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Early Exposure to Mercuric Chloride or Methylmercury Alters Zebrafish Embryo (Danio rerio) Development

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Abstract

Environmental mercury contamination is ubiquitous and toxic to developing embryos. Zebra fish embryos (ZFEs) were exposed for 24 h to different concentrations of mercuric chloride or methyl mercury. Exposure to 100 µg/L methyl mercury and 1000 µg/L mercuric chloride resulted in 100% mortality. ZFEs exposed to 100 µg/L or higher mercuric chloride exhibited decreased body length, deformed tails and reduced eye volume. Using Proliferating Cell Nuclear Antigen (PCNA) immunohistochemistry assessment at 30 h post fertilization, we determined that exposure to mercuric chloride did not alter cell proliferation in the developing brain. These ZFE data were compared to results obtained with methyl mercury exposure; both compounds produced delayed toxicity and methyl mercury is more toxic than mercuric chloride at equal concentrations.

Keywords: Mercuric chloride; Delayed toxicity; Early development; Morphology; Zebra fish embryo; PCNA

Introduction

Mercury is a widespread environmental toxicant found in freshwater and ocean fish, shellfish and other foods [1-3]. Both natural (30%) and manmade (70%) sources are responsible for mercury that is found in the environment [4-5].

Mercury usually exists in one of three major forms, each with different bio-availabilities and toxicities: 1) the metal or elemental form; 2) as an inorganic salt (mercurous mercury (Hg1+ or mercuric mercury (Hg2+)); and 3) several possible organic compounds, including methyl mercury, methyl mercury, and phenylmercury [6]. Both inorganic and organic forms of mercury impair normal function of multiple tissues, including the kidney, gastrointestinal tract, heart and especially the central nervous system [7-14]. Once elemental mercury is airborne (either from burning fossil fuels or from volcanic eruptions) it can be carried over wide distances and deposited into soil and water. Elemental mercury is transformed primarily by bacteria into methyl mercury (Guimaraes et al. [15]), which is biomagnified, particularly in the food chain found in the oceans, and reaches highest concentrations in predatory ocean fish such as swordfish, tuna and shark [16-18]. Most human exposure to methyl mercury results from consuming contaminated fish. However, contamination of other foods with various forms of mercury including Rice [19], have been reported.

A confounding factor studying mercury toxicity is the occurrence of delayed signs of toxicity that can occur with developmental exposure to methyl mercury [19]. This delay in clinical signs of mercury toxicity, while well known, is not well understood. One hypothesis states that when methyl mercury is sequestered into CNS tissues, it is slowly converted to inorganic forms of mercury and the inorganic form of mercury causes the delayed neurologic signs. However, this theory has yet to be documented with substantial experimental evidence.

Exposure to mercury is still a serious concern in our environment today [20]. Warnings have been issued by the Environmental Protection Agency, Food and Drug Administration and National Academy of Science/National Research Council to pregnant women and women considering to become pregnant advising them to avoid eating significant amounts high-risk fish such as tuna. Most fish found in US waters contain less than 0.5 ppm methyl mercury, but large predatory fish can contain more than 1 ppm mercury. Although most health concerns are associated with gestational exposure, it also is possible that infants and children can be exposed postnatally to methyl mercury due to contaminated breast milk, should lactating mothers consume foods containing high levels of methyl mercury [21].

It has been reported that autism spectrum disorders may affect as many as 1 in 68 children (Centers for Disease Control, 2014). While the association of autism and exposure to mercury compounds pre- and postnatally is controversial (Van Wijngaarden et al. [22]), there are reports that a combination of genetic and biochemical susceptibilities including reduced ability to excrete mercury and/or increased environmental exposure at important developmental time points have been associated with some cases of autism [23-24]. Thus, developing embryos and fetuses exposed to relatively high levels of mercury could be adversely affected even if they eliminate mercury at normal levels. However, if the developing embryo/fetus or the pregnant mother were genetically or biochemically unable to eliminate mercury effectively, then exposure to lower levels of mercury also could be detrimental to the developing fetus.

Non-mammalian models that are used to study toxicity such as *C. elegans* (Martinez-Finley et al. [25]) and Zebrafish (Danio rerio) (Carvalho et al. [26]) are becoming more commonly used to study mechanisms of toxicity and as alternatives to mammalian animal testing. Some advantages of using zebrafish embryos (ZFEs) in toxicity assessment include: a transparent "shell" or chorion that encloses the developing ZFEs during early development; embryos whose tissues also are transparent early in development; rapid ex utero development; and the ability to directly and accurately deliver chemicals of interest to developing ZFEs [27-29]. Zebrafish genetics also are well-documented [30-32].

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Numerous studies have been published that examine effects of methyl mercury on developing mammalian embryos [33-36]. However, many studies have examined the effects of exposure to only relatively high levels of methyl mercury [37-41]. On the other hand, most human exposure takes place at low to moderate methyl mercury exposure levels. Only a few studies are available concerning methyl mercury neurotoxicity at low exposure doses, and even with respect to these few studies, scientists do not agree on the interpretations [2,42-43]. In this study we assessed toxicity of inorganic mercury (mercuric chloride) using a zebrafish (Danio rerio) embryo assay and compared results of exposure to mercuric chloride with exposure to methyl mercury, an organic form of mercury. ZFEs were exposed to different concentrations of mercuric chloride or methyl mercury for 24 h (6-30 hpf) and subsequently observed until 96 hpf for various parameters, including: mortality, hatching, morphologic changes, movement and neural tube cellular proliferation.

Materials and Methods

Animals

Wild type zebrafish (AB strain) were housed at the Department of Biology at Texas A&M University under standard laboratory conditions that included an average ambient temperature of 28.5°C [27]. Mature male and female zebrafish were paired in late afternoon and fertilized zebrafish embryos (ZFEs) were obtained at approximately 9:00 am the following morning, which was near the beginning of the light cycle (14 h of light and 10 h of darkness). ZFE medium, was made using ultrapure water to which was added low concentrations of specific ions and adjusted to pH 7.2 [27]. Developing ZFEs were maintained in ZFE medium that was freshly prepared for each experiment. Criteria published by Kimmel et al. (1995) were used to stage ZFE development. The ZFEs were anesthetized and fixed at specific hours post fertilization (hpf). All procedures for animal use were approved by the Texas A&M University Laboratory Animal Use Committee and all zebrafish were maintained and used according to protocols consistent with the Information Resources on Zebrafish, Animal Welfare Information Center Resource Series, No. 46, August, 2010, U.S.D.A.

Preparation of and exposure to mercuric chloride and methyl mercury

Mercuric chloride (99.5% purity; $HgCl_2$) was obtained from Sigma-Aldrich (St. Louis, MO, USA). Methyl mercuric chloride (95% purity; CH_3HgCl) was purchased from Alfa Aesar (Ward Hill, MA, USA). Each compound was dissolved in sterile, deionized water to yield stock solutions of 0.1 mg/mL, and stored at 4°C until needed. The stock solutions were diluted with ZFE medium to the desired final concentrations. Exposure of ZFEs was conducted in 24-well, flat bottom, polystyrene plates with low evaporation lids (BD Biosciences, San Jose, CA, USA). Negative controls consisted of ZFEs exposed to just ZFE medium (0 parts per billion (ppb) or µg/L=control ZFEs). Each 24-well plate contained 2 mL ZFE medium with one of 11 different concentrations of mercuric chloride ($HgCl_2$; 0, 5, 10, 50, 80, 100, 200, 500, 600, 700, and 1500 ppb (µg/L) or one of five different concentrations of methyl mercury: 0, 5, 10, 50 or 80 ppb (µg/L). Two to four ZFEs were placed in each well and the 24-well plates were prepared in duplicate or triplicate for each experiment. The plates were held for a maximum of 96 h at 28.5°C in an incubator (Thelco Laboratory Incubator; Cole-Palmer Instruments, Vernon Hills, IL, USA). After 24 h exposure to mercuric chloride, surviving ZFEs were transferred to new ZFE medium that did not contain mercury.

Morphologic assessment

Effects of 24 h or exposure to mercuric chloride or methyl mercury on ZFE morphology were assessed at 30, 48, 72, or 96 hpf. A SZ-40 binocular microscope (Olympus, Center Valley, PA, USA) was used to examine the ZFEs. Spontaneous movement and response to touch was assessed starting at 30 h of development. An Eclipse E400 microscope with a 2× objective (Nikon Instruments, Melville, NY, USA), a DXM1200 digital camera, and ACTI imaging software (Nikon Instruments) was used to evaluate pigmentation, larval length and yolk sac area. ZFEs were examined at room temperature (RT; 25°C) to assess developmental stage, mortality, hatching, spontaneous and elicited movement (response to touch) of hatched ZFEs, and the presence of any deformities. ZFEs were kept at RT for a maximum of 20 minutes before returning living ZFEs to the 28.5°C incubator.

The presence of any dead ZFEs was noted and the dead ZFEs were removed at each assessment time period. Due to the small size and the shape of the ZFE eye, the volume of the ZFE eyes exposed to different concentrations of mercuric chloride were determined from measurements of serial sections through each eye. At 96 hpf, 10 to 20 ZFEs from each experimental group were chilled on ice for euthanasia before they were preserved in 4% phosphate-buffered paraformaldehyde (pH 7.4) for 1 h at room temperature (RT). Fixed ZFEs were processed and embedded in paraffin, sectioned at a thickness of 5 µm, stained with hematoxylin and eosin (H&E) and cover slipped using Permount (VWR, West Chester, PA, USA). Images were captured on an Eclipse E400 microscope using a 4× objective (Nikon Instruments) and equipped with a DXM1200 digital camera and ACTI imaging software (Nikon Instruments). Every 5th section through each eye was measured for area and then the volume calculated based on number of sections measured and number of sections skipped. All morphological measurements were made using NIH Image J [44].

Movement assessment

Effect of mercuric chloride ($HgCl_2$) on spontaneous and elicited ZFE movement was assessed. All ZFEs were exposed to $HgCl_2$ for 24 h (6-30 hpf). At 30 hpf, each ZFE was observed in the 24 well plates for one minute to record spontaneous movement before the ZFE medium was changed to fresh embryo medium without mercury. ZFEs were observed for spontaneous movement using an Olympus SZ40 dissecting microscope (Olympus) with an attached Nikon DXM1200 camera and ACTI imaging software (Nikon Instruments). To observe elicited movement each hatched ZFE was placed in a 100 × 15 mm polystyrene petri dish containing fresh ZFE medium at 28.5°C and each ZFE was gently touched once near the tip of the tail with a fine needle. The distance each ZFE moved after being touched was recorded.

Immunohistochemistry

At 30 hpf, 10 to 20 ZFEs from the 0 to 200 ppb mercuric chloride exposure groups were placed on ice for euthanasia then fixed for 1 hour at RT in 4% phosphate-buffered paraformaldehyde (pH 7.4). Fixed ZFEs were immersed in 20% sucrose in phosphate-buffered saline (PBS) for cryoprotection. Then the ZFEs were submerged in cryogel mounting medium (Electron Microscopy Sciences, Hatfield, PA, USA), rapidly frozen with powdered dry ice and sectioned coronally at 15 µm, using a SLEE cryostat (SLEE Medical, Mainz, Germany). The frozen sections were thaw-mounted onto gelatin-coated slides and stored at -70°C until used. Unless noted otherwise, subsequent procedures were carried out at RT. We used an antibody to Proliferating Cell Nuclear Antigen (PCNA) to label proliferating neural tube cells in ZFEs exposed

to mercuric chloride. This same procedure was performed previously in this lab for ZFEs exposed to methyl mercury [45].

Sections of ZFEs were stained using a standard immunohistochemistry protocol as described by Abbott and Jacobowitz [46]. Briefly, sections (on slides) were incubated in 0.3% Triton in PBS (1 h), rinsed with PBS, placed in 5% normal horse serum in PBS (1 h), then exposed to mouse anti-PCNA primary antibody in PBS (1:40,000, Sigma) overnight at 4°C. Following extensive washes with PBS, sections were incubated in biotinylated horse anti-mouse secondary antibody (1:400; Vector Laboratories, Burlingame, CA, USA; 2 h) followed by multiple PBS washes and then incubated overnight at 4°C in horseradish peroxidase conjugated to strept-avidin (1:5000; Kirkegaard & Perry Laboratories, Inc. Gaithersburg, MD, USA). Following several PBS washes and one last wash with 0.05 M Tris- HCl buffer (pH 7.6), a solution of 0.06% diaminobenzidine (DAB; Sigma), 0.6% nickel ammonium sulfate and 0.02% hydrogen peroxide in 0.05 M Tris-HCl buffer (pH 7.6) was used to reveal tissue-bound peroxidase activity. The DAB reaction was terminated after two to four minutes by transferring the sections to fresh 0.05 M Tris-HCl buffer (pH 7.6) then rinsed in PBS. The sections were dehydrated through an ascending series of ethyl alcohol and counterstained with eosin in 95% ethyl alcohol. Dehydration was completed in 100% ethyl alcohol followed by several rinses in xylene, then Permount (VWR) was used to coverslip the sections.

Images of stained ZFE sections exposed to mercuric chloride for 24 h and fixed at 30 hpf were captured using an Eclipse E400 microscope equipped with a 40x objective, a Nikon DXM1200 digital camera, and ACTI imaging software. PCNA staining in the ZFE neural tube was assessed using NIH Image J (Abramoff et al. 2004).

Statistical analysis

Approximately 90-100 ZFEs per group for mercuric chloride analyses of mortality and hatching and 40-50 ZFEs per group were included in methyl mercury analyses for the same parameters. Using SPSS 15 software, one-way analysis of variance (ANOVA) was used to determine whether differences existed among responses to the different concentrations of mercuric chloride or methyl mercury. Results are expressed as percentages (for mortality) or as mean ± standard error of the mean (SEM). For movement assessment, 20 ZFEs per group were assessed. For PCNA staining 8 to 10 ZFEs were sectioned and stained for each concentration of $HgCl_2$ analyzed. For body length, eye volume, and yolk sac area, 8 to 20 ZFEs were measured for each concentration of $HgCl_2$ analyzed. Comparisons between means for each treatment were made using ANOVA or multiple comparisons Duncan's difference procedure (Duncan, 1955). When applicable, the Student's t-test was used and the Bonferroni correction was applied as needed. For movement assessment, Chi-Square analysis was used to compare the number of ZFEs in each movement category. Significance for all analyses was set at $p \leq 0.05$.

Results

Mercuric chloride ($HgCl_2$) exposure

ZFEs were exposed for 24 h (from 5 to 30 h post fertilization (hpf)) to one of ten different concentrations (0 to 1500 µg/L) of mercuric chloride ($HgCl_2$) and mortality was first assessed at 30 hpf, as shown in Table 1, 100% of the control ZFEs (0 µg/L $HgCl_2$) and 99% of ZFEs exposed to 5 µg/L $HgCl_2$ were alive at 30 hpf. Mortality remained low (approximately 11% or less) for ZFEs exposed to 10, 50 or 80 µg/L $HgCl_2$. Increased numbers of ZFEs died as development proceeded to 96 hpf, starting with exposure to 100 µg/L $HgCl_2$.

Exposure of ZFEs to higher $HgCl_2$ concentrations, even though exposure ended at 30 hpf resulted in substantial delayed toxicity. Exposure to the highest concentration of $HgCl_2$ in this study, 1500 µg/L, resulted in 100% mortality by 30 hpf (Table 1). At 96 hpf there was no difference between mortality rates seen at 500 µg/L and 1000 and 1500 µg/L $HgCl_2$ concentrations (Table 1). However, mortality at 96 hpf was significantly higher than that observed at earlier times when compared within the same exposure concentration for 100 through 1000 µg/L $HgCl_2$ (Figure 1A). As seen in Figure 2, mortality exhibited by ZFEs exposed for 24 h to 100 µg/L and higher concentrations of $HgCl_2$ exhibited gradual increases in mortality over time, with the highest mortality always at 96 hpf, with the exception of exposure to 1500 µg/L, where 100% mortality was observed at the earliest time of assessment (30 hpf).

Zebrafish embryos normally begin to hatch from their chorion at approximately 48 hpf.

$HgCl_2$ (µg/L)	30 hpf	48 hpf	72 hpf	96 hpf
0	0	0	0	0
5	0	0	0	0.9
10	4.6	3.9	3.9	6.6
50	2.2	2.8	2.8	4.49
80	5	5	5	10.7
100*	10.1	12.2	12.2	28.5§
200*	12.3	15.1	20.4	33.3§
500*	13.9	25.1	51.2	94.5§
1000*	22.1	55	77.3	100§
1500*	100	100	100	100

Effect of mercuric chloride ($HgCl_2$) on ZFE mortality at 30, 48, 72, and 96 hpf.

ANOVA, p=0.008. N=96 ZFEs exposed to each concentration of mercuric chloride for 24 hours (6- 30 hpf; ANOVA, p=0.03) Substantial delayed toxicity was observed, starting at an exposure concentration of 100 ppb (µg/L).

*Compared to control ZFEs increased mortality was observed beginning at 100 µg/L mercuric chloride at 30 hpf.

§Mortality at 96 hpf is significantly higher compared to mortality at earlier times within the same exposure level.

p <0.05, Student's t-test was used as the post hoc test (using the Bonferroni correction)

Table 1: Per cent mortality.

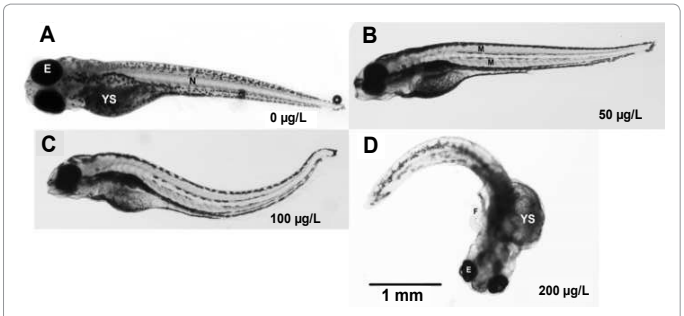


Figure 1: 96 hpf embryos exposed to different concentrations of mercuric chloride ($HgCl_2$) for 24 h, starting at 6 hpf: A) control (0 µg/L); B) 50 µg/L; C) 100 µg/L; D) 200 µg/L. Labeled structures: E=eye; YS=yolk sac; M=myotome; N=notochord. Note the reduced pigmentation in ZFEs exposed to concentrations of $HgCl_2$ at 50 µg/L and higher. ZFEs exposed to 100 µg/L $HgCl_2$ for 24 h began to consistently display curved tails. ZFEs exposed to 200 µg/L $HgCl_2$ for 24 h exhibited greatly reduced eyes, much larger yolk sacs, reduced body length and highly curved tails. Scale bar in D=1.0 mm and applies to all photomicrographs.

No significant differences in hatching success were observed at 48 hpf for any of the concentrations of $HgCl_2$ tested (Table 2). There was a trend towards higher hatching rates at 72 hpf for ZFEs exposed to 50 and 80 µg/L, but this trend was reversed with exposure to 200 µg/L and higher concentrations of $HgCl_2$ (Table 2). No surviving ZFEs exposed to 500 or 1000 µg/L Cl_2Hg hatched by 72 or 96 hpf.

Reduced pigmentation was apparent in ZFEs exposed to 50 µg/L and higher concentrations of $HgCl_2$ (Figure 1B-D). ZFEs exposed to 100 µg/L $HgCl_2$ for 24 h began to consistently display curved tails Figure 1C-D and also presented significant reduction in body length (Figure 3A). A dose response was observed in that higher concentrations of $HgCl_2$ exposure resulted in significantly shorter body lengths when compared to ZFEs exposed to 200 µg/L and 100 µg/L $HgCl_2$, but not for ZFEs exposed to 80 µg/L $HgCl_2$ versus 100 µg/L $HgCl_2$. Eye volume was significantly reduced in ZFEs exposed to 50 µg/L, 100 µg/L and 200 µg/L $HgCl_2$ compared to control ZFEs (Figure 1D and Figure 3B), but no difference in eye volume was observed between these three groups of ZFEs. While a trend towards increased yolk sac area was observed starting with ZFEs exposed to 100 µg/L $HgCl_2$ compared to control ZFEs, the only significant difference was observed when the yolk sac area from ZFEs exposed to 200µg/L $HgCl_2$ was compared to ZFEs exposed to 0 µg/L, 10 µg/L and 50 µg/L $HgCl_2$ (Figure 3C and Figure 1D).

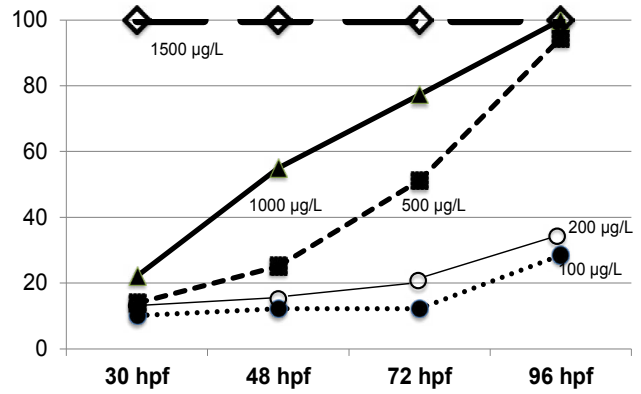
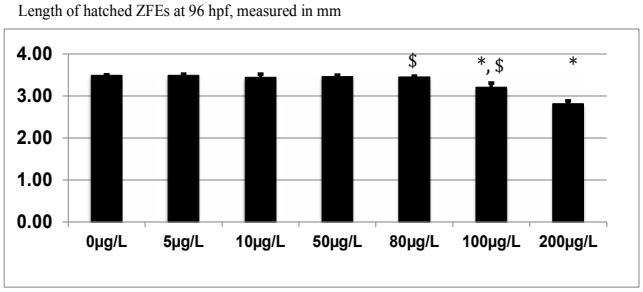


Figure 2: Graph showing mortality exhibited by ZFEs exposed for 24 h to four different concentrations of $HgCl_2$. Concentrations of 100 to 1000 µg/L $HgCl_2$ showed increases in mortality over time, with the highest mortality always at 96 hpf.

$HgCl_2$ (µg/L)	48 hpf	72 hpf	96 hpf
0	1.9	33.2	72.9
5	0	37.5	67.7
10	1.9	33.5	62.8
50	2.7	46.3	77.7
80	2.9	49	83.6
100	2.9	34.5	62.5
200*	0.9	15.2	38.3
500*	0	0	0
1000*	0	0	0

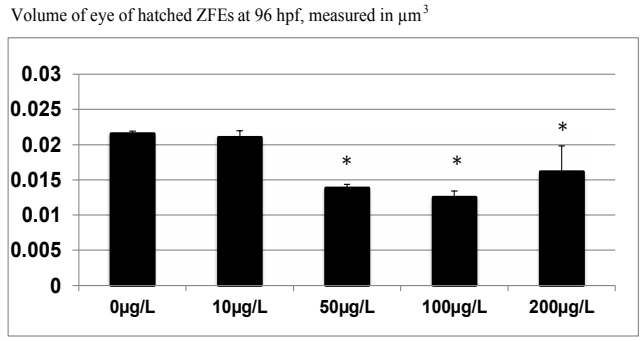
Effect of mercuric chloride ($HgCl_2$) on ZFE hatching at 48, 72, and 96 hpf. (ANOVA, p=0.002)
N=96 ZFEs exposed to each concentration. All ZFEs were exposed to $HgCl_2$ for 24 hours (6-30 hpf). Data from ZFEs exposed to 1500 µg/L $HgCl_2$ were not included in Table 2 because all ZFEs exposed to 1500 µg/L $HgCl_2$ died by 30 hpf.
*Compared to control ZFEs, significantly fewer ZFEs hatched starting at exposure to 200 µg/L or PPB).
p<0.05, Student's t-test was used as the post hoc test (using the Bonferroni correction)

Table 2: ZFE hatching success (based on percent of live ZFES) with 24 h exposure to different concentrations of mercuric chloride.



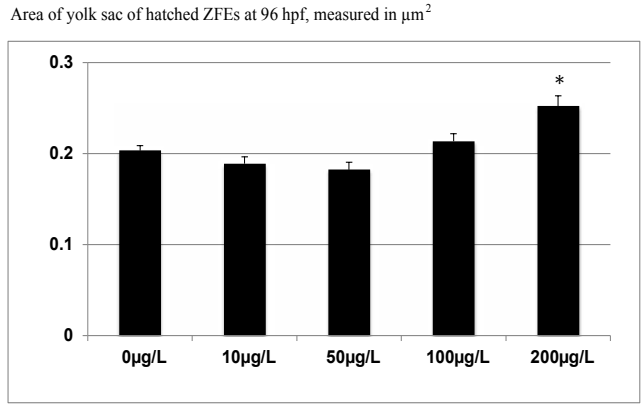
*=different from control (0 µg/L), p<0.01; $=different from 200 µg/L, p<0.03 (post hoc test using Bonferroni correction).

Figure 3A: Average length of hatched ZFEs at 96 hpf. All ZFEs were exposed to $HgCl_2$ for 24 h (6-30 hpf) and then transferred to fresh embryo medium without $HgCl_2$. Error bars indicate SEM. ANOVA was significant. (ANOVA, p<0.001) Sample size=8-20 for each group).



*=different from 0 µg/L and 10 µg/L, p<0.01. (post hoc test using Bonferroni correction).

Figure 3B: Average volume of the eye in hatched ZFEs at 96 hpf, measured in µm3. All ZFEs were exposed to $HgCl_2$ for 24 h (6-30 hpf) and then transferred to fresh embryo medium without $HgCl_2$. Error bars indicate SEM. (ANOVA, p<0.001).



*=different from 0 µg/L, 10 µg/L and 50 µg/L, p<0.01. (post hoc test using Bonferroni correction).

Figure 3C: Average area of the yolk sac in hatched ZFEs at 96 hpf, measured in µm2. All ZFEs were exposed to $HgCl_2$ for 24 h (6-30 hpf) and then transferred to fresh embryo medium without $HgCl_2$. Error bars indicate SEM. (ANOVA, p<0.001).

Beginning at 24 hpf, ZFEs start to display stereotypic spontaneous movement of the body and hatched ZFEs will exhibit stereotypic motor responses to touch [47]. Observations of spontaneous and elicited movement can be used to assess effects of neurotoxicity. Spontaneous movements included rapid coiling of the tail, which was immediately followed by a slower relaxation phase [48-50]. When observed at 30 hpf, approximately 35% of control ZFEs (0 µg/L $HgCl_2$) demonstrated spontaneous movements during a one minute observation window. ZFEs exposed to 24 h of 10 µg/L $HgCl_2$ exhibited spontaneous movements that were similar to control ZFEs. However, ZFEs exposed to 24 h of 50 µg/L, 80 µg/L or 100 µg/L $HgCl_2$ actually exhibited increased spontaneous movement (Table 3). In contrast, spontaneous movements observed for ZFEs exposed to 200 µg/L $HgCl_2$ for 24 h were not statistically different from control ZFEs (Table 3). Elicited movement as a response to a light touch of the tail was assessed in hatched ZFEs at 72 hpf (Table 4). In contrast to changes observed in spontaneous movement at 30 hpf, by 72 hpf, all hatched ZFEs exposed to any concentration of $HgCl_2$ exhibited decreased elicited movement compared to control ZFEs. A dose response was observed when elicited movement of ZFEs exposed to lower concentrations of $HgCl_2$ was compared to elicited movement of ZFEs exposed to higher concentrations of $HgCl_2$. No ZFEs exposed to 200 µg/L $HgCl_2$ for 24 h exhibited any movement in response to touch (Table 4), even though it was clear that the ZFEs were alive based on the presence of a beating heart.

At 30 hpf we examined Proliferating Cell Nuclear Antigen (PCNA) immune histochemical staining of neuronal nuclei to assess relative extent of cell division taking place in developing neurons in the brains of developing ZFEs (Figure 4). A one-way ANOVA indicated significant differences existed in the data set (p=0.0002). Post hoc analysis indicated a significant increase in PCNA expression in the neural tubes of ZFEs exposed to 80 µg/L and 200 µg/L $HgCl_2$, but no different in PCNA expression in the neural tubes of ZFEs exposed to 100 µg/L $HgCl_2$ (Figure 4). In contrast, exposure of ZFEs to 500 µg/L $HgCl_2$ resulted in no difference in cell proliferation when compared to PCNA expression in control ZFE (Figure 4).

Methyl mercury exposure

In the present study, we focused on the effects of exposure of ZFEs to low levels of methyl mercury. At 30 hpf after 24 h of exposure to both 50 µg/L and 80 µg/L methyl mercury, the number of hatched ZFEs decreased from the expected rate of greater than 95% at 96 h as seen for control ZFEs (0 µg/L methyl mercury), to 70.8% and 1.3% hatched ZFEs, respectively (Table 5). No significant difference was observed between hatching at 72 hpf and 96 hpf for ZFEs exposed to 80 µg/L methyl mercury (p=0.89).

We assessed the morphology of ZFEs exposed to methyl mercury, starting at 30 hpf and at 48, 72 and 96 hpf. Starting with hatching and continuing through to 96 hpf, ZFEs exposed to 80 µg/L methyl mercury for 24 h developed significant tail deformities (Figure 5). ZFEs exposed to 10, 50 and 80 µg/L methyl mercury displayed less pigmentation at 72 hpf compared to age-matched control ZFEs (Figure 5). ZFEs continued to display less pigmentation at 96 hpf (data not shown). Compared to age matched control embryos, ZFEs exposed to 80 µg/L methyl mercury exhibited significantly smaller eyes, larger yolk sacs, reduced body length and highly curved tails that the ZFEs could not straighten (Figure 1D).

These morphological changes have been described in greater detail in Hassan et al. [45]. ZFEs exposed to 0, 10 and 50 µg/L methyl mercury all exhibited normal spontaneous movements at 30 hpf, but ZFEs exposed to 80 µg/L methyl mercury showed little to no spontaneous movement (data not shown). At 48 hpf (Table 6A) and even more so at 72 hpf (Table 6B), hatched ZFEs exposed to either 10 µg/L or 50 µg/L showed significantly decreased response to touch even though they were alive, which was confirmed by the presence of a beating heart.

Number of movements in one minute	0 µg/L	10 µg/L	50 µg/L*	80 µg/L*	100 µg/L*	200 µg/L
0	65	59	56	58	80	75
1	22	24	19	19	17	17
2	8	12	23	13	0	4
3	5	5	2	10	3	4

Effect of mercuric chloride ($HgCl_2$) on spontaneous movement of ZFEs.
N=24 to 60 ZFEs in each group. Data are presented as percent of total number of live ZFEs that exhibited spontaneous movements in each movement category for each treatment group, using a one-minute observation time.
*significant difference compared to controls, using Chi-square analysis, p<0.05

Table 3: Percent of total number of ZFE embryos that exhibited spontaneous movement when observed at 30 hpf after 24 h exposure to mercuric chloride.

Mercuric chloride concentration	Response to touch of the tail at 72 hpf			
	no movement	moved 5-15 mm	moved 15-30 mm	Moved > 30 mm
0 µg/L (control; n=29)*	0	10	38	52
10 µg/L (n=20) #	0	45	50	5
50 µg/L (n=20) &	25	50	25	0
80 µg/L (n =38) &	71	13	5	11
100 µg/L (n=16) &	75	25	0	0
200 µg/L (n=10)¥	100	0	0	0

Effect of mercuric chloride ($HgCl_2$) on elicited ZFE movement at 72 hpf (A). All ZFEs were exposed to $HgCl_2$ for 24 hours (6-30 hpf). Each ZFE was touched on the tail with a fine needle and the distance the ZFE moved after being touched was recorded. Chi-Square analysis was used to compare the number of ZFEs in each movement category in each exposure range and control ZFEs. With the control group (0 µg/L $HgCl_2$) set as the expected range, movement of control ZFES was significantly different compared to all the experimental groups. (*, p<o.001) With 10 µg/L $HgCl_2$ group set as the expected range, movement of control ZFES was significantly different compared to all other experimental groups. (#, p<0.001) The same was true for each of the experimental groups when they were compared to the remaining groups exposed to higher concentrations of mercuric chloride. (&, p<0.001)

¥ZFEs exposed to 200 µg/L were not assessed as they exhibited no elicited movement.

Table 4: Elicited ZFE movement observed at 72 hpf after 24 h exposure to mercuric chloride.

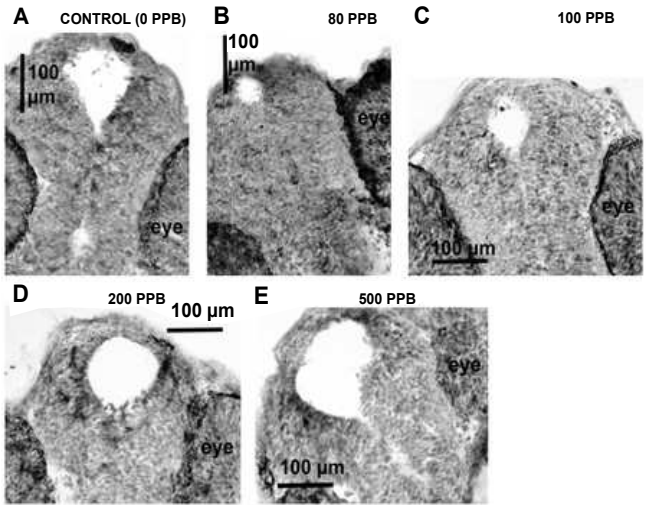


Figure 4: Zebrafish embryos examined at 30 hpf and sectioned through the region of the forebrain; the developing eyes are seen on either side of the brain. The sections were stained with PCNA immunohistochemistry. (A) ZFE exposed to 0 µg/L $HgCl_2$ (control; 0 PPB); (B) ZFE exposed to 80 µg/L $HgCl_2$ (80 PPB); (C) ZFE exposed to 100 µg/L $HgCl_2$ (100 PPB); (D) ZFE exposed to 200 µg/L $HgCl_2$ (200 PPB); (E) ZFE exposed to 500 µg/L $HgCl_2$ (500 PPB); (F) is a graph showing relative density of immune histochemical staining for PCN of the ZFE CNS exposed to different concentrations of $HgCl_2$ for 24 h. (ANOVA, $p<0.05$; post hoc test was the Student's t-test, using the Bonferroni correction).

Methyl mercury concentration	% hatched at 72 hpf	% hatched at 96 hpf
0 µg/L (control)	100	100
5 µg/L	100	100
10 µg/L	100	100
50 µg/L	58.3	70.8
80 µg/L	0	1.3
Hatching success observed for live ZFEs exposed to different concentrations of methylmercury for 24 hours (5 to 30 hpf). N=96 for each methylmercury concentration. No decrease in ability to hatch was noted until ZFEs were exposed to 50 µg/L methylmercury for 24 hours.		

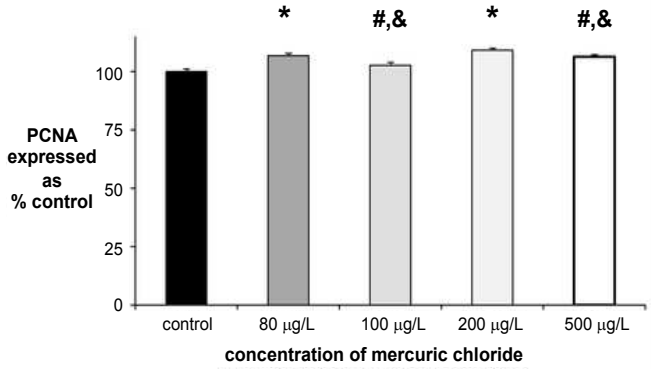
Table 5: ZFE hatching success (based on percent of live ZFES) with 24 h exposure to different concentrations of methyl mercury.

Methyl mercury concentration (24 hours exposure)	Response to touch of tail at 48 hpf			
	no movement	moved 5-15 mm	moved 15-30 mm	Moved more than 30 mm
0 µg/L (control)	0	5	10	5
10 µg/L *	0	9	10	1
50 µg/L *	5	10	5	0
N=20 ZFEs assessed at each exposure concentration *Chi-Square analysis comparing: control to 10 µg/L, p=0.041 and control to 50 µg/L, p=0.002				

Table 6A: Elicited ZFE movement observed at 48 hpf after 24 h exposure to methyl mercury.

Methyl mercury concentration (24 hours exposure)	Response to touch of tail at 72 hpf			
	no movement	moved 5-15 mm	moved 15-30 mm	Moved more than 30 mm
0 µg/L (control)	0	5	10	5
10 µg/L *	2	10	8	0
50 µg/L *	7	11	2	0
N=20 ZFEs assessed at each exposure concentration *Chi-Square analysis comparing: control to 10 µg/L, p=0.006 and control to 50 µg/L, p<0.001				

Table 6B: Elicited ZFE movement observed at 72 hpf after 24 h exposure to methyl mercury.



Labeled structures: E=eye; YS=yolk sac; M=myotome; N=notochord.

Note the reduced pigmentation in ZFEs exposed to 10 µg/L and higher concentrations of methylmercury. ZFEs exposed to 80 µg/L methylmercury for 24 h exhibit greatly reduced eyes, much larger yolk sacs, reduced body length and highly curved tails.

Scale bar in D=0.5 mm and applies to all photomicrographs.

*=significantly different from control (0 µg/L, $p<0.001$).

#=significantly different from 80 PPB exposure ($p<0.02$).

&=significantly different from 200 PPB exposure ($p<0.002$) Scale bars in photomicrographs=100 µm.

Figure 5: 72 hpf embryos exposed to different concentrations of methylmercury for 24 h, starting at 6 hpf: A) control (0 µg/L); B) 10 µg/L; C) 50 µg/L; D) 80 µg/L.

Discussion

There is significant concern for women about exposure to mercury compounds while they are pregnant or lactating [51-52]. Developing embryonic, fetal and neonatal brains are more susceptible to mercury toxicity than the fully formed (mature) adult brain. While a great amount of information has been published concerning the effects of exposure to high concentrations of mercury on the nervous system, much less is known about exposure of the developing brain to very low concentrations of mercury, in both its organic and inorganic forms. We used developing ZFEs as a high-throughput animal model to learn more about the toxicity of low concentration mercury exposure on the developing embryo in general and specifically on the developing nervous system. We examined the effects of 24 h of exposure to inorganic (mercuric chloride or $HgCl_2$) and organic (methyl mercury) mercury on developing ZFEs.

Delayed development was observed with exposure to mercuric chloride as well as methyl mercury, as demonstrated by decreased hatching rates and in decreases in both spontaneous and elicited movement. Previous studies of effects of methyl mercury from our lab support these observations [45,53].

Kimmel et al. (1995) defined seven distinct periods of zebrafish development between fertilization (0 h post fertilization or hpf) and initiation of the early larval period. These seven periods of ZFE development are: zygote (0-1 hpf), cleavage (1-2 hpf), blastula (2-5 hpf), gastrula (5-10 hpf), segmentation (10-24 hpf), pharyngula (24-48) and hatching (48-72 hpf). In this study ZFEs were initially exposed to the different mercury compounds and concentrations beginning during the gastrula stage (at 6 hpf) and exposure ended during the early pharyngula stage (at 30 hpf). When ZFEs are raised at 28.5°C, 24 hpf is the time point separating the segmentation and pharyngula phases. The pharyngula phase is named based when the pharyngeal (or branchial) arches develop. Hatching normally occurs from 48 t0 72 hpf.

As with other developing vertebrates, brain development begins during gastrulation. Because development is so rapid in ZFEs, initial neural tube formation is completed by 24 hpf, resulting in a brain that is at the five-vesicle stage. Also by 30 hpf, the eyes are well developed, with a distinct retina and lens present.

It has been proposed that zebrafish, as both adults and developing embryos, can be used to detect low levels of environmental contaminants including heavy metals. Previous investigations in this laboratory examined the effects of different doses of methyl mercury on mortality, hatching, body length and eye area in ZFEs [45,53]. These investigations determined that the lethal concentration of methyl mercury is 200 µg/L and greater for ZFEs exposed for 24 h and exposure to concentrations of 50 µg/L methyl mercury and greater significantly delayed ZFEs from hatching out of their chorion.

Adult zebrafish exposed to a range of low concentrations of mercuric chloride ($HgCl_2$) exhibited significantly higher reporter gene expression under conditions of oxidative stress, but did not result in obvious morphological defects [54]. Low concentrations of methyl mercury also have adverse affects on ZFE development [45,55-57]. Similar studies examined the use of ZFEs specifically to assess environmental water contamination Yang et al. [58] observations that ZFEs are highly sensitive to adverse effects of exposure to mercury and the Lahnsteiner [59] study also reported that the first 24 h of ZFE development was the most sensitive period for exposure to various environmental contaminants, including $HgCl_2$.

Delayed toxicity effects were observed with 24 h exposure to both methyl mercury and $HgCl_2$ in this study. In an earlier study by Samson et al. [60] ZFEs exposed to 0, 5, 10 or 15 µg/L methyl mercury for different lengths of time also exhibited delayed embryonic death, referred to as "delayed mortality syndrome"; ZFEs were given continuous exposure to very low doses of methyl mercury and died by 144 hpf (6 days) of development. We did not extend our experimental trial as long as the Sampson et al. [60] study, having ended our study at 96 hpf, but we did observe significantly decreased elicited movement from ZFEs exposed to 10 µg/L methyl mercury for only 24 h, which indicates that the ZFEs were adversely affected. We do not know whether these ZFEs would have died at a later time due to earlier exposure to 10 µg/L methyl mercury for 24 h. Delayed toxicity was also observed for $HgCl_2$ in this study. Finally, Sipes et al. [61] reported that ZFEs and mammalian embryos respond quite similarly to developmental toxicity, on average about 85%. These observations indicate that ZFEs are an excellent vertebrate model to investigate the mechanisms underlying why exposure to mercury during development results in delayed toxicity.

It was interesting to note that relatively low $HgCl_2$ concentrations of 50 and 80 µg/L had a mild positive effect on ZFE hatching, in that slightly more ZFEs hatched than control ZFEs at the same time period, but exposure to higher $HgCl_2$ concentrations (200 µg/L and higher) clearly was detrimental to hatching. This appears to be a possible example of hormesis, whereby exposure to low concentrations of a toxicant have a positive effect, while exposure to higher concentrations of the same toxicant have clear detrimental effects. We also observed increased amounts of spontaneous movement at 30 hpf in ZFEs exposed to 50, 80 or 100µg/L $HgCl_2$ but not with exposure to 200 µg/L $HgCl_2$. It is not clear if the increased spontaneous movements should be viewed as positive or beneficial and an example of hormesis or as detrimental to overall ZFE development. Further study on muscle development and neuromuscular junction formation is warranted. The concept of hormesis is still controversial in the field of toxicology. However, possible examples of this phenomenon do merit further investigation.

We observed deformed tails in ZFEs exposed to both $HgCl_2$ and methyl mercury. Other studies also support the observation that exposure to mercury results in tail deformities in ZFEs [57,58,62]. The study by Yang et al. [57] suggests that methyl mercury specifically impairs tail development in part through activation of matrix metalloproteases 9 and 13, which are active in tissue remodelling. It also is possible that the occurrence of tail deformities are associated with decreased movements that occurred with exposure to both methyl mercury and $HgCl_2$. Decreased ability to move the tail and the tail deformities could be due to abnormal skeletal muscle development or to impaired innervation of the developing muscle. Usuki et al. [63] reported that chronic treatment of rats with low dose methyl mercury produced pathologic changes in skeletal muscle, including changes in fiber size and altered mitochondrial enzyme activity. Several other authors have reported that methyl mercury exposure can affect gene expression in zebrafish skeletal muscle [29,64-65].

Both methyl mercury and $HgCl_2$ inhibit action potentials when applied at a concentration of ~40 µg/L or higher in an isolated rat phrenic nerve-diaphragm preparation [66]. Methyl mercury also increases spontaneous release of acetylcholine at the neuromuscular junction [67]. An earlier study that used an in vitro frog sciatic nerve-sartorius muscle preparation, reported that mercury caused reduced voltage-gated calcium ion entry into presynaptic nerve terminals Cooper et al. [68]. These reports indicate that exposure to either methyl mercury or $HgCl_2$ could adversely affect muscle innervation resulting in the observed abnormalities. Future experiments will be carried out to determine whether exposure to mercury at low concentrations adversely affects muscle development, neuromuscular junction formation and function or both.

We reported previously that ZFEs exposed to 50 µg/L and 80 µg/L methyl mercury for 24 h displayed less pigmentation than control ZFEs (Hassan et al. [53]) and this study corroborated that observation with 24 h of exposure to 50 µg/L and higher concentrations of both methyl mercury and mercuric chloride. Pigmentation in zebrafish is produced by chromatophores derived from neural crest cells. Pigmentation can be affected through several mechanisms, including: chromatophore cell number, pattern, morphology or intensity of pigmentation production [69]. We did not examine the chromatophores quantitatively in this study, but based our morphological observations it would appear that the chromatophores were either fewer in number or smaller in size, resulting in overall less pigmentation in experimental ZFEs. In the estuarine crab, Chasmagnathus granulatus, exposure to 100 µg/L mercury during embryonic development resulted in hypopigmentation of body chromatophores (Sanchez et al. [70]), but no specific effects of mercury exposure on melanocytes in mammals or chromatophores in other species have been published. Thus, additional investigation of the effects of mercury exposure on zebrafish chromatophore and mammalian melanocyte development is needed.

As part of the developing CNS, the visual system is particularly susceptible to mercury toxicity. Korbas et al. [55] demonstrated that the developing ZFE lens and retinal pigmented epithelium can accumulate mercury and that mercury levels in the lens of the eye continue to increase even after ZFEs are placed in non-contaminated medium. According to Weber et al. [71], ZFEs exposed for 24 h to $HgCl_2$ at 75 µg/L, similar to concentrations used in this study, exhibited dose-dependent visual deficits as adults, but the authors did not comment on the overall size of the ZFE eye during development.

We previously reported that exposing ZFEs to methyl mercury concentrations as low as 10 µg/L resulted in decreased PCNA staining in

cells of the central nervous system (Hassan et al. [45]), which indirectly suggests that cell proliferation was decreased at the time of assessment. Perry et al. [72] reported a decreased mitotic index in killifish embryos exposed to methyl mercury. Decreased cell division also has been demonstrated for rodent embryonic cells exposed to methyl mercury [73]. Thus, it is likely that decreased cell division is occurring in methyl mercury treated ZFEs, and decreased cell division may be occurring in other tissues as well, including developing skeletal muscle, which could be another reason for the observed reduction in movement and tail abnormalities, due to delayed muscle development. However, exposure to increasing concentrations of $HgCl_2$ from 80 to 500 µg/L resulted in either no significant difference from PCNA expression in the brains of control ZFEs or only slightly increased PCNA expression in brains of ZFEs exposed to 80 or 200 µg/L $HgCl_2$. These data suggest that decreased cell proliferation is not likely to be a primary cause for the observed abnormalities that occur in common with exposure to methyl mercury and $HgCl_2$. Mercury has a multitude of adverse affects on developing cells, including adverse affects on mitochondrial function (Cambier et al. [65]) increased production of reactive oxygen species (Gonzalez et al. [29]) and other cell stress responses [74]. A recent study that carried out a genome wide transcriptional analysis of the central nervous system of ZFEs exposed to methyl mercury emphasized that genes associated with oxidative stress, transport and cell protection were the most affected [74]. These data suggest that cellular stress may be an important component of mercury toxicity.

In summary, this study examined the affect of exposure of developing ZFEs to a range of concentrations of mercuric chloride ($HgCl_2$) or to methyl mercury [75]. Delayed or decreased hatching from the chorion, increased mortality and delayed toxicity were observed with exposure to both mercury compounds, with methyl mercury exhibiting more toxic effects compared to equivalent concentrations of $HgCl_2$ [76-78]. Both compounds resulted in reduced body length, abnormalities in tail morphology, reduced eye size and decreased movement. The rate of cell proliferation in the ZFE CNS was not decreased at the concentrations of $HgCl_2$ assessed in this study (80 to 500 µg/L), while we previously reported that cell proliferation in the ZFE central nervous system was reduced with exposure to methyl mercury concentrations as low as 10 µg/L. These data confirm the potent toxicity of methyl mercury and that $HgCl_2$, while slightly less toxic, also is a potent toxicant [79-80]. These data suggest that decreases in cell proliferation may not be the primary mechanism of toxicity for mercuric chloride.

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