Available online on www.ijtpr.com
International Journal of Toxicological and Pharmacological Research 2016; 8(2); 83-93

ISSN: 0975-5160

Research Article

Neurotoxic Effects of Gestational Exposure of Methyl Mercury on Different Brain Regions of F_1 Generation; Neurobehavioural, Biochemical and Histological Study During Weaning Period of Rat.

Sherin J, Sumathi T*

Department of Medical Biochemistry, Dr. ALM Post Graduate Institute of Basic Medical Sciences, University of Madras, Taramani Campus, Chennai –600 113, Tamil Nadu, India.

Available Online:1st May, 2016

ABSTRACT

MeHg is an environmental neurotoxin. Exposure to MeHg during gestation causes cognitive and motor imbalance in the rat pups. No studies are available on the differential effect of transplacental MeHg exposure on oxidative stress responses and cellular architecture in the specific motor and cognitive centers of rat offspring. Hence our study aimed to evaluate the changes in oxidative stress markers and histology in cerebral cortex, cerebellum and hippocampus of F_1 generation due to prenatal MeHg exposure. We have tried four different doses viz, 0.5, 1.5, 2.5 and 3.5 mg/kg of MeHg in drinking water to the pregnant rats from GD 5 till parturition. Pups of 2.5mg/kg and 3.5 mg/kg group were not survived. In 0.5 and 1.5 mg/kg MeHg dosage group, on PND 21, the pups showed neurobehavioural toxicity in morris water maze, forced swim, rotarod, hotplate and open field behaviour tests. Increased TBARS levels and decreased GSH, GPx, Catalase and SOD were found in cerebral cortex, cerebellum and hippocampus. Appearance of gliosis and extensive neuronal degeneration in both 0.5mg/kg and 1.5mg/kg doses were observed. Among the doses, oxidative, histological and behavioural changes were significant in 1.5mg/kg group. Hence we conclude that developmental exposure of MeHg causes oxidative stress changes and histological damages in the cerebral cortex, cerebellum and hippocampus of neonatal brain around weaning period (PND 21) and is manifested as postnatal neurobehavioural impairment. Further molecular studies are needed in each brain regions to explore therapeutic targets of MeHg neurotoxicity in future.

Keywords: Methyl mercury, Gestational, oxidative stress response, cerebral cortex, cerebellum, hippocampus.

INTRODUCTION

Methyl mercury (MeHg) is a highly neurotoxic environmental contaminant[1]. This hazardous global pollutant is a by product of vast developing industrialisation and technologies like mining, smelting coal combustion etc[2,3]. Mercury from industrial effluents and other anthropogenic sources gets accumulated in the upper sedimentary layers of sea or lake beds. Mercury undergoes methylation by microorganisms to its organic form, methy mercury, in water bodies. It is rapidly taken up by the aquatic organisms and is biomagnified through food chain. It reaches humans through fish and sea food consumption[4]. Even in the midst of various safeguard measures, MeHg is becoming a serious threat to the planet due to its fast spreading nature in sea[5,6]. The catastrophic effect of MeHg was reported several years before when large outbreak of human poisoning occurred in Minamata and Niigata, Japan and in Iraq[7-9]. MeHg can readily pass blood brain barrier and placental barrier[10,11]. The main target for MeHg toxicity is the central nervous system (CNS), and cause ultra structural and biochemical alterations in neurons[12,13]. As the developing offspring are highly vulnerable to xenobiotics, MeHg has the potential to cause developmental neurotoxicity. Epidemiological studies on pregnant women with fish consumption habits showed impairment of child motor and cognitive behaviour due to low-level of prenatal MeHg exposure[14]. The mechanism behind MeHg neurotoxicity remains poorly under-stood. One of the major known mechanisms for the action of this neurotoxin is by increased generation of reactive oxygen species (ROS)[15]. Earlier studies have demonstrated that cells exposed to MeHg exhibited decreased antioxidant status, suggesting that this could play a role in the pathogenesis of neurobehavioral toxicity due to MeHg[16]. As the cognitive or motor centers of brain play an important role in behavioural outcomes, our study mainly focused on these specific brain regions, cerebral cortex, cerebellum and hippocampus that are proved to be involved in regulation of memory, motor co-ordination, learning etc. Cerebral cortex is specialized for different types of learning. Cerebellum is involved in motor control and cognitive functions. Behavioral training can cause structural changes in task relevant areas of cortex or cerebellum in experimental rats. The hippocampus has been studied as the center for spatial memory and navigation[17-20]. We adopted a protocol which has been successfully implemented in previous studies in which MeHg was diluted in drinking water of the animal and allowed to drink, ad libitum. Oral exposure represents a major form by which human population is exposed to

**Author for Correspondence*

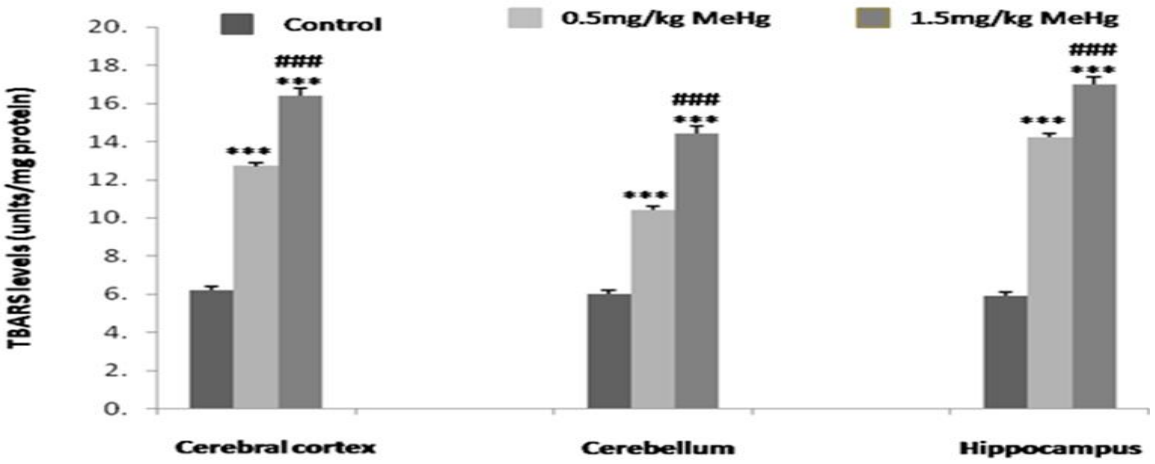

Figure 1: Effect of MeHg on TBARS levels in specific regions of the offspring brain on PND 21.

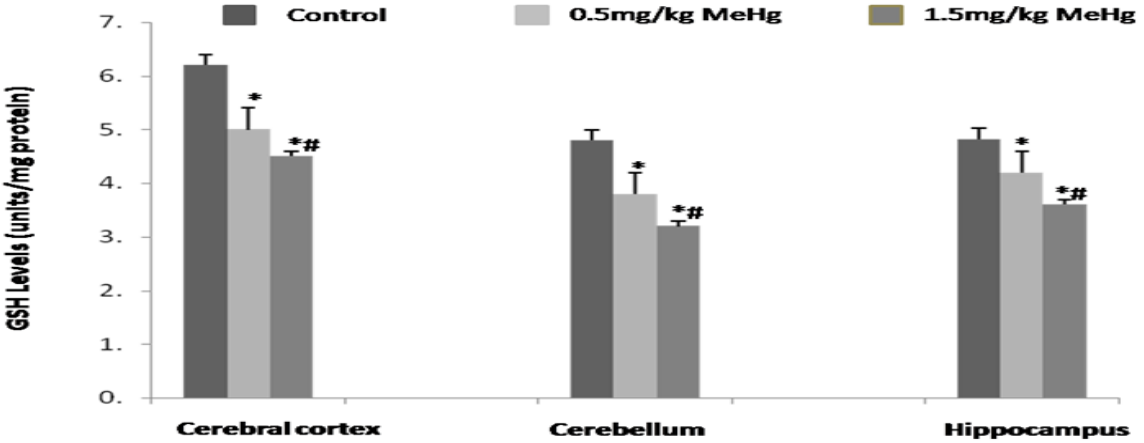

Figure 2: Effect of MeHg on reduced Glutathione (GSH) levels in specific regions of the offspring brain on PND 21.

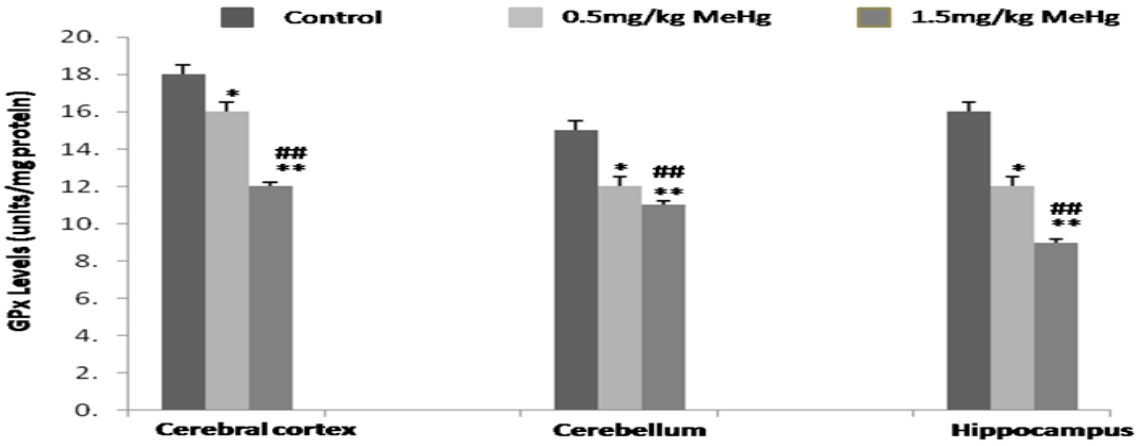

Figure 3: Effect of MeHg on GPx levels in specific regions of the offspring brain on PND 21.

MeHg[21]. Previous researches in this area have revealed that cerebral cortex, cerebellum and hippocampus are one of the most affected regions of brain in MeHg neurotoxicity in rats[22]. However, a differential study of effect of gestational exposure of MeHg in the specific motor and cognitive centers of F1 generation has not been greatly explored. Limited studies are available on the differential analysis of oxidative stress markers and morphometric changes due to transplacental exposure of MeHg in cerebral cortex, cerebellum and hippocampus of F1 generation. Accordingly, the present study aimed at investigating the effects of gestational MeHg exposure on the oxidative stress responses and histopathological changes in the cerebral cortex, cerebellum and hippocampus during the postnatal development of rat offspring brain and its associated neurobehavioral disorders. Objectives of the present study were (i) to study the cognitive, motor and psychological disorders in the F_1

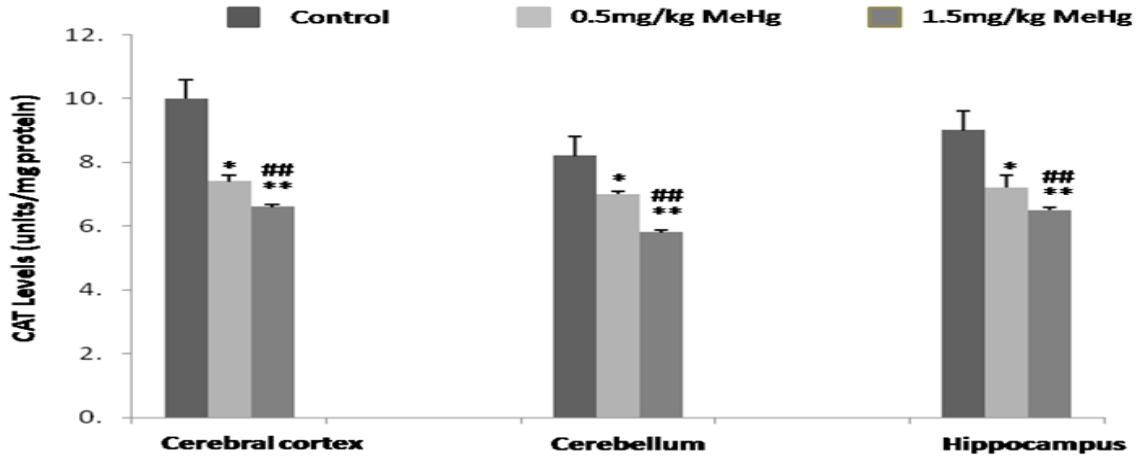

Figure 4: Effect of MeHg on CAT levels in specific regions of the offspring brain on PND 21.

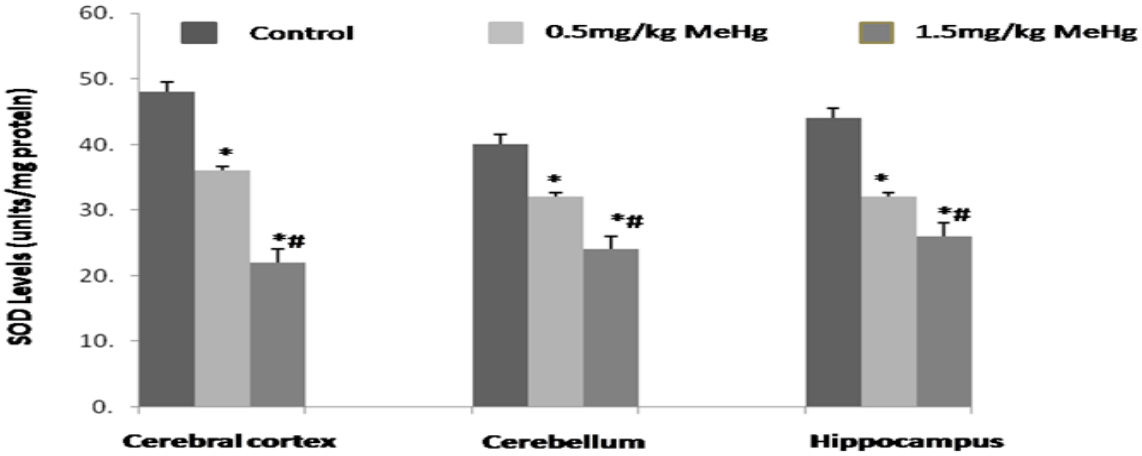

Figure 5: Effect of MeHg on SOD levels in specific regions of the offspring brain on PND 21.

generation due to transplacental exposure of MeHg. (ii) to evaluate the levels of oxidative stress markers like TBARS, GSH, GPx, catalase and SOD in the three specific brain centers (cerebral cortex, cerebellum and hippocampus) (iii) to study the differential histopathological changes in the three specific brain centers (cerebral cortex, cerebellum and hippocampus) of the rat offspring.

MATERIALS AND METHODS

Chemicals

Methyl mercury (II) chloride used for inducing toxicity was obtained from Sigma Aldrich Company, CAS No: 115-09-03. All the chemicals used in the study were of AR Grade.

Animals

Female Albino rats of Wistar strain, 12-14 weeks of age, weighing 180−200g obtained from Central Animal House, Dr. ALMPGIBMS, University of Madras, Taramani campus, Chennai-113, Tamil Nadu, India, were used for the study. Rats were housed separately in polypropylene cages in hygienic conditions and fed a standard pellet diet purchased from Hindustan Lever. All individual cages were kept in a temperature controlled room at 23 ± 3° C with relative humidity of 55 ± 15% on a 12 h light/dark cycle and 10 to 15 air changes / hr and given ad libitum free access to food pallets. Before the start of experiment, animals were kept in laboratory conditions for a period of 7 days for acclimatization. After one week of acclimatization all the animals were allowed to mate with proven fertile male rats overnight (1:2). The day of mating was confirmed by the presence of sperm positive vaginal smears and was designated as gestational day (GD) 0. Experimental animals were handled according to the University and Institutional legislation, regulated by the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Ministry of Environment and Forest (Animal welfare Division), Government of India (IAEC No. 01/09/2015).

MeHg Exposure Paradigm and dosage

Methyl mercury chloride was given to the study groups dissolved in drinking water at four different doses (0.5, 1.5, 2.5 and 3.5 mg/kg in drinking water). Methyl mercury were administered to the study groups from gestational day (GD 5), the day after uterine implantation, in order to reduce the chance of abortion/ miscarriage and continued till parturition (GD 21). GD0 pregnant females (F_0 Generation) were categorized in to five experimental groups of 6 animals each. Vehicle Control (n=6); 0.5 mg MeHg⁄kg (n= 6); 1.5 mg MeHg⁄kg (n= 6); 2.5 mg MeHg⁄kg (n= 6); 3.5 mg MeHg⁄kg (n= 6).

Tissue Preparation

Table 1: Mercury accumulation in brain and changes in brain weight and body weight due to prenatal exposure to MeHg in rat pups.

		PND 21		
MeHg Dosage (mg/Kg during Gestation		Control	0.5mg/kg	1.5mg/kg
Hg levels in Brain (mg/kg)		0.01±0.002	0.03±0.02^{NS}	0.05±0.38 ^{NS}
Average Brain Weight of pups (g)		1.04	1.01 ^{NS}	1.02 ^{NS}
Average Body Weight of pups (g)	PND 14	19.42±0.66	18.01±0.87 ^{NS}	17.26±0.66 ^{NS}
	PND 21	28.21±0.21	28.26±0.62 ^{NS}	29.81±0.52 ^{NS}

During weaning period, on postnatal day 21 (PND 21), the offspring were euthanized by cervical dislocation. Brains were immediately taken out and washed with ice-cold saline to remove blood. The brain regions (cerebral cortex, cerebellum, and hippocampus) were promptly isolated from the intact brain carefully on ice plate for biochemical assays. The whole brain from six randomly selected litters from each dose group was used for both Hg measurement and histopathological examination. For histopathological examination the tissues were stored in 10% formalin solution untill analysis and for Hg estimation, tissues were immediately frozen and maintained at −80 °C until analysis.

Analysis of Neurobehavioral toxicity

Offspring were randomly selected from each litter for conducting a series of behavioural tests. All the behavioural parameters were performed at room temperature in a calm room without any outside interference. All the neurobehavioral tests were performed between 10.00 A.M and 5.00 P.M ensuring proper rest for the rats in between every trial and probe tests.

Rotarod Task

Motor activity was assessed in rotarod using the protocol from previous studies. The apparatus consisted of a bar with a diameter of 2.5 cm, subdivided into four compartments by equally spaced 4 disks placed along the length of the rod disks, 25 cm in diameter. The rotation speed is increased slowly the velocity was controlled and fixed at 10rpm. The time spent by the rat on the rod till it fell down was measured as its motor activity. Four trials were carried out for 5 days prior to PND 21. The maximum time allowed on the rotarod apparatus was 60 seconds and two minutes of resting time was given in between each trial. The first three trials were considered as training trials and the latency to fall from the rod (in seconds) on fourth trial of each day was chosen for the statistical analysis[23, 24].

Open Field Test

General locomotor and explorative activity was measured on PND 21 by an open field test. A 60cmx90cm floor covered with an opaque wall (height: 30cm) was chosen as the open field. The floor was divided into six equal squares. The experimental setup focussed on an extremely calm and undisturbing surrounding to ensure the normal loco motor and explorative behaviour of the animals. On PND 21, an hour was fixed for testing open field activity. The rats were placed at the centre of the open field and behaviours were observed for 5 minutes. The parameters studied were the total number of rears (rearing), time spend without any motion (resting time in seconds) and the total distance travelled[25].

The nociceptive testing (Hot Plate Test)

Pain thresholds of the offspring were assessed using a hot plate test. Gass beakers (suitable for the rat size) were kept on a hot plate maintained at 55ºC and the rats were kept in it. The latency period was determined as the time taken for the rats to react to the thermal pain by licking their paws or jumping from the beaker[26]. The latency time for pain sensation was kept to a maximum of 45 seconds to prevent burning of skin.

Morris water maze test

Spatial learning was studied using Morris water maze test. A circular pool (180 cm diameter × 75 cm high) was filled to a depth of 45 cm with black opaque water at 22°C. A plexiglass (14 × 14 cm), painted black was placed 1.5cm below water level. The maze was divided in four virtual quadrants and the platform was placed in the middle of one of the quadrant and followed the same position throughout the trial test and probe test. Four trials per each day (5 days prior to PND 21) with a resting time of 1minute time interval between every trial were given the entire offspring group to ensure correct result.

The pups from each group were released in to one of the four quadrants facing the wall of the maze. Throughout the experiment the release positions were changed systematically. When the rat could not find the platform within 60 seconds from the time of release, it was lead to the platform and placed on it for 20 seconds before being removed. In such cases, the escape latency time were recorded as 60 seconds. The escape latency of the entire study group was recorded and after the test all the rats were wiped with dried cloth and were allowed to dry[27].

Forced Swim Test

The forced swim test was done by placing rat in an inescapable situation. The rat was put in a beaker of water so that the rat could not escape by itself. A vertical glass cylinder (25cm high, 14cm in diameter) was filled with water (30ºC) to a depth of 20 cm. The water depth was adjusted in such a way that the hind limbs and tail was not touching the base while swimming. Each animal was placed in the cylinder for 6 minutes. After a series of try to escape from the beaker, the rat remained inactive and started floating on the top.

The floating time is calculated as the immobility time, which is a measure of depressive like behaviour[28,29].

Analysis of oxidative stress response

Antioxidant status of each brain region (cerebral cortex, cerebellum and hippocampus) was done by assessing the levels of oxidative stress markers like TBARS (LPO), antioxidant GSH and antioxidant enzymes (GPx, SOD and CAT) by using methods published in previous literatures. All the antioxidants were assayed within a few minutes after the sacrifice to avoid the chances of errors.

Thiobarbituric acid-reactive substances (TBARS), an index of lipid peroxidation, were estimated by the method of Okhawa *et al*. The amount of TBARS was determined spectrophotometrically at 532 nm. The level of reduced glutathione (GSH) was measured by the method of Moron *et al* on the basis of the reaction of 5, 5'-dithiobis-2-nitrobenzoic acid which is readily reduced by sulfhydryls forming a yellow substance which was measured at 412 nm in spectrophotometer. The enzyme glutathione peroxidase (GPx) was assayed according to the method of Rotruck *et al*. The assay takes the advantage of concomitant oxidation of NADPH by GR, which was measured at 340 nm in spectrophotometer. The antioxidant enzyme, Superoxide dismutase (SOD) activity and Catalase (CAT) were expressed as units/mg of protein. The method was based on the inhibition of superoxide radical reaction with pyrogallol. Catalase (CAT) activity was determined by consequent decrease in absorption of hydrogen peroxide (H2O2) at 240 nm using spectrophotometer. The total protein content of the samples was measured using nano drop spectrophotometer[30-33].

Histopathology

After sacrifice of all animals, the whole brain samples were fixed in 10% neutral formalin. The tissues were processed and the paraffin embedding and sections were mainly stained with Hematoxylin and Eosin (H&E) for histopathology (400x).

Mercury estimation

Accumulation of total Hg in brain tissues of the offspring were measured using Atomic absorption spectroscopy (AAS).

Statistical analysis

Data represents mean ± SD. Statistical comparisons were performed by one way analysis of variance (ANOVA) using SPSS 20 software. If ANOVA analysis indicated significant differences, Tukey's post-hoc test was performed to compare mean values between the study groups and control. A value of *p<0.05 was considered as statistically significant.

RESULTS

Oxidative stress responses in cerebral cortex, cerebellum and hippocampus of rat offspring brain on PND21 due to developmental exposure of MeHg.

Oxidative stress responses were measured in the cerebral cortex, cerebellum and hippocampus regions of PND21 offspring. The antioxidant status was found to be affected significantly by MeHg dosage level but not in a region specific manner. MeHg induced an increase in the levels of the TBARS in cerebral cortex, cerebellum and hippocampus when compared to the control. It was found significantly increased (p<0.001) in both 0.5mg/kg and 1.5mg/kg group when compared to control. Levels of reduced Glutathione (GSH) and the antioxidant enzymes (GPx, SOD, and CAT) were found to be significantly reduced (p<0.001) in both 0.5mg/kg and 1.5mg/kg when compared to the controls in all the three specific cognitive centers (Fig 1-5). Significant changes in antioxidant status were found in 1.5mg/kg when compared to 0.5 mg/kg dosage groups.

Data represents the mean±SD (n=6/group). Levels of TBARS in cerebral cortex, cerebellum and hippocampus is shown. ***p< 0.001 is significantly different from control group and $^{###}p$< 0.001 is significantly different between 0.5mg/kg and 1.5mg/kg dosage groups, using one way ANOVA with Tukey's *post hoc* test. Data represents the mean±SD (n=6/group). Levels of GSH in cerebral cortex, cerebellum and hippocampus is shown. *p< 0.05 is significantly different from control group and $^{#}p$< 0.05 is significantly different between 0.5mg/kg and 1.5mg/kg dosage groups, using one way ANOVA with Tukey's *post hoc* test

Data represents the mean±SD (n=6/group). Levels of GSH in cerebral cortex, cerebellum and hippocampus are shown. *p< 0.05, **p< 0.01 is significantly different from control group and $^{##}p$< 0.01 is significantly different between 0.5mg/kg and 1.5mg/kg dosage groups, using one way ANOVA with Tukey's *post hoc* test.

Data represents the mean±SD (n=6/group). Levels of GSH in cerebral cortex, cerebellum and hippocampus are shown. *p< 0.05, **p< 0.01 is significantly different from control group and $^{##}$p< 0.01 is significantly different between 0.5mg/kg and 1.5mg/kg dosage groups, using one way ANOVA with Tukey's *post hoc* test

Data represents the mean±SD (n=6/group). Levels of GSH in cerebral cortex, cerebellum and hippocampus are shown. *p< 0.05 is significantly different from control group and $^{#}p$< 0.05 is significantly different between 0.5mg/kg and 1.5mg/kg dosage groups, using one way ANOVA with Tukey's *post hoc* test.

Effect of developmental exposure of MeHg on brain weight and body weight of the offspring

There were no significant differences in brain and body weight between the progeny of the control and MeHg exposed groups on PND 21 *(Table 1).*

Hg accumulation

Total Hg concentrations in whole brain of offspring at PND 21 showed a slight increase (though statistically non-significant) depending upon the dosage group in comparison to the normal control rats (Table 1). Data represents the mean±SD). NS represents no significant difference from control group and between the groups, using one way ANOVA with Tukey's *post hoc* test.

Maternal Toxicity

Maternal weight gain of dams during gestation was significantly reduced in the high MeHg dosage (3.5 mg/kg) group. In the low dosage groups (0.5 and 1.5 mg/kg) there were no noticeable signs of maternal toxicity were found and in the high dosage groups (2.5 and 3.5 mg/kg/day) gait alterations and ataxic movements were found. Similar findings were reported in a study conducted by three dosages of MeHg given through oral gavage treatment (Gandhi DN, 2013). Pregnant dams of 2.5mg/kg/day MeHg treatment group showed dystocia or total litter loss and 3.5mg/kg/day MeHg treatment group showed extended period of gestation (> 28 days) and death of all the pups after two days of delivery. As a result of the poor

Table 2: Effect of MeHg on maternal parameters.

MeHg Dosage (mg/kg) during Pregnancy	Control	0.5	1.5	2.5	3.5
Gestational length (days)	21.00±00	21.00±00	21.00±00	29.00±00	>29.00±00
Maternal weight on GD 0 (%)	230.68±10.67	228.46±5.8	219.44±8.2	215.32±6.9	211.27±7.12
Maternal weight gain to GD 21(%)	104.44±8.68	106.38±9.21	79.19±9.61*	66.24±15.01*	52.84±10.47*
Delay in delivery (no. of days delayed)	0	0	0	8	-
Total resorption or no delivery (%)	0	0	0	98	100
Total no. of pups born	48	30	24	-	-
Live births (%)	100	100	100	-	-
No. of pups alive	48	29	24	-	-
Viability (%)	100	98.6	98.5	-	-
Males	20	12	10	-	-
Females	24	18	14	-	-
Sex ratio (M/M +F)	0.83	0.66	0.71		
Dead pups on PND1 to PND5	0	0	0	-	-

Table 3: Effect of developmental exposure of MeHg on spontaneous locomotor activity (Distance travelled, Rearing and Resting time) in the open field on PND21.

MeHg Dosage (mg/kg/day) during gestation	Control	0.5mg/kg	1.5mg/kg
Distance Travelled (cm)	61±14.3	138±26.1*	175±9.2*#
Rearing time (seconds)	3.3±1.2	7.2±1.0*	12.1±2.1*#
Resting time (seconds)	9.5±1.5	4.2±1.2*	1.4±0.2*#

condition of these animals, these were euthenized in the expected lactation (LD 0 or 1) period *(Table 2).*

Embryo/Foetal Toxicity

In 1.5 mg/kg dosage MeHg treated group, there were significant numbers ($p<0.05$) of post-implantation loss, which resulted in total resorptions or dead foetuses when compared to control *(Table 2).* Data represents the mean±SD). *$p<0.05$, Significantly different from control, using one way ANOVA with Tukey's *post hoc* test.

Neurobehavioural toxicity.

The assessment of motor coordination was done by using rota rod on PND 21. The time spent on the rod was found significantly less in MeHg induced offspring in comparison to the control group ($p<0.001$). Latency time to fall was significantly reduced ($p<0.001$) in 1.5mg/kg when compared to 0.5mg/kg *(Fig.6).* Nociception or the ability for sensation of pain was studied using hot plate test. The time required to show symptoms of pain sensation like paw licking, jumping out of beaker etc was more for MeHg induced offspring when compared to control group ($p< 0.01$ and $p<0.001$ for 0.5mg/kg and 1.5 mg/kg MeHg dosage group respectively in comparison to control group). Among the doses higher dose (1.5 mg/kg) showed significant impairment of nociception ($p<0.001$) *(Fig 7).*

Rota rod test data represents the mean±SD . ***$p< 0.001$ is significantly different from control group and $^{###}p< 0.001$ is significantly different between 0.5mg/kg and 1.5mg/kg dosage groups, using one way ANOVA with Tukey's *post hoc* test.

Data represents the mean±SD. ***$p< 0.001$ is significantly different from control group and $^{###}p< 0.001$ is significantly different between 0.5mg/kg and 1.5mg/kg dosage groups, using one way ANOVA with Tukey's *post hoc* test. The open field is a test of spontaneous locomotor activity. Three measures of activity were assessed in this test during the PND21: distance travelled (in cm), number of rearing movements (in numbers), and resting time (RT) spent in the centre of the chamber (in seconds). Distance travelled and rearing movements were significantly increased in MeHg exposed offspring than the controls ($p<0.05$) The resting time was found to be significantly decreased ($p<0.05$) in both 0.5mg/kg and 1.5mg/kg dosage group in comparison to control offspring. The results of open field test were significant in 1.5mg/kg dosage group than 0.5mg/kg ($p<0.05$) *(Table 3).* Data represents the mean±SD). *$p<0.05$, Significantly different from control, $^{\#}p<0.05$, significantly different between 0.5mg/kg and 1.5mg/kg dosage groups, using one way ANOVA with Tukey's *post hoc test.*

Impairment in spatial learning and memory was studied using morris water maze test. The escape latency time taken by the MeHg exposed offspring was more than the control offspring. The time required to find out the hidden platform was significantly increased in both 0.5mg/kg dosage ($p< 0.01$) and 1.5mg/kg dosage group ($p<0.001$) when compared to control offspring. 1.5mg/kg dose caused high escape latency time than 0.5mg/kg dosage group ($p<0.001$) *(Fig8).* Forced swim test measured the depression level of the animal. MeHg induced offspring group, both in 0.5 and 1.5mg/kg dosage showed significantly longer immobility time ($p< 0.01$) than the control pups. 1.5mg/kg dosage group showed significant immobility time than 0.5mg/kg dosage group *(Fig9).*

Morris water maze test data represents the mean±SD Data. **$p< 0.01$, ***$p< 0.001$ is significantly different from control group and $^{###}p< 0.001$ is significantly different

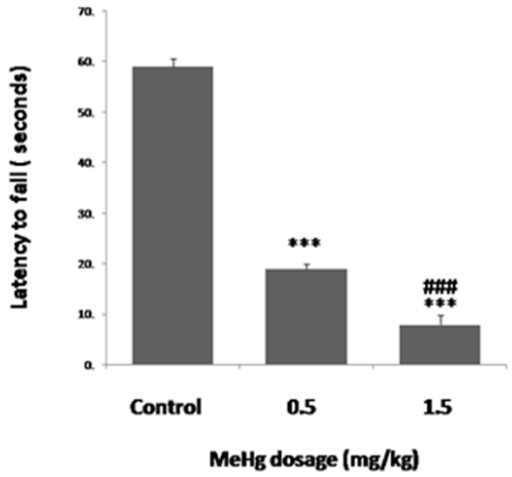

Figure 6: Effects of gestational MeHg exposure on motor co-ordination of the rat offspring on PND21.

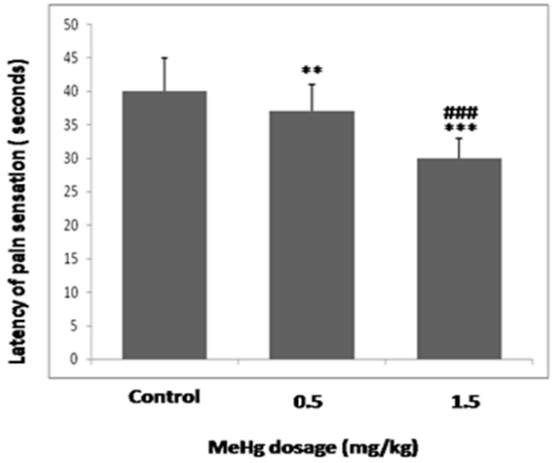

Figure 7: Effects of gestational MeHg exposure on nociception of the rat offspring on PND21.

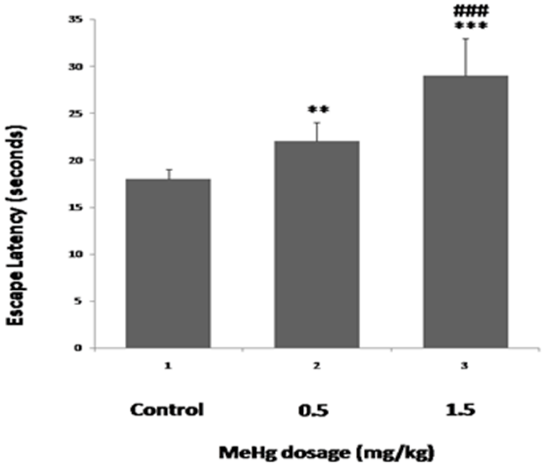

Figure 8: Effects of gestational MeHg exposure on learning and memory of the rat offspring on PND21.

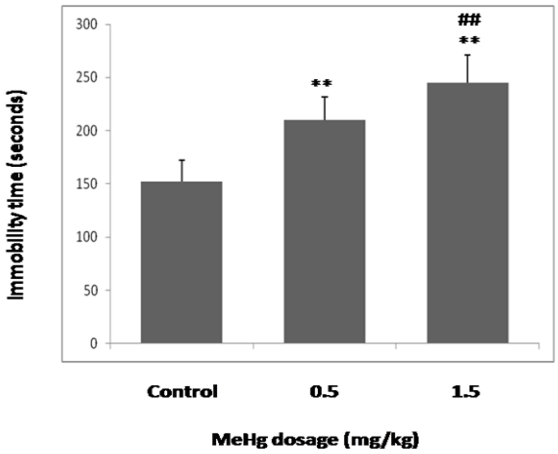

Figure 9: Effects of gestational MeHg exposure on depression symptoms of the rat offspring on PND21.

between 0.5mg/kg and 1.5mg/kg dosage groups, using one way ANOVA with Tukey's *post hoc* test. Forced swim test data represents the mean±SD $^{**}p< 0.01$ is significantly different from control group and $^{\#\#}p< 0.001$ is significantly different between 0.5mg/kg and 1.5mg/kg dosage groups, using one way ANOVA with Tukey's *post hoc* test.

Histopathology

In control rat offspring, cytoarchitecture was preserved and there was no significant deformalities. In 0.5mg/kg group, there was moderate neuronal degeneration which was reflected as focal neuronal loss in cerebral cortex, cerebellum and hippocampus. Where as in the higher dosage (1.5mg/kg) group, there was significant extensive neuronal degeneration characterised by wide spread neuronal loss. Cerebral cortex showed appearance of gliosis in a 1.5mg/kg dosage group *(Fig10).* Changes in cellular architecture of cerebral cortex, cerebellum and hippocampus due to developmental exposure of MeHg are shown. Prominently seen lesion was the appearance of gliosis in 0.5mg/kg and was more significant in 1.5mg/kg dosage group (showed in arrow). Cellular architecture was preserved in the control rats. In 0.5mg/kg group, there was slight neuronal degeneration characterized by focal neuronal loss in cerebral cortex, cerebellum and hippocampus. Where as in the higher dosage (1.5mg/kg), there was significant neuronal degeneration characterised by wide spread neuronal loss (showed in arrow).

DISCUSSION

The current study focussed on the developmental neurotoxicity caused by MeHg in developing rat offspring brain. We administered four different doses of MeHg in drinking water to the pregnant wistar rats from GD 5 till parturition. The dosage was fixed after conducting preliminary experiments based on the literatures showing that at this level of exposure, the mercury concentration in newborn rat is comparable to that one found in human infants born in populations that highly consume mercury contaminated fish[35]. The exposure paradigm used in the present study exactly mimicked the ways of human exposure like chronic consumption of contaminated sea foods, fish and mercury contaminated ground water exposure etc[36, 37]. Four different doses were selected ranging from less toxicity (0.5mg/kg and 1.5mg/kg) to significant toxicity (2.5mg/kg and 3.5mg/kg) to study the dose dependent effects in the offspring brain[38]. The highest dosage of 3.5 mg/kg caused 100% death of the foetus in the womb itself (total resorption of pups) whereas 2.5

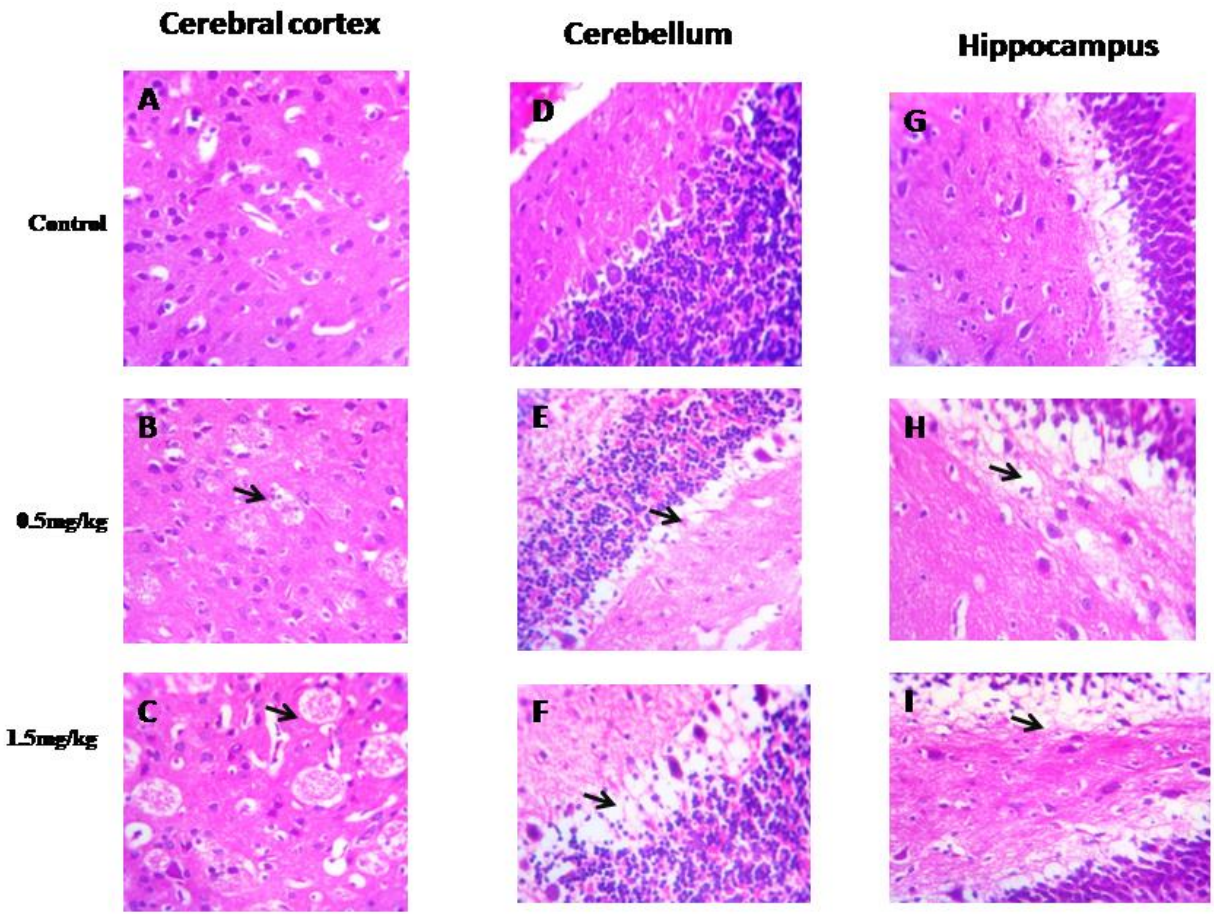

Figure 10: Effect of prenatal exposure of MeHg on the cellular architecture of the rat offspring on PND21.

mg/kg dosage group showed a significant extension of gestational length, (28 days) and all the pups were died within two days of delivery. Failure to deliver in time or unsustainability of live pups emphasizes the extreme toxicity at 2.5 mg/kg dosage of MeHg, which is in concordance with the study of Gandhi DN, 2013[34]. The present study outcomes can be related to the human population study that showed still birth, miscarriages or no delivery of pups due to exposure to high doses of MeHg through excessive intake of contaminated fish and sea foods[35]. In a previous study, total resorption of pups occurred at a dosage of 2 mg/kg of MeHg through oral gavage, in contrast to that, our study showed total resorption of pups at 3.5 mg/kg through drinking water exposure paradigm. This in turn proves the idea that the selection of dosage, pattern of administration and time period of exposure of MeHg can cause differences in the study results. We couldn't find any significant changes in brain and body weight of pups in comparison to control which is supported by the past studies in this area[34]. The impact of transplacental exposure of MeHg on anatomical, functional and biochemical changes were studied on the weaning day of rat offspring. Weaning is the period in which the rats are not dependent on mother's milk anymore and it happens after 21 days of birth[36]. Effectiveness of the MeHg exposure paradigm used in the experiment was confirmed by measuring the accumulation of mercury in whole brain of the offspring during weaning (PND 21). Transplacental exposure of MeHg via drinking water caused accumulation of mercury, though the values were near normal, in the offspring brain compared to control even around weaning age. This is an evidence for the fact that drinking water exposure paradigm used in the present study was effective for methyl mercury to cross the placental and blood brain barriers of the developing offspring. Previous metal toxicity studies have showed that long half-lives of elimination of the metal from brain causes its accumulation in brain and the consequent nervous system damage results in behavioural manifestations which are often silent, significantly delayed, and irreversible[37,39,40]. The first and very obvious consequences exhibited by the developmental exposure of MeHg are neurobehavioural disorders in the progeny of the exposed mother. Morris water maze test is for assessing learning and memory of the animal. Increased time latency taken to find out the hidden platform in MeHg administered group showed impairment of memory and learning process. Hippocampus is found to be the center for regulation of cognitive functions. Hippocampus development occurs during the first two weeks of postnatal days in rats[27]. Thus weak performance in morris water maze test in both 0.5mg/kg and 1.5 mg/kg MeHg dosage group shows impact of MeHg toxicity even after the stoppage of exposure. Time required to find out the hidden platform was more for the MeHg treated groups when compared to the control groups. An increase in the

immobility time in forced swim test showed alteration of brain center controlling the psychological behaviour like depression. Motor activity was significantly altered in MeHg exposed offspring than control offspring. The decreased motor activity due to prenatal exposure of MeHg in rat offspring when compared to control is supported by the previous studies[32]. The nociception of the rats were also affected due to gestational exposure of MeHg as the rats showed increased time latency in the hot plate test. Our results on nociception test goes hand in hand with the earlier findings based on tail flick test in prenatally exposed rat progeny [26]. Locomotion frequency were measured as distance travelled, immobility or resting time and rearing in the open field and is been used as an index of both locomotor activity and emotionality. In male rat offspring, a significant increase in spontaneous motility was observed and a significant increase in rearing was observed in either sex of the MeHg induced rat pups. Taken all together, these findings shows that during development, low dosage of MeHg cause neurotoxic effects that are manifested during the commencement of adolescence, and that susceptibility is gender-dependent. These results are matching with the findings of studies conducted by prenatal exposure to MeHg in rat pups given through gavage[25,34,]. Abnormal neurobehavioral development made us to study the alterations in the ultrastructure of specific brain regions due to transplacental exposure of MeHg. Histopathological changes observed in the cerebral cortex, cerebellum and hippocampus can be attributed to the motor and cognitive behavioural deficits shown by the offspring. Extensive neuronal degeneration was reflected as widespread neuronal loss especially in the purkinje layer of cerebellum and hippocampus in a dose dependent manner and it supports the similar findings of previous studies[41, 42]. The Appearance of gliosis in the cerebral cortex proves the deleterious effect of MeHg on astrocytes during development. The severity of the lesions due to gliosis seemed to increase in proportion to the administered dose of methyl mercury, a highest occurance of gliosis occurred at 1.5mg/kg. Significant gliosis was found in the rat offspring brain in the earlier studies using different doses of MeHg in drinking water[43]. Evidence of morphological damage in the specific cognitive and motor centers of brain made us to further assess the biochemical changes elicited by MeHg in these regions during development. Reactive oxygen species (ROS) formation is one of the toxic effects of MeHg exposure. However, cells can neutralize the harmful effects of ROS and detoxify themselves via a variety of cellular antioxidative defense systems that employ enzymatic and non-enzymatic components to prevent lipid peroxidation damage[44]. Antioxidants such as glutathione (GSH), glutathione peroxidase (GPx), superoxide dismutase (SOD), and catalase (CAT) are widely used as antioxidative biomarkers to investigate the toxic effect of metals[45]. In the present study an elevated level of LPO in all the three specific regions proves the abnormal ROS generation in the pups due to prenatal MeHg exposure. The present data together with our previously published results suggests that the deleterious effects of MeHg during development may be related to increased oxidative stress caused by inhibition of non-enzymatic antioxidant GSH and enzymatic antioxidants like GPx, SOD, CAT in cerebral cortex, cerebellum and hippocampus. Studies on MeHg developmental toxicity gives contradictory results. When most of the results of the present study goes parallel to many previous studies, a few literatures give slightly different outcomes of MeHg developmental neurotoxcity. Histopathological changes in the cerebellum showed contradictory findings in a few studies, similarly some previous studies showed no change in motor activity of the offspring. This difference in the study results are because, of the differences in selection of dose levels, manner of administration, duration of exposure etc which will directly impact the outcomes being measured.

CONCLUSION

The findings of the present study suggest that transplacental exposure of MeHg alters antioxidant balance of the offspring brain but cause no significant differential changes in the oxidative stress responses between cerebral cortex, cerebellum and hippocampus around the time of weaning (PND 21). Also gestational exposure of MeHg changes the cellular morphology of motor and cognitive centers of the weaning rat. We couldn't grade the severity of cellular lesions to a particular brain region among cerebral cortex, cerebellum and hippocampus regions, but showed appearance of gliosis in cerebral cortex of the F1 generation of high MeHg dosage group (1.5mg/kg). Our findings help to conduct more molecular and gene level studies which can help to elucidate MeHg developmental neurotoxicity mechanism in future. The neurobehavioural toxicity exhibited by the rat offspring suggest that even low concentration of prenatal methyl mercury exposure (0.5mg/kg) can affect the motor and cognitive centers of the brain. Understanding the cellular mechanisms and the behavioral outcomes of methyl mercury is helpful to reduce the morbidity and dysfunction associated with methylmercury exposure by creating new avenues of prevention and treatment.

ACKNOWLEDGEMENT

The first author acknowledges the DST-PURSE for funding the Research and awarding Junior Research fellow (JRF) to the author under this project.

Conflict of interests

The authors declare that they have no conflict of interest.

REFERENCES

1. Jiang GB, Shi JB, Feng XB (2006) Mercury pollution in China. Environmental science and technology 2006; 40: 3672–3678
2. Mergler D, Anderson HA, Chan LHM et al. Methylmercury exposure and health effects in humans: a worldwide concern. Ambio 2007; 36: 3–11
3. Kocman, D, Horvat, M, Kotnik, J. Mercury fractionation in contaminated soils from the Idrija

mercury mine region. Journal of Environmental Monitoring. 2004; 6:696-703
4. Heileen Hsu-Kim, Katarzyna H. Kucharzyk, Tong Zhang, Marc A. Deshusses. Mechanisms regulating mercury bioavailability for methylating microorganisms in the aquatic environment: A critical review. Environ. Sci. Technol. 2013; 47: 2441–2456
5. Madenjian C.P, O'Connor D.V. Trophic transfer efficiency of mercury to lake whitefish coregonus clupeaformis from its prey. Bull Environ Contam Toxicol. 2008; 81: 566-570
6. Daniel Cossa, Lars-Eric Heimbürger, Delphine Lannuzel. Mercury in the Southern Ocean. Geochimica et Cosmochimica Acta. 2011; 75: 4037–4052
7. Kimio Maruyama,Takashi Yorifuji, Toshihide Tsuda, Tomoko Sekikawa, Hiroto Nakadaira, Hisashi Saito. Methyl Mercury Exposure at Niigata, Japan: Results of Neurological Examinations of 103 Adults. Journal of Biomedicine and Biotechnology. 2012; doi:10.1155/2012/635075
8. Harada M. Minamata disease: methylmercury poisoning in Japan caused by environmental pollution. Crit Rev Toxicol. 1995; 25: 1-24
9. Amin-Zaki L, Elhassani S, Majeed MA, Clarkson TW, Doherty RA, Greenwood M. Intra-uterine methylmercury poisoning in Iraq. Pediatrics. 1974;54:587-95
10. Cheng-Kuan Su, Cheng-Hung Yang, Cheng-Hsing Lin, Yuh-Chang Sun. In-vivo evaluation of the permeability of the blood–brain barrier to arsenicals, molybdate, and methylmercury by use of online microdialysis–packed minicolumn–inductively coupled plasma mass spectrometry. Analytical and Bioanalytical Chemistry.2014; 406: 239-247
11. Kajiwara Y, Yasutake A, Adachi T, Hirayama K . Methylmercury transport across the placenta via neutral amino acid carrier. Arch Toxicol . 1996;70:310-4
12. Ceccatelli S, Dare E, Moors M. Methylmercury-induced neurotoxicity and apoptosis. Chem Biol Interact. 2010;188: 301–308
13. Clarkson TW, Magos L, Myers GJ. The toxicology of mercury–current exposures and clinical manifestations. N Engl J Med. 2001; 349: 1731–1737
14. Oken E, Radesky JS, Wright RO, Bellinger DC, Amarasiriwardena CJ, Kleinman KP, Hu H, Gillman MW. Maternal fish intake during pregnancy, blood mercury levels, and child cognition at age 3 years in a US cohort. Am J Epidemiol. 2008; 167:1171–1181
15. Ali SF, LeBel CP, Bondy SC. Reactive oxygen species formation as a biomarker of methylmercury and trimethyltin neurotoxicity. Neurotoxicology. 1992; 13:637-648
16. Yee S, Choi BH. Oxidative stress in neurotoxic effects of methylmercury poisoning.Neurotoxicology. 1996; 17: 17–26
17. O'Reilly, Randall C, Rudy, Jerry W. Conjunctive representations in learning and memory: Principles of cortical and hippocampal function. Psychological Review. 2001;108:311-345
18. Catherine J. Stoodley, Jeremy D. Schmahmann. Evidence for topographic organization in the cerebellum of motor control versus cognitive and affective processing. Cortex. 2010; 46:831–844
19. W Singer. Synchronization of Cortical Activity and its Putative Role in Information Processing and Learning. Annual Review of Physiology. 1993; 55: 349-374
20. Tereza Nekovarova, Jan Nedvidek, Daniel Klement, Jan Bures . Spatial decisions and cognitive strategies of monkeys and humans based on abstract spatial stimuli in rotation test. Proc Natl Acad Sci 2009; 106: 15478–15482.
21. Emily B. Bisen-Hersh, Marcelo Farina, Fernando Barbosa et al. Behavioral Effects of Developmental Methylmercury Drinking Water Exposure in Rodents. J Trace Elem Med Biol 2014; 28: 117–124
22. Zhang Y, Jiang X, Zhao X et al. Time-course effect and region-specificity of endoplasmic reticulum stress in rat brains acutely exposed by methylmercury. Wei Sheng Yan Jiu 2010; 39:271-4
23. Rustay NR, Wahlsten D, Crabbe JC. Influence of task parameters on rotarod performance and sensitivity to ethanol in mice. Behav Brain Res. 2003;141:237-49
24. Hamm RJ, Pike BR, O'Dell DM et al. The rotarod test: an evaluation of its effectiveness in assessing motor deficits following traumatic brain injury. J Neurotrauma. 1994;11: 187–96
25. Gandhi D. N, Dhull Dinesh K . Postnatal Behavioural Effects on the Progeny of Rat after Prenatal Exposure to Methylmercury. American Journal of Experimental Biology 2014;1:31-51
26. Beyrouty P, Stamler CJ, Liu JN, Loua KM, Kubow S, Chan HM. Effects of prenatal methylmercury exposure on brain monoamine oxidase activity and neurobehaviour of rats. Neurotoxicol Teratol.2006; 28:251-9
27. Falluel-Morel A, Sokolowski K, Sisti HM et al. Developmental mercury exposure elicits acute hippocampal cell death, reductions in neurogenesis, and severe learning deficits during puberty. J Neurochem. 2007; 103:1968-81
28. Porsolt RD, Bertin A, Jalfre, M. Behavioral despair in mice: A primary screening test for antidepressants. Arch. Int. Pharmacodyn. Ther. 1977;229: 327–336
29. Natalia Onishchenko, Christoffer Tamm, Marie Vahter et al. Developmental exposure to methylmercury alters learning and induces depression-like behavior in male mice. Toxicological sciences. 2007; 97:428–437.
30. Okhawa H, Ohishi N, Yagi K. Assay for lipid peroxides in animal tissues by thiobarbituric acid reaction. Anal Biochem. 1979; 95: 351–358.
31. Moron M, Depierre J W, Mannervik BT. Levels of glutathione, glutathione reductase and glutathione S-transferase activities in rat lung and liver. Biochim Biophys Acta. 1979; 582:67–78.
32. Rotruck JT et al. Selenium: biochemical role as a component of GPx. Science. 1973;179:588–590.
33. Marklund. S, Marklund, G. Involvement of the superoxide anion radical in the autoxidation of

pyrogallol and a convenient assay for superoxide dismutase. Eur.J Biochem. 1974; 47: 469–474.
34. Gandhi D.N, Panchal G.M, Dinesh K.Dhull. Influence of gestational exposure on the effects of prenatal exposure to methyl mercury on postnatal development in rats. Cent Eur J Public Health. 2013; 21: 239–243.
35. Harada M. Minamata disease: methylmercury poisoning in Japan caused by environmental pollution. Crit Rev Toxicol. 1995; 25:1-24.
36. Catherine P. Crameredda Thiels Jeffrey R. Alberts. Weaning in Rats: I. Maternal Behavior Developmental Psychobiology. 1990; 23:479-493.
37. Seongil Jo, Hae Dong Woo, Ho-Jang Kwon. Estimation of the Biological Half-Life of Methylmercury Using a Population Toxicokinetic Model. Int J Environ Res Public Health. 2015; 12: 9054–9067.
38. Amin-Zaki L, Majeed MA, Elhassani SB, Clarkson TW, Greenwood MR, Doherty RA. Prenatal methylmercury poisoning. Clinical observations over five years. Am J Dis Child. 1979; 133:172-7.
39. Rossi AD, Ahlbom E, Ogren SO, Nicotera P, Ceccatelli S. Prenatal exposure to methylmercury alters locomotor activity of male but not female rats. Exp Brain Res. 1997;117:428-36.
40. Nielsen JB, Andersen O. The toxicokinetics of mercury in mice offspring after maternal exposure to methylmercury–effect of selenomethionine. Toxicology. 1992;74: 233–41.
41. Mineshi Sakamoto, Koichi Wakabayashi, Akiyoshi Kakita. Widespread neuronal degeneration in rats following oral administration of methylmercury during the postnatal developing phase: a model of fetal-type Minamata disease.Brain Research. 1998; 784 :351–354.
42. Akiyoshi Kakita, Koichi Wakabayashi, Mu Su. Distinct pattern of neuronal degeneration in the fetal rat brain induced by consecutive transplacental administration of methylmercury Brain Research.2000;859:233–239.
43. Kakita, Akiyoshi MD, Wakabayashi, Koichi MD et al. Experimentally Induced Leptomeningeal Glioneuronal Heterotopia and Underlying Cortical Dysplasia of the Lateral Limbic Area in Rats Treated Transplacentally with Methylmercury. Journal of Neuropathology & Experimental Neurology. 2001; 60:768–777.
44. Sarvajeet Singh Gill, Narendra Tuteja. Reactive oxygen species and antioxidant machinery in abiotic stress tolerance in crop plants. Plant Physiology and Biochemistry. 2010; 48: 909-930.
45. John Sushma, S. Priyanka, K. Jayantha Rao. Neuroprotective role of Melatonin against aluminum-induced oxidative stress in the hippocampus of mouse brain. Journal of Applied Pharmaceutical Science. 2011; 10:126-133.