology on the safety of low-dose exposure to TCV.

Parental attitude towards perception of vaccine safety has changed over the last decade in some of the most developed countries. Freed et al. [15] have just reported that a "disturbingly high proportion of parents (25%)" believe vaccines can cause neurodevelopment problems, adding that current public health campaigns have not been effective. Parental-guidance reference books advise expecting mothers to avoid Hg exposure from sources that include TCV [16]. Meanwhile, there are demands for regulatory agencies to control residual Thimerosal in countries that are no longer using it in infant's vaccines [17]. There is a clear need to address uncertainties related to vaccine preservatives, and it centers on Thimerosal [18]. Therefore, this research focuses on the emerging experimental studies (in vitro and in vivo) that have addressed the effects of small doses of Thimerosal on neural cells and animal tissues and motor and behavioural functions. This review aims to integrate experimental (in vitro and in vivo) studies on the potential impact of Thimerosal in vaccines still liberally used in pregnant women and infants. Table 1 shows some of the neurotoxic mechanisms at cellular level, whereas Table 2 summarizes toxicokinetic and toxcodynamic information relevant to TCV-Hg.

In Vitro Tests

Although Thimerosal is the preservative of choice for multidose vaccine vials, it may not be the most effective. Thimerosal may fail to prevent short-term bacterial contamination [19] and it can also destabilize antigens [20, 21]. Geier et al. [22] tested several compounds routinely used in the US; they reported that the concentration of Thimerosal necessary to induce bacterial cell-death was higher than that actually found in the US products. Furthermore, the phenol-preserved vaccine showed less proteolytic activity than the Thimerosal-preserved one [23]. Such relative limitations are now coupled with experimental studies consistently showing neural-cell toxicity caused by Thimerosal at concentrations relevant to vaccines. Compared to other vaccine preservatives, Thimerosal showed a relatively higher toxicity (phenol < 2-phenoxyethanol < benzethonium chloride

Table 1 Summary of toxicity studies of low-dose Thimerosal (or ethylmercury) and aluminum in human and animal cultured-neural-cells

Reference	Species	Cell type	Compound	Dose	Measured outcomes
Geier et al. [22]	Human	Neuroblastoma (SH-SY-5Y)	Thimerosal compared to other vaccine preservatives	1 μM–10 μM	Relative toxicity: phenol < 2-phenoxyethanol < benzethonium chloride < Thimerosal
Geier et al. [33]	Human	Neuroblastoma (SH-SY-5Y), astrocytoma (1321N1); fetal (nontransformed) model systems	Thimerosal	10 nM–10 μM	Time-dependent mitochondrial damage; reduced oxidative–reduction activity; cellular degeneration; and cell death
James et al. [36]	Human	Lymphoblastoid derived from children with autism	Thimerosal	0.156–2.5 μM	Decreased the reduced glutathione/oxidized disulfide glutathione ratio and increased free radical generation in autism compared to control cells
Herdman et al. [32]	Human	Neuroblastoma SK-N-SH line	Thimerosal, compared to thiosalicylate	0–2.5 μM	Neurotoxicity occurs through the JNK-signaling pathway, independent of cJun activation, leading to apoptotic cell death
Parran et al. [34]	Human	Neuroblastoma (SH-SY5Y)	Thimerosal	1 nM–10 μM	Alter nerve growth factor signal transduction; causes cell death and elevated levels of fragmented DNA
Yel et al. [30]	Human	Neuroblastoma, CRL-2268	Thimerosal	0.025–5.0 μM	Neuronal cell death through the mitochondrial pathway (depolarization of mitochondria, generation of reactive oxygen species, release of cytochrome c and apoptosis-inducing factor)
James et al. [27]	Human	Neuroblastoma (SH-SY5Y CRL 2266) and glioblastoma (CRL 2020)	Thimerosal	15 μM	<50% decrease in intracellular glutathione levels in the glioblastoma cells but more than eightfold-decrease in the neuroblastoma cells
Humphrey et al. [31]	Human	Neuroblastoma, SK-N-SH line	Thimerosal	5 μM	Deleterious effects on the cytoarchitecture leading to mitochondrial-mediated apoptosis and oncosis/necrosis
Waly et al. [35]	Human	SH-SY5Y neuroblastoma	Thimerosal	1 nM	Inhibition of both IGF-1- and dopamine-stimulated methylation with an IC50 of 1 nM and eliminated methylating activity
Toimela and Tahti [42]	Human	SH-SY5Y neuroblastoma, U 373MG glioblastoma	Aluminum	0.01–1,000 μM	Al was effective in inducing apoptosis of glioblastoma
Baskin et al. [29]	Human	Cortical neurons	Thimerosal	1–250 μM	Changes in cell membrane permeability; induction of DNA breaks; apoptosis
Lawton et al. [39]	Mouse and rat	Respectively N2a neuroblastoma and C6 glioma cells	Thimerosal	1 μM	Inhibition of neurite process outgrowth in differentiating N2a and C6 cells

Table 1 continued

Reference	Species	Cell type	Compound	Dose	Measured outcomes
Ueha-Ishibashi et al. [26]	Rat	Cerebellar neurons	Thimerosal compared to methylmercury	0.3–10 μM	Increased the intracellular concentration of Ca2+ ([Ca2+]i); the potency of 10 μM thimerosal < methylmercury in decreasing the cellular content of glutathione in a concentration-dependent manner
Jin et al. [37]	Rat	Cultured sensory neurons	Thimerosal	0.3–300 μM	Altered cellular function by decreasing transient receptor potential V1 activity through oxidation of extracellular sulfhydryl residues
Song et al. [38]	Rat	Dorsal root ganglion	Thimerosal	100 μM	Inhibition of sodium channels in sensory neurons
Chanez et al. [24]	Rat	Brain homogenate, synaptosomes and myelin	Thimerosal compared to mercury chloride	50 μM	The toxicity, in terms of inhibition of Na+K+ATPase activity was greater with mercuric chloride than with thimerosal In myelin fraction. added serotonin increased inhibition caused by thimerosal
Wyrembek et al. [41]	Rat	Hippocampal neurons	Thimerosal compared to mercury cholride	1, 10, 100 μM	Complex interactions of thimerosal and mercuric ions with the GABA(A) and NMDA receptors
Minami et al. [28]	Mice	Cerebellum microglia C8-B4 cells, neuroblastoma, rat glioma cells	Thimerosal, ethylmercuric, Thiosalicylic acid	2.5 μM of solutions	Increased expression of MT-1 mRNA in mouse neuroblastoma after incubation with thimerosal; decreased MT-1 mRNA in C8-B4 cells after thiosalicylate addition; ethylmercury induced MT-1 mRNA expression
Rush et al. [25]	Mice	Primary cortical cultures (neuronal and glial cells)	Thimerosal and MeHg	0.1–5 μM	MeHg and thimerosal produced similar toxicity profiles, both causing approximately 40% neuronal death at 5 μM

Table 2 Animal studies of neurotoxic preservative-thimerosal and adjuvant-Al at doses relevant to vaccines

Reference	Species	Postnatal age	Dose of metal	Test	Measured effects
Blair et al. [49]*	Squirrel monkeys	Adult	Thimerosal: 2.2–12.0 μg/days intranasally for 6 months	Clinical signs, tissue Hg concentrations	Only the high dose had significantly higher levels in brain compared to controls, but no clinical signs of toxicity
Burbacher et al. [50]	Monkeys	Infant birth and 1, 2, and 3 weeks	TCV: 20 μg Hg/kg	Brain distribution of total and inorganic mercury	A higher percentage of the total Hg in the brain was in the form of inorganic Hg for the thimerosal-exposed monkeys (34 vs. 7%)
Hewitson et al. [74]	Rhesus macaque	Infants	USA vaccine schedule (1994–1999)	Volumetric analyses	Maturational changes over time in amygdale volume was different in exposed animals
Hewitson et al. [75]	Rhesus macaque	Infants	Hepatitis B vaccine at birth	Acquisition of neonatal reflexes and sensorimotor skills	Exposed animals showed a significant delay in the acquisition of three survival reflexes: root, snout and suck, compared with unexposed animals
Gassett et al. (1975–[52])*	Rabbits; rats	Pregnant; pregnant	Intraperitoneal injection of Thimerosal tagged with radioactive mercury	Autoradiography of different tissues	Thimerosal was found to cross the blood–brain and placenta barriers; accumulation of mercury was noted by histopathological and histochemical studies
Olczak et al. [73]	Suckling Wistar and Lewis rats	7, 9, 11 and 15 days	Thimerosal: 12–3,000 μg Hg/kg (Wistar) 54–1,080 μg Hg/kg (Lewis)	Pain sensitivity using the hot plate test and tissue Hg accumulation	Impairs sensitivity to pain, apparently due to activation the endogenous opioid system. Hg from thimerosal accumulates in the rat brain in significant amounts. Wistar rats were more sensitive to this effect than Lewis rats
Olczak et al. [69]	Suckling Wistar rats	7, 9, 11 and 15 days	Thimerosal: 12–3,000 μg Hg/kg	Analysis of brain regions rich in opioid receptors	A dose dependent increase in Mu-opioid-receptor densities in the periaqueductal gray and caudate putamen, but a decrease in the dentate gyrus, with the presence of degenerating neurons and loss of synaptic vesicle marker (synaptophysin)
Olczak et al. [70]	Suckling Wistar rats	7, 9, 11 and 15 days	Thimerosal: 12 and 240 μg Hg/kg	Neuro-pathological and morphological alterations in brain tissues	"Ischaemic degeneration of neurons and 'dark' neurons in the prefrontal and temporal cortex, the hippocampus and the cerebellum, pathological changes of the blood vessels in the temporal cortex, diminished synaptophysin reaction in the hippocampus, atrophy of astroglia in the hippocampus and cerebellum, and positive caspase-3 reaction in Bergmann astroglia"
Orct et al. [55]	Suckling rats	7, 9 and 11 days	Thimerosal: 0.81 μM Hg/kg	Tissue Hg concentrations	Brain and blood concentrations of mercury were higher in the thiomersal exposed compared to inorganic Hg group
Minami et al. [54]	Mouse	42 weeks	Thimerosal: 12 μg/kg	MT-1 mRNA expression	MT-1 and MT-3 mRNAs but not MT-2 mRNA are expressed in the cerebellum rather than in the cerebrum

Table 2 continued

Reference	Species	Postnatal age	Dose of metal	Test	Measured effects
Minami et al. [53]	Mouse	35 weeks	Thimerosal: 60 μgHg/kg	Hg contents in the cerebrum	Increased tissue-Hg after damage to the blood–brain-barrier
Hornig et al. [71]	Mice (SJL/J)	7, 9, 11 and 15 days	Thimerosal: 5.6–14.2 μg EtHg/kg	Autoimmune propensity to influence neuro-behavioral outcomes	Growth delay; reduced locomotion; exaggerated response to novelty; and densely packed, hyperchromic hippocampal neurons with altered glutamate receptors and transporters
Berman et al. [72]	Mice (SJL/J)	7, 9, 11, and 15 days	Thimerosal: 5.6–14.2 μg Hg/kg	Behavioral tests selected to assess domains relevant to core déficits	The majority of behaviors were unaffected by thimerosal injection; female mice showed increased time in the margin of an open field at 4 weeks of age
Petrik et al. [66]	Mice	3 months	Aluminium hydroxide (30–34 μg/kg), commercial squalene	Behavioral testing and motor deficits	Al treated group expressed a progressive decrease in strength measured by the wire-mesh hang test (final deficit at 24 weeks; about 50%)
Shaw and Petrik [68]	Mice	3 months	Aluminium: 30–34 μg/kg	Motor and cognitive behaviours	Aluminum-treated mice showed significantly increased apoptosis of motor neurons and increases in reactive astrocytes and microglial proliferation within the spinal cord and cortex
Hunter et al. [81]	Neuroglin-deficient *C. elegans*	Young adults	Thimerosal: 91 nM in incubating plates	Sensory processing and oxidative stress	Hypersensitive to oxidative stress and heavy metal toxicity

* Thimerosal modeled intranasal and intraocular medication, not vaccines

< Thimerosal) in human neuroblastoma [22]; however, compared with other mercuric compounds, Thimerosal was shown to be less toxic than mercury chloride [24] but, depending on the parameter tested, it was similarly [25] or less toxic than MeHg [26]. Thimerosal has shown more neurotoxicity towards neuroblastoma than glioblastoma cells [27]. However, Minami et al. [28] showed that Thimerosal and its metabolites can express metallothionein mRNAs in mouse cerebellum microglia cells; cell viability depended on the metabolite tested (Thimerosal, thiosalicylate, and ethyl mercury), dose and incubation time.

Studies showing the toxic effects of low (nano- and micromolar) Thimerosal concentrations in human and animal cell-cultures are summarized in Table 1. Thimerosal concentrations ranging from 0.16 to 10 μM cause cell death in cultured human cortical neurons [29], neuroblastoma [30–33], astrocytoma, and in a foetal non-transformed system [33]. In some studies, cytotoxicity was present at concentrations lower than those found in TCV [29, 34, 35]. Indeed, cultured lymphoblastoid cells derived from autistic children and unaffected controls were studied by James et al. [36]; they found that exposure to Thimerosal resulted in a greater decrease in the glutathione and oxidized disulfide glutathione ratio and an increase in free radical generation in autism-derived cells than in control cells [36].

Other aspects of the neuropathology of Thimerosal-Hg toxicity have also been revealed by animal-cell studies of cerebellar [26], sensory neurons [37, 38], dorsal root ganglion [38], neuroblastoma and glioma [39], and microglia tissues [28]. Additionally, Zieminska et al. [40] recently demonstrated in cerebellar cell-cultures a neuroprotection mechanism against Thimerosal toxicity that is modulated by sulphur-containing compounds. The excitatory and inhibitory neurotransmiter systems has been studied by electrophysiological recordings of cultured hippocampal neurons from rats; Wyrembek et al. [41] reported that there was a significant decrease in NMDA-induced currents and GABAergic currents following exposure for 60–90 min to 1 or 10 μM Thimerosal. However, after brief (3–10 min) exposure to Thimerosal at concentrations up to 100 μM no significant effects were noted. However, it was noticed that Thimerosal was also neurotoxic, damaging a significant proportion of neurons after 60–90 min exposure in the healthiest looking neurons [41].

Although TCVs are mostly used in developing countries, both TCVs and Thimerosal-free vaccines are adjuvanted with Al salts (aluminum phosphate and aluminum hydroxide), which are also neurotoxic. Despite the scarcity of comparative studies, it seems that Thimerosal is more toxic to human neuroblastoma and glioblastoma cells than adjuvant-Al [42]. Indeed Geier et al. [33] reported that Thimerosal toxicity (as measured by mitochondrial dysfunction) was higher than that of Al sulphate. Waly et al. [35] reported that aluminium inhibited insulin-like growth factor-1-stimulated phospholipid methylation in human neuroblastoma cells. Campbell et al. [43] speculated that glial cells are the main neurotoxic target of Al; then, after compromising these cells, there could be a secondary impact on the neuronal population. It should be noted, however, that the effects of both TCV-Hg and Adjuvant-Al (as a binary mixture) have not yet been studied.

Animal Models

A full TCV schedule can expose newborns and infants to acute doses of Hg above the maximum limit recommended by the WHO [5]. Indeed, Redwood et al. [44] estimated the total Hg exposure from multidose-vial vaccines based on the U.S. Centers for Disease Control and Prevention recommended schedule; cumulative Hg at six and 18 months were 187.5 and 237.5 μg, respectively [44]. However, a combination of vaccines in one shot or single visit can cause even higher EtHg exposures. Additionally, before birth and depending on the country, immunizing pregnant mothers with TCVs exposes foetuses to EtHg. Regardless of pregnancy stage, perinatal CNS-maturity or body weight, each dose of a TCV exposes a foetus or a young infant to a fixed (non-adjusted) dose of EtHg (and adjuvant-Al). To complicate matters, considering countries per se, not all vaccination schedules are alike, which adds further complexity to animal models. In some countries, including Brazil, pregnant mothers can be immunized with TCVs against tetanus, hepatitis B, and seasonal flu. Because of the recent H1N1 pandemic, specific vaccination of pregnant mothers with this TCV can add even more EtHg to the foetus.

If one considers EU countries as examples of hepatitis B immunization, some vaccinate only infants who have at-risk mothers, while others vaccinate all infants at the age of 2 months [5]. In the USA and many countries around the world hepatitis B vaccines are given at birth. These differences in exposure time (and attendant dose) are nearly impossible to model. In Table 2 the earliest exposure time in mice was equivalent to 2 months (of infant's age), which in no way reflects the neonate hepatitis B vaccine per se or after maternal vaccination during pregnancy. Furthermore, the few animal studies of adjuvant-Al have been done as a single exposure, not as a binary mixture with Thimerosal as it normally occurs in TCVs. Therefore, animal studies summarized in Table 2 can only capture part of the complex exposure to vaccine neurotoxic preservative-Hg and adjuvant-Al during early life.

Tissue-Hg Concentrations and Biomarkers

We have learned that mercury toxicity is modulated by many factors, including mercury chemical forms, brain-mercury concentrations, nutritional cofactors as well as numerous genetic polymorphisms [45, 46]. Binding of Hg to sulphur-containing molecules [40] and to blood cells modulates the toxicokinetics (and toxicodynamics); a stronger binding of Hg to blood cells retards its diffusion for brain uptake or faecal elimination [47]. Indeed the ability to excrete inorganic mercury is lacking or diminished in the suckling animal [48].

Neural-tissue concentrations of toxic metals in TCVs have been studied across animal species: monkeys, rats, mice, zebra-fish, and nematodes (Table 2); these studies have shown a highly localized affinity of Thimerosal for neural tissues and impaired sensory functions. The monkey studies that measured brain-Hg after Thimerosal exposure showed differences in brain Hg accumulation between infant and adult animals. The blood–brain barrier of adult monkeys showed more functional efficiency towards Thimerosal than that of infant monkeys [49]. However, when comparing organic forms of Hg (MeHg and EtHg) in infant monkeys, there was significantly more inorganic Hg in the brain of infants exposed to TCV [50]; nevertheless, EtHg was found primarily in the kidneys. After inorganic-Hg enters the brain it has the potential to accumulate because of its longer half-life [51].

Biomarkers related to CNS integrity in relation to Thimerosal have been studied across animal species. Depending on the organic mercury form, the brain-to-blood ratio is highest for primates and lowest for rats [47]. Early studies had shown that Thimerosal can penetrate the blood–brain barrier [52]. Depending on the integrity of the blood–brain barrier Thimerosal-Hg can penetrate the mouse cerebrum relatively quickly [53] and express metallothionein messenger RNA even at low concentrations [54]. Observations of mercury retention in mice brain have also been reported at low [53, 55] and high doses of Thimerosal [56, 57]. The mice model is both strain [58] and gender sensitive to Thimerosal-Hg [58, 59]. In this species, Thimerosal-Hg also remained unchanged in the brain while levels decreased in the blood after intramuscular injections; however, when compared to methylmercury, there was proportionally less Hg (derived from EtHg and Thimerosal) in brain tissue [60].

Zareba et al. [56] showed that mice grafted with human tissue incorporate EtHg into growing hair in a similar manner to methyl-mercury. Indeed, EtHg has been found in the hair of nursing staff resulting from occupational exposure [61] and it can be measured in hair of post-vaccinated infants [62].

It is recognized that brain mercury may also increase the pathological influence of other neurotoxic metals [63]. It is worth noting that most TCVs are also adjuvanted with aluminium compounds [64]. Aluminium is a neurotoxic element of significance for infants' exposure [64, 65] but the binary mixture in TCVs has not yet been fully addressed. Nevertheless, it is worth mentioning that the brain of adult mice can accumulate substantial amounts of Al derived from vaccines [66]. Flarend et al. [67] have shown a difference in metabolism between Al species (oxide and phosphate forms) in adjuvants; although the brain accumulated less of the radio-labelled-Al, the phosphate form was retained in proportionately larger amounts than the oxide. In adult mice, adjuvant-Al showed apoptotic neurons and increased activated caspase-3 labelling in lumbar spinal cord and primary motor cortex [66]; indeed, adjuvant-Al provoked significant impairments in motor functions and diminished spatial memory capacity [68]. In light of these findings, we are left with pressing questions related to the binary (and frequently combined) serial exposure of Thimerosal-Hg and adjuvant-Al.

Neurobehavioural Outcomes

The occurrence of various CNS toxicity outcomes of in vitro studies (Table 1) as well as lasting neuropathological changes in animal brains (Table 2) can result in losses of neural functions, such as learning and sensory impairments. Recently, Olczak et al. [69] showed a dose dependent increase in rat mu-opioid receptors. This research group also showed lasting neuropathological changes in rat brain after intermittent neonatal administration of Thimerosal [70]; their findings documented "ischaemic degeneration of neurons and 'dark' neurons in the prefrontal and temporal cortex, the hippocampus and the cerebellum, pathological changes of the blood vessels in the temporal cortex, diminished synaptophysin reaction in the hippocampus, atrophy of astroglia in the hippocampus and cerebellum, and positive caspase-3 reaction in Bergmann astroglia."

The neurobehavioural effects of vaccine-Thimerosal (and aduvant-Al) on infant animal models (Table 2) are very limited: two monkeys and four rodent studies (two in mice and two in rats). When tested for performance in behavioural domains, mice exposed to Thimerosal may [71] or may not be significantly affected [72]. Rats treated with Thimerosal doses equivalent to those expected for infants showed significantly elevated pain (latency for paw licking, jumping) threshold on a hot plate [73]. A recent study by Hewitson et al. [74] reported maturational changes in infant rhesus monkeys that were submitted to a vaccine schedule. In a previous paper, the same group showed several adverse neurodevelopmental outcomes from neonatal TCVs; animals exposed to hepatitis B vaccine (preserved with Thimerosal) had a significant delay in

the acquisition of three survival reflexes: *root, snout and suck* when compared with unexposed animals [75].

As a result of increased awareness about MeHg exposure, which has only recently been extended to EtHg, neurobehavioural studies of vaccine-Thimerosal exposure in children are emerging. Collectively, population studies summarized elsewhere [76] addressing TCV and the risks of subtle/mild neurodevelopment outcomes (explicitly excluding autism) suggest that the risk of TCV-Hg effects on the CNS are not dismissed. Regarding combined exposure of preservative-Hg and adjuvant-Al during pregnancy, a relatively small set of breastfed infants ($n = 82$) showed neurodevelopmental sensitivity at 6 months of age [77]; however, perinatal and postnatal neurodevelopmental delays associated with TCV-Hg were overcome at 5 years [78].

Overview and Research Interpretation

The most critical CNS developmental window of vulnerability to neurotoxic substances extends from foetal stages until 6 months of age. Pregnant mothers and infants around the world are currently immunized with TCVs. While expecting mothers are routinely immunized with TCVs, after birth the infant is subjected to repeated loads of TCV-Hg. This cumulative exposure to Thimerosal is a likely risk factor for neurodevelopmental delays that has yet to be defined. Because of the wide variation in infant development at the time of immunization, cell-culture and animal experiments (Tables 1, 2) cannot model the full complexity of variable interactions related to time of dosing (cumulative pre- and post-natal exposure) and neurodevelopment of young human infants. Additionally, subtle neurodevelopment delays in susceptible infants (as measured in most tests) are multifactorial in origin and may not be perceived in routine medical examinations.

Clements [10] discussed issues related to Thimerosal safety for vaccines used in developing countries. Thimerosal-safety issues did not include pregnant women ("Thiomersal is a safe preservative to use in vaccines administered to infants, children and non-pregnant adults"). Furthermore, in Clements's [10] discussion there were clear uncertainties related to premature and low-birth-weight newborns. Even in born-at-term babies, a 1-day-old still carries most of its vulnerable foetal characteristics; depending on gestational age (37–42 weeks) or stage of foetal maturity newborns have a wide range of organ development, biochemical and physiological functions. Because the concentrations of preservative-Hg are constant, extreme difference in birth weight exposes babies to an attendant wide range of exposure to Thimerosal [79]; this causes a disproportionate EtHg (combined with adjuvant-Al) exposure (per unit of body mass) compared to adults, children, and even older infants.

Both in vitro (human cells) and animal studies (Tables 1, 2) provide unequivocal evidence that low doses of Thimerosal relevant to vaccines can affect neural tissues and functions and that the pathophysiological processes can be understood through pathways and doses already known to occur with MeHg. These cell-based assays (Table 1) captured relevant information on pathway perturbations caused by Thimerosal (and EtHg) that were compatible with the results experimentally observed in vivo (Table 2).

Different outcomes of neural cell challenges with Thimerosal imply different hazards in terms of animal neurodevelopment; animal models did differentiate some of these complex outcomes which have implications for translating such results to risks (or risk severity for vulnerable subgroups) of suboptimal neurodevelopment of human infants. Indeed, Judson et al. [80] showed that a statistically significant inverse association exists between the number of pathways perturbed by a chemical at low in vitro concentrations and the lowest in vivo dose at which a chemical causes toxicity. Therefore, concurrent with the conventional thinking of neurodevelopmental toxicology, early exposure to Hg is detrimental to the CNS, and the increasing pattern of TCV-Hg exposure during pregnancy and infancy has the potential to contribute to an elevated risk of neurotoxicity.

Concluding Remarks

- Without vaccination it would be impossible to eradicate or control infectious disease that otherwise would be devastating to children, causing unnecessary suffering and waste of human and material resources. However, the use of thimerosal in vaccines should be reconsidered by public health authorities, especially in those vaccines intended for pregnant women and children.
- In vitro and animal studies have shown consistently that low dose of Thimerosal (or ethylmercury) is active against brain cells. Animal studies with Thimerosal at concentrations used in vaccines have demonstrated toxicity compatible with low-dose Hg exposure. Thus, from observed changes in animal behaviour it is reasonable to expect biological consequences in terms of neurodevelopment in susceptible infants.
- Despite demonstrable toxicity of EtHg, TCV are still used in large scale in developing countries; however, because of global actions to reduce Hg exposure we need to extend such concerns to pregnant women, newborns, and young children still receiving TCV.
- We cannot compare the risk of tangible deadly diseases (preventable by immunization) with plausible neurodevelopment delays (clinically undefined) which can be

transient and mostly unperceived in the majority of children (as a result of low-dose of Thimerosal). Nevertheless, we know for sure that Thimerosal-Hg (and Al as a binary mixture) in the child's brain is an issue of concern, and that an ever increasing pattern of exposure (from vaccine schedule) deserves special attention.

- We urgently need studies that address TCV-EtHg exposure in pregnant mothers, neonates, and young children of less developed nations where immunization programs are most needed and where confounding factors related to endemic undernutrition and co-exposure to intestinal parasites and other toxic substances are more prevalent.
- The persisting use of TCV (in developing countries) is counterintuitive to global efforts to lower Hg exposure and to ban Hg in medical products; its continued use in TCV requires evaluation of a sufficiently nontoxic level of ethylmercury compatible with repeated exposure (co-occurring with adjuvant-Al) during early life.

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