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## Research Article

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

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Due to the facts that thiomersal-containing vaccine is still in use in many developing countries, and all forms of mercury have recognised neurotoxic, nephrotoxic, and other toxic effects, studies on disposition of ethylmercury and other mercury forms are still justified, especially at young age. Our investigation aimed at comparing mercury distribution and rate of excretion in the early period of life following exposure to either thiomersal (TM) or mercuric chloride ( $\text{HgCl}_2$ ) in suckling rats. Three experimental groups were studied: control, TM, and  $\text{HgCl}_2$ , with 12 to 18 pups in each. Both forms of mercury were administered subcutaneously in equimolar quantities ( $0.81 \mu\text{mol/kg b.w.}$ ) three times during the suckling period (on the days of birth 7, 9, and 11) to mimic the vaccination regimen in infants. After the last administration of TM or  $\text{HgCl}_2$ , total mercury retention and excretion was assessed during following six days. In TM-exposed group mercury retention was higher in the brain, enteral excretion was similar, and urinary excretion was much lower compared to  $\text{HgCl}_2$ -exposed sucklings. More research is still needed to elucidate all aspects of toxicokinetics and most harmful neurotoxic potential of various forms of mercury, especially in the earliest period of life.

## 1. Introduction

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

The molecule of thiomersal is sodium ethylmercury-thiosalicylate that dissociates to ethylmercury and thiosalicylate [7]. Ethylmercury is acting as a preservative against bacterial and fungal contamination of the vaccines that are repeatedly given to infants (Diphtheria-Tetanus-acellular-Pertussis vaccine, 3 to 7 times) up to 6 months of age. A potential threat of neurodevelopmental toxic effect of

mercury lies in the fact that the exposure occurs in the most vulnerable period of life, when the brain is developing and growing [8]. Organic forms of mercury are more easily absorbed when ingested and are less readily eliminated from the body than its inorganic forms [1].

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

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

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

## 2. Material and Methods

**2.1. Experimental Animals.** Experimental rats (Wistar strain reared in the Laboratory Animal Unit of the Institute for Medical Research and Occupational Health in Zagreb, Croatia) were supplied with feed for small laboratory animals (Mucedola, Milano, Italy) and tap water *ad libitum*. Animal facility was kept under constant indoor conditions (20–22°C, constant humidity of 40%, and 12 h light/dark cycles). Animal cages were provided by sterilised pine shaving beddings. Two weeks after mating (in ratio male:female 1:3 over a week), pregnant rats were placed into small individual polycarbonate cages (20.7 × 26.5 × 14.0 cm, Ehret, Germany) where they gave birth and reared the litters. Six mother rats with the litters that delivered on the same day were designated for the experiment. The litters were normalized

to eight male pups per litter one day after birth. Pups' body weights were recorded every morning throughout the experiment. All procedures with animals were carried out in accordance with national Law on the Protection of Animal Welfare. The experimental protocol was approved by the Institute's Bioethical Committee and was conducted within the framework of the research project granted by the Croatian Ministry of Science, Education and Sports. The implementation of the protocol was officially permitted by the Veterinary Administration of the Croatian Ministry of Agriculture, Forestry and Water Management.

**2.2. Experimental Design.** The pups were assigned into three experimental groups: Control, Thiomersal, and  $\text{HgCl}_2$  with two to three pups per group within each litter, with total number of pups per group 12, 18, and 18. The experiment started at pups' age of seven days by subcutaneous injection of deionised water, thiomersal (ethylmercurythiosalicylic acid sodium salt, SERVA Electrophoresis, Germany), or mercuric chloride ( $\text{HgCl}_2$  *p.a.*, Kemika, Croatia), depending on the assignment to the experimental group. The dose of administered mercury in both forms was equimolar, that is, 0.81  $\mu\text{mol/kg}$  b.w. and was given in the volume of 0.05 mL. Compounds were dissolved in deionised water and freshly prepared each time before injection. The dose of mercury used in the experiment was calculated to be 10% of  $\text{LD}_{50}$  dose based on a previous finding for mercuric chloride in suckling rats provided by our Unit. Subcutaneous administration of both mercury forms was repeated three times; first time on the day of birth 7 (as described above), second time on day 9, and third time on the day of birth 11. The morning after the last parenteral exposure of either mercury form, from day of birth 12 through 17, during six consecutive days, we euthanized all pups from one litter of each experimental group after intraperitoneal administration of the combination of anaesthetics Narketan plus Xylapan (Vetoquinol AG, Switzerland) in doses 0.8 plus 0.6 mL/kg b.w. We then sampled the blood, selected organs, urine, and gut with its content for mercury analysis. Blood was collected from the heart in heparinised syringes. Urine was collected from the urinary bladder with a syringe immediately after opening the abdominal cavity. The brain, kidneys, liver, and entire small and large intestine were dissected after exsanguination from the abdominal aorta.

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

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

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

### 3. Results

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

The concentrations in whole blood and urine and mass fractions of mercury in the selected organs of two mercury-exposed groups are presented graphically to show the differences and daily course during six-day collection of samples (Figure 1). In all sampling points, concentrations and mass fractions of total mercury in urine and kidney in  $\text{HgCl}_2$ -exposed group were significantly higher than in TM-exposed group. In the liver and in both small and large intestine, total mercury values were lower, although not always statistically significant. However, total mercury in TM-exposed group was significantly higher both in blood and in brain than in  $\text{HgCl}_2$ -exposed group. During six-day period of sampling, only whole blood showed a decline with time in both mercury-exposed groups (Figure 1).

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

### 4. Discussion

We investigated disposition of two different forms of mercury other than methylmercury during the critical period of brain maturation process, which occurs in rats during early postnatal period up to age of 3-4 weeks after birth [8]. Our

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

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

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

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

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

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

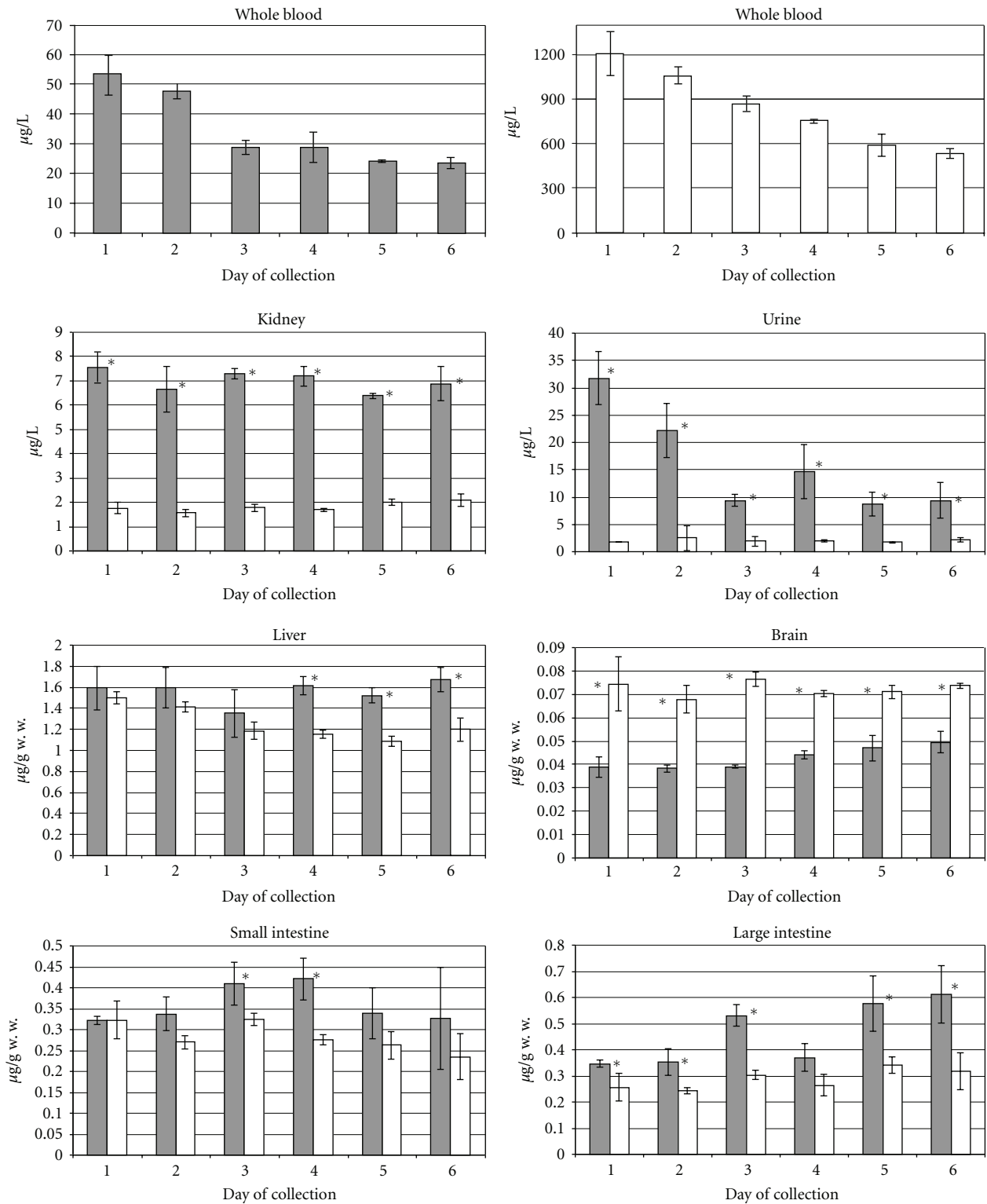


FIGURE 1: Concentrations in whole blood and urine and mass fractions of mercury in organs (kidneys, liver, brain, small intestine, and large intestine) of suckling rats exposed to mercuric chloride ( $\text{HgCl}_2$ ; grey bars) or thiomersal (ethylmercury; white bars). Data are presented within six days after administration of either form of mercury as means  $\pm$  SD; \*statistically significant difference between exposed groups (at  $P < 0.05$ ).

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

Study group	Samples	Mean	Std. dev.	N	P value	Pearson's <i>r</i>
HgCl <sub>2</sub>	Whole blood	33.2	11.0	18	0.000	0.899
Thiomersal		831	257	18		
HgCl <sub>2</sub>	Urine	15.9	9.10	18	0.742	0.089
Thiomersal		2.06	0.95	18		
HgCl <sub>2</sub>	Liver	1546	172	18	0.77	0.079
Thiomersal		1251	158	18		
HgCl <sub>2</sub>	Kidneys	6958	589.6	18	0.746	-0.088
Thiomersal		1818	253.5	18		
HgCl <sub>2</sub>	Small intestine	369.9	70.76	18	0.51	0.178
Thiomersal		276.9	42.43	18		
HgCl <sub>2</sub>	Large intestine	471.7	134.4	18	0.026	0.554
Thiomersal		284.5	48.72	18		
HgCl <sub>2</sub>	Brain	42.7	5.69	18	0.444	0.206
Thiomersal		72.0	5.48	18		

N = Number of rats.

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

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

In conclusion, although analytical methods that we used did not allow discerning between different mercury species,

our experimental design showed that parenterally administered mercury in the form of thiomersal during the suckling period underwent different distribution, retention, and elimination compared to inorganic mercury given under same experimental conditions. In the case of thiomersal exposure, mercury retention is evidently higher in the brain, its urinary excretion is much lower, and enteral excretion is similar to that of inorganic mercury. Our results contribute to the evidence on mercury disposition in the early period of life, comparing in a simple original experimental design the distribution and retention in the brain and other tissues, and elimination of two types of mercury: thiomersal and mercuric chloride mercury. Both mercury forms are present in real life, including the most vulnerable period of growth and development. Our findings are in line with the overall conclusion reached so far in the research initiatives in this area that more work is still needed to elucidate especially neurodevelopmental toxic potential of various forms of mercury and their fate in body in the earliest period of human life.

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