Ann Ist Super Sanità 2014 | Vol. 50, No. 1: 28-37
DOI: 10.4415/ANN_14_01_05

Neurobehavioral toxicity in progeny of rat mothers exposed to methylmercury during gestation

Dinesh N. Gandhi[a], Govind M. Panchal[a] and Dinesh K. Dhull[b]

[a] Department of Neurobehavioral Toxicology, National Institute of Occupational Health (ICMR), Meghaninagar, Ahmedabad, Gujarat, India
[b] Department of Neurophysiology (Glial Cells), National Institute of Mental Health and Neurosciences (NIMHANS), Bangalore, India

Abstract

Introduction. Methylmercury (MeHg) is recognized as one of the most hazardous environmental pollutants. This may be a concern to long-term consumption of contaminated fish and seafood for health risk to pregnant women and their children.

Aim. An animal study was conducted to assess the effect of MeHg exposure on rodent offspring following *in utero* exposure.

Methods. Pregnant Wister rats were treated by gavage with MeHg at dose levels of 0.5, 1.0 and 2.0 mg/kg/day from gestation day (GD) 5 till parturition, and then were allowed to deliver.

Results. Dams treated with 2.0 mg/kg/day MeHg group showed signs of toxicity such as gait alterations and hyperactivity resulting in the failure to deliver sustainable viable pups. MeHg had significant effects on body weight gain of dams during GD 5 till parturition. MeHg had no significant effects on the ages of physical developments such as pinna detachment, incisor eruptions or eye opening as well as alter cliff avoidance, surface righting, swimming ontogeny, startle reflex, pivoting, negative geotaxis, or forelimb and hindlimb grip strength in either sex. Exposure to 1.0 mg/kg/day MeHg treatment group prolonged gestation period, retard mid-air righting in male pups, shortened forelimb grip strength measured on rotating rod in either sex and enhanced open field behaviour in male pups. Data obtained from Functional Observation Battery (FOB) also revealed impairment of neuromotor performance in male pups. The male pups appeared to be more susceptible than the female pups.

Conclusion. Overall, the dose level of MeHg in the present study produced a few adverse effects on the neurobehavioral parameters, and it may alter neuromotor performance of the male pups.

Key words
- methylmercury
- neurobehavioral toxicity
- developmental
- prenatal
- rat

INTRODUCTION

In the 1950s and '60s neurological disease was noted in many people living around Minamata Bay in Japan. The disease was traced to methylmercury (MeHg) pollution that accumulated in fish (10-40 ppm). Principal sources of exposure to mercury compound in the general population are ingestion and inhalation of mercury from dental amalgams, and ingestion of fish (fresh water and marine) and seafood. The effect of low-dose *in utero* exposure to mercury on neurological development in school- age children in the Faroe Islands was reported [1]. MeHg is toxic to embryotoxic and fetal tissues and can induce embryonal and teratogenic effects in golden hamsters [2], cats [3], rats [4], and mice [5, 6]. Exposure to toxic elements such as mercury [7, 8] or arsenic [9] during gestation and lactation may potentially cause adverse effects on the development of foetuses and neonates. Development delays in acquiring motor skills associated with low to moderate prenatal MeHg exposure are known [10]. Behavioural alterations were observed, even at MeHg levels below those causing morphological abnormalities [11].

There is increased interest towards animal models in developmental neurotoxicity, after having revealed that prenatal administration of different drugs, neurotoxic agents such as alcohol, amphetamine, morphine, heroin, methadone, pesticides and metals, induces subtle neurobehavioral impairments and delayed development of nervous system functions without any morphological malformation. Researchers have focused on the functions that should be included in behavioural test battery such as sensory systems, neuromotor development,

Address for correspondence: Dinesh N. Gandhi, Scientist 'D' & Head, Department of Neurobehavioral Toxicology, National Institute of Occupational Health (ICMR), Meghaninagar, Ahmedabad-380 016, Gujarat, India. E-mail: d_gandhi28@yahoo.co.in.

locomotor activity, learning and memory, reactivity and habituation. Using this Functional Observation Battery (FOB) [12] can be tested in rat pups in different postnatal days in order to assess the properties of neurotoxicity.

Therefore, the objectives of the present study was three-fold: (a) to establish the experimental design that can be better representative of possible human exposures, (b) to investigate at which developmental stage, MeHg causes neurotoxicity to the rat's fetuses and (c) to assess whether *in utero*/gestational MeHg exposure has a detrimental impact on early physical and neurobehavioral outcomes. Within this context, we performed an *in vivo* evaluation of behavioural toxicity on some endpoints, in order to understand its further developmental effects.

MATERIALS AND METHOD

Animals and maintenance: Albino rats of Wistar strain (12-14 weeks of age, 180-200 g) were procured from Animal House, National Institute of Occupational Health (NIOH), Ahmedabad, India. Before starting the experiment, the animals were kept in laboratory conditions for a period of 7 days for acclimatization. Wistar Albino GD 0 female rats were housed in individual shoebox size polypropylene cages with sterilize bedding. 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 (Pranav Agro Industries Ltd., ISO9001 Certified Company, Maharastra, India) and Kent RO water. Each food pallet contains: 22-23% protein; 4.20% fat; 3.50% fibre; 2.10% calcium; 1.05% phosphorus; 7.50% total ash; 8.68% moisture and 56% carbohydrate. All experiments were performed between 9.00 and 17.00hr. All animal experiments were performed according to the ethical guidelines suggested by the Institutional Animal Ethics Committee (IAEC) of the National Institute of Occupational Health and Committee for the Purpose of Control and Supervision of Experiments on Animals, Ministry of Environment and Forests, Government of India (CPCSEA, 2003, Registration No.111/RO/c/1999/CPCSEA) and were conducted according to the Indian National Science Academy guidelines (INSA, New Delhi, India) for the use and care of experimental animals, chemical, dose and treatment schedule.

Pregnant rats: after one week of acclimatization, proestrus virgin female rats, weighted 200 ± 15 g, were mated with proven fertile male rats (2:1) overnight from our Institutional (National Institute Of Occupational Health, Ahmedabad, India) Animal House Breeding Colony. The day of mating, confirmed by the presence of sperm positive vaginal smears, was designated as gestational day (GD) 0. Pregnant animals were randomly assigned to 4 groups of 7-10 rats each in a house individually. Date of birth was designated as postnatal day (PND) 0.

Chemicals and dosing: pregnant animals were treated with MeHg (Sigma Aldrich, USA) at doses of 0.5, 1.0 and 2.0 mg/kg/day by gavages from GD 5 till parturition. Control group received 0.9% saline water throughout treatment period.

Data collection: all pregnant rats were allowed to deliver. At birth, the following data were recorded: live births, still birth, litter size, sex ratio, dead pups on PND 1, body weight of pups on PND 1, body length (PND 1), Tail length (PND 1), gestational age, maternal weight (GD 0), maternal weight on GD 20, maternal weight gain (%) and any malformations.

Observation

Neurobehavioral evaluation of pups: confirmed, 29 GD 0 female dams were evaluated in the present study and assigned to one of the following four treatment groups: Group I: Control- No treatment (0.9% saline water; n = 7); Group II (MeHg, 0.5 mg/kg/day; n = 8); Group III (MeHg, 1.0 mg/kg/day; n = 7) and Group IV (MeHg, 2.0 mg.kg/day; n = 7). Dams were weighed and dose daily from GD5 to till parturition. Each group was allowed to deliver the pups. Group I Control dams (n = 7) delivered 66; Group II MeHg 0.5 mg/kg/day dams (n = 8) delivered 80; Group III MeHg 1.0 mg/kg/day dams (n = 7) delivered 73 and Group IV MeHg 2.0 mg/kg/day dams (n = 7) delivered 0 pups respectively. Out of 29 GD 0 female dams, 22 pregnant female dams delivered the pups in observed hundred percentage of resorption of the pups, in the rest of the 7 pregnant female dams treated with MeHg 2.0 mg/kg/day. In the present study, finally 22 pregnant female dams (Control n = 7; MeHg 0.5 mg/kg/day, n = 8 and MeHg 1.0 mg/kg/day, n = 7) delivered total 219 pups respectively. Out of 219 pups, twenty pups (20) either sex (male = 10 and female = 10) from each treatment groups were randomly selected to perform functional and behavioural development measurements on certain postnatal days. Constant experimenters who were blind to the exposure doses completed all procedures. The selected parameters and score methods are described below:

Somatic growth and maturation (PND 1-30): from GD 5, the pregnant rats were daily examined for overt signs of toxicity. Ages (PND) on which pups in each group were observed at first appearance of pinna detachment (PND 2-5); incisor irruption (PND 6-7); eye opening (PND 11-16); development of fur (PND 9); ear unfolding (PND 2) [13, 14]; testes descent (PND 25) and vaginal opening (PND 30) [15].

Neuromotor and reflex development: Surface righting reflex (PND 4, 6, 8) – the pup's ability to turn over from supine position at surface level; Mid-air righting reflex – the pup's ability to turn over in mid-air from supine position; Pivoting – pup's circular movement with no forward or backward propulsion; Swimming ontogeny (PND 6) – the pup's ability to swim; Negative geotaxis (PND 10) – the pup's ability to turn 180° on a 25° incline placed head down; Forelimb and hind limb grip strength (PND 10) – ability to hold on to a thin wire; Forelimb grip strength (PND 10) – performance on rotating rod (PND 20) were assessed (Columbus Instrument, Columbus, Ohio, USA).

Sensory function: Startle reflex (PND 7, 15) – the presence or absence of sensorimotor reaction (jerks) to auditory stimulus.

Activity and emotional reactivity: exploratory and stereotypic behaviour were assessed by an open field activity monitor system (Columbus Instrument, Columbus, Ohio, USA), measuring intensity of motor activity, ambulatory activity, rearing and stereotype behaviours of pups.

Functional Observation Battery (FOB)

Detailed clinical observations: detailed clinical examination included identification of clinical signs related to: general appearance, body position and posture, autonomic nervous system function, motor coordination, ambulatory abnormalities, reaction to being handled and to environmental stimulation, nervous system (*e.g.*, tremor, convulsion, muscular contractions), changes in exploratory behaviour, abnormal behaviour (*e.g.*, autophagia, backward motion, abnormal vocalization) and aggression.

Neurobehavioral assessment on PND 29: FOB [16, 17] and motor activity tests were conducted on all rats assigned to each dosage level on the 4th week of the postnatal period. During each of the test periods, the behavioural tests were conducted on the rats. Dosage groups and gender (n = 10 in either sex) were counterbalanced across the test sessions. The motor activity and FOB evaluations were conducted at approximately the same time of day, across all test sessions.

A single trained observer unaware of the group assignment of each rat conducted the FOB. The order in which rats from different dosage groups were tested was randomised. Evaluation of each individual rat was conducted at the home cage during handling of the rat, for a 2-min period in an open field (85 cm x 50 cm x 13 cm), and following reactivity and sensitivity testing. The FOB evaluation lasted approximately one to one and a half hours, and included the following parameters:

1 lacrimation, salivation, palpebral closure, prominence of the eye, pupillary reaction to light, piloerection, respiration, and urination and defecation (autonomic functions);
2 sensorimotor responses to visual, acoustic, tactile, and painful stimuli (reactivity and sensitivity);
3 reactions to handling and behaviour in the open field (excitability);
4 gait pattern in the open field, severity of gait abnormalities, air-righting reaction, and visual placing response;
5 landing foot splay (gait and sensorimotor coordination);
6 forelimb and hindlimb grip tests.

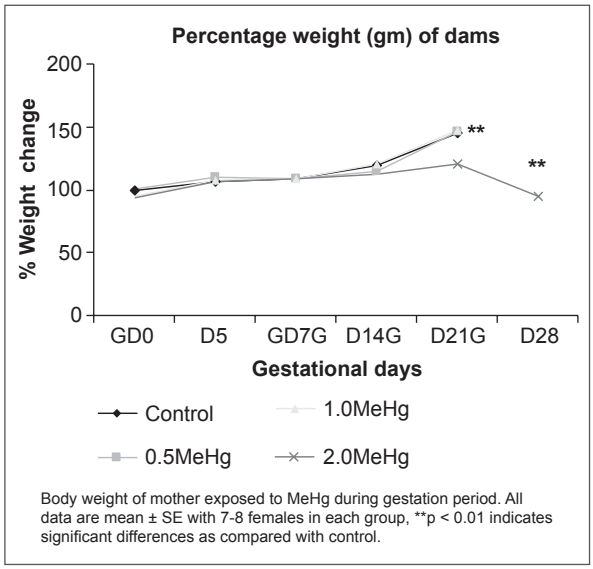
Statistical analysis

Data from non-pregnant F0 (exposed pregnant dams) animals were excluded from statistical analysis. With the exception of adult measurements (body weights and neurobehavioral assessments) on the F1 (first generation of FO dams, *i.e.* pups/offspring) generation, when a statistical analysis included measurements on multiple offspring from the same litter, the litter was used as the experimental unit and accounted for in the statistical analysis. A number of end points were evaluated using different statistical models *(Table 1)*. Descriptive statistics were calculated for each variable. The data obtained from either sex (10 males; 10 females) in the experiments were expressed as the mean ± SEM, unless otherwise stated. F0 body weight gain GD 0-20; gestational length and litter size data were analyzed by 3 (MeHg or water) x 2 (sex) x 7 (measures) ANOVA with the measures factor treated as within subject factor using the litter weight gain, day and litter size respectively. The data from the date of appearance of each reflex and physical developments were analyzed by 3 (MeHg or water) x 2 (sex) x Time ANOVA using the mean litter score (seconds) in either sex except cliff avoidance, startle reflex and swimming ontogeny ANOVA data the percentage. Negative geotaxis data were analyzed by 3 (MeHg or water) x 2 (sex) x 10 males/10 females with repeated measures ANOVA with the treatment as a between-subjects factor and that day as the repeated measures factor, followed by Tukey's *post-hoc* test. Latency to rim escape was analyzed by 3 (MeHg or water) x 2 (sex) ANOVA using the mean litter score (seconds). Open field data such as distance travel (DT in cm); resting time (RT in time); rearing or jumps (V1C in number) as well as FOB data (forelimb grip strength(g); hindlimb grip strength(g); hindlimb splay(cm); rearing(no.) were analyzed by 3 (MeHg or water) x 2 (sex) X repeated ANOVA using the mean litter score for either sex F1 offspring. The comparison among control and MeHg exposed groups was carried out by means of One-way ANOVA, followed by Tukey's HSD *post-hoc* test [1-6]. We used Kruskal-Wallis H test for comparing pup viability: males per litter. MeHg 0.05 was considered significant.

RESULTS

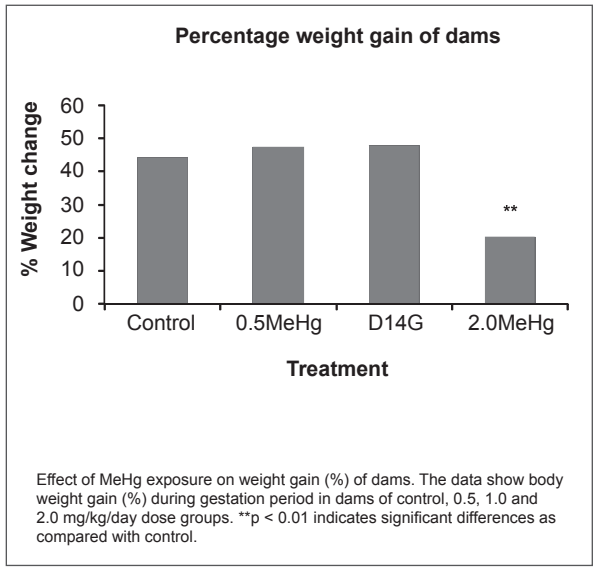
Maternal health status and reproduction outcome

Pregnant females (dams) were divided into four groups of 20 animals; Control (with free access to fresh 0.9% saline water); 0.5 mg/kg/day MeHg; 1.0 mg/kg/day MeHg and 2.0 mg/kg/day MeHg by oral gavages. Dam's body weight as well as weight gain was noted during gestation. After birth the number of pups for each group was as follow: Control (n = 66), 0.5 mg/kg/day MeHg (n = 80), 1.0 mg.kg/day MeHg (n = 73) and 2.0 mg/kg/day MeHg (n = 0). We have randomly selected twenty per litter to achieve the behavioral test (10 males and 10 females). During pregnancy the exposed groups did not differ in water and food intake and in the rate of the body mass increase in dam gestational periods GD 5 till parturition. The 0.5 and 1.0 mg/kg/day MeHg treatment groups did not differ from the control group in the level of food and water consumption (nata not shown) and body weight gain, whereas maternal body weight during gestational period (GD 0-20) *(Figure 1A)* and percentage maternal weight gain *(Figure 1B)* was drastically reduced with higher MeHg treatment group. Commencing between GD 5 till parturition, at dose level 2.0 mg/kg/day MeHg group, animals developed gait alterations and incoordination. The animals later became difficult to handle and showed limb abnormalities including exaggerated movements, and limited usage of the hind limbs. As a result of the poor



Body weight of mother exposed to MeHg during gestation period. All data are mean ± SE with 7-8 females in each group, **p < 0.01 indicates significant differences as compared with control.

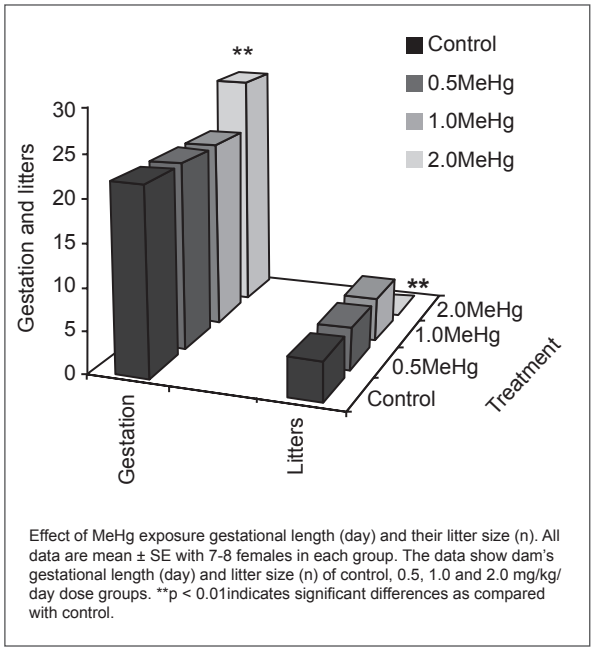
Figure 1A
Effect of methylmercury (MeHg) on maternal body weight during gestational period.



Effect of MeHg exposure on weight gain (%) of dams. The data show body weight gain (%) during gestation period in dams of control, 0.5, 1.0 and 2.0 mg/kg/day dose groups. **p < 0.01 indicates significant differences as compared with control.

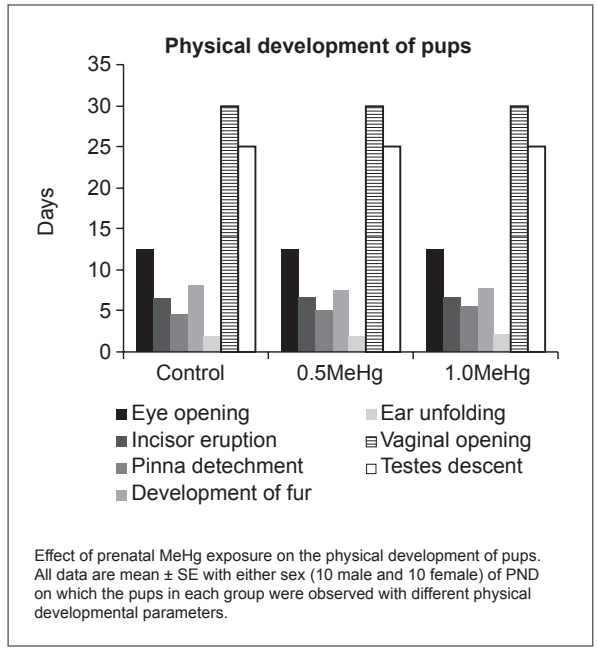
Figure 1B
Effect of methylmercury (MeHg) exposure on body weight gain (%) during pregnancy.

condition of these animals, they were euthanised early in lactation (LD 0 or 1) due to dystocia, delivery of dead pups or total litter loss. Gross pathological examination of animals to litter or complete parturition indicated undelivered dead fetuses or resorptions in the uterus. All other animals in the 0.5 and 1.0 mg/kg/day MeHg groups appeared normal and no adverse clinical signs were observed. There were no deaths, resorbed or late deliveries due to MeHg with 0.5 and 1.0 mg/kg/day exposure groups. In contrast, treatment of rat with 2.0 mg/kg/day MeHg caused hundred percent of resorbed pups *(Figure 2)*. The length of gestation was not statistically different between the control and MeHg – treated groups, however, there was an indication of an extended length by 0.14 for the 1.0 and 2.0 mg/kg/day MeHg treated dams *(Figure 2)*. The low and medium dose groups did not differ from the control group in body weight gain, the number of pups per litter, male/female ratio, the number of still births or the values of the viability index (*i.e.* percent of pups surviving beyond PND 4), were notably lower up to 100%, during treatment



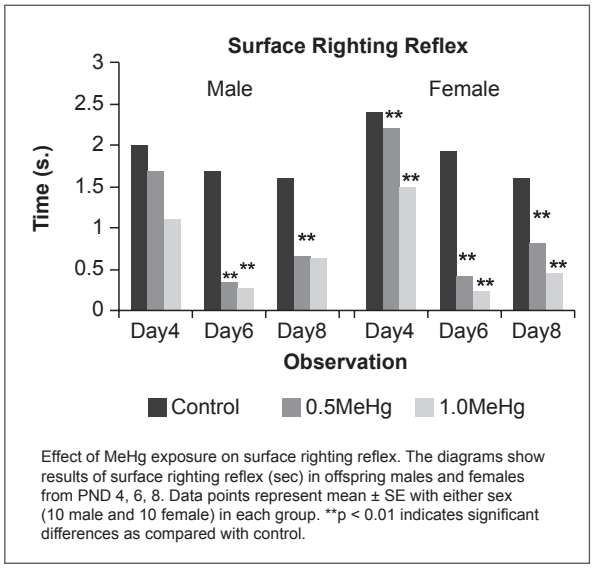
Effect of MeHg exposure gestational length (day) and their litter size (n). All data are mean ± SE with 7-8 females in each group. The data show dam's gestational length (day) and litter size (n) of control, 0.5, 1.0 and 2.0 mg/kg/day dose groups. **p < 0.01indicates significant differences as compared with control.

Figure 2
Effect of methylmercury (MeHg) exposure on gestational period and litter size of dams



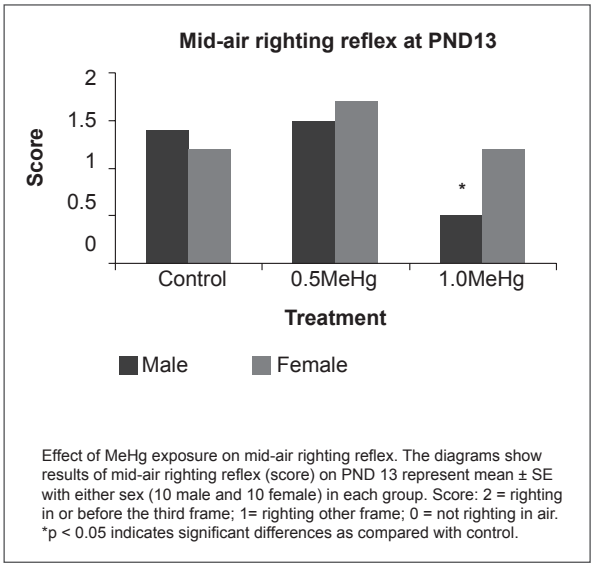
Effect of prenatal MeHg exposure on the physical development of pups. All data are mean ± SE with either sex (10 male and 10 female) of PND on which the pups in each group were observed with different physical developmental parameters.

Figure 3
Effect of methylmercury (MeHg) exposure on gestational day GD 5 till parturition on physical development of pups.



Effect of MeHg exposure on surface righting reflex. The diagrams show results of surface righting reflex (sec) in offspring males and females from PND 4, 6, 8. Data points represent mean ± SE with either sex (10 male and 10 female) in each group. **p < 0.01 indicates significant differences as compared with control.

Figure 4
Effect of methylmercury (MeHg) exposure on gestational day GD 5 till parturition on surface righting reflex on PND 4, 6, and 8.



Effect of MeHg exposure on mid-air righting reflex. The diagrams show results of mid-air righting reflex (score) on PND 13 represent mean ± SE with either sex (10 male and 10 female) in each group. Score: 2 = righting in or before the third frame; 1= righting other frame; 0 = not righting in air. *p < 0.05 indicates significant differences as compared with control.

Figure 5
Effect of methylmercury (MeHg) exposure on gestational day GD 5 till parturition on mid-air righting reflex on PND13.

periods. There were no significant differences in body weight of male offspring [F (2,30) = 0.42. P < 0.066] and female offspring [F (2,30) = 0. P < 1.00] between the progeny of the control and low doses of MeHg exposed groups at PND 1.

Somatic growth and maturation

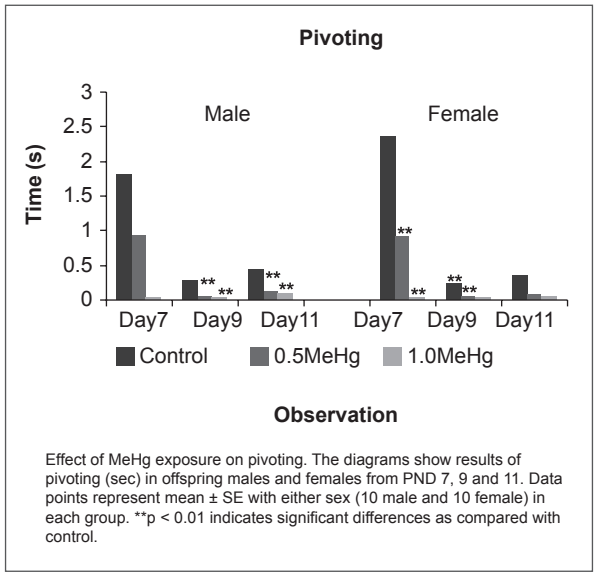
There were no significant differences among the control, 0.5 and 1.0 mg/kg/day MeHg treatment groups in the fetal body length, and tail length and body weights at PND 1, 4, 7, 21 in either sex. The numbers of total resorption and dead fetuses, as well as the percentage of post implantation loss were only significantly affected with 2.0 mg/kg/day MeHg treatment group. There was no overt main effect of treatment or sex on the age at first appearance of pinnadetechment [F (2,30) = 0.93. P < 0.405], eye opening [F (2,30) = 0.05. P < 0.951], auditory startle reflex [F (2,30) = 0.01. P < 0.990], incisor eruption [F (2,30) = 0. P < 1.000], development of fur [F (2,30) = 0. P < 1.000], testes descent [F (2,30) = 0. P < 1.000] or vaginal opening [F (2,30) = 0. P < 1.000] in either sex *(Figure 3)*.

Neuromotor, sensory function and reflex development

There were non-significant adverse effect of MeHg exposure on, cliff avoidance (PND 10), startle reflex (PND 7, 15), swimming ontogeny (PND 6), forelimb and hindlimb grip strength of male [F (2,30) = 0.69. P < 0.509], [F (2,30) = 0.29. P < 0.750] and female offspring [F (2,30) = 1.4. P < 0.262], [F (2,30) = 2.48. P < 0.100] respectively. As seen in *Figure 4*, surface righting on PND 4 of male [F (2,30) = 2.82. P < 0.075] were scarcely altered in all the exposure groups, whereas of female [F (2,30) = 2.91. P < 0.069; Tukey *post-hoc* test: control *vs* 0.5 and 1.0 mg/kg MeHg, p < 0.01]. However, significant slowness of surface righting reflex either on PND 6 of male [F (2,30) = 37.46. P < 0.0001; Tukey *post-hoc* test: control *vs* 0.5 and 1.0 mg/kg MeHg, p < 0.01] and female [F (2,30) = 41.57. P < 0.0001; Tukey *post-hoc* test: control *vs* 0.5 and 1.0 mg/kg MeHg, p < 0.01] or on PND 8 of male [F (2,30) = 30.3. P < 0.0001; Tukey *post-hoc* test: control *vs* 0.5 and 1.0 mg/kg MeHg, p < 0.01] and female [F (2,30) = 50.09. P < 0.0001; Tukey *post-hoc* test: control *vs* 0.5 and 1.0 mg/kg MeHg, p < 0.01] were altered in all the exposure groups in either sex. Subsequently One-Way ANOVAs and *post-hoc* comparisons revealed that both highest and lowest exposure groups as well as PND 6 and PND 8 had significantly reduced the surface righting time in either sex as compare with control *(Figure 4)*.

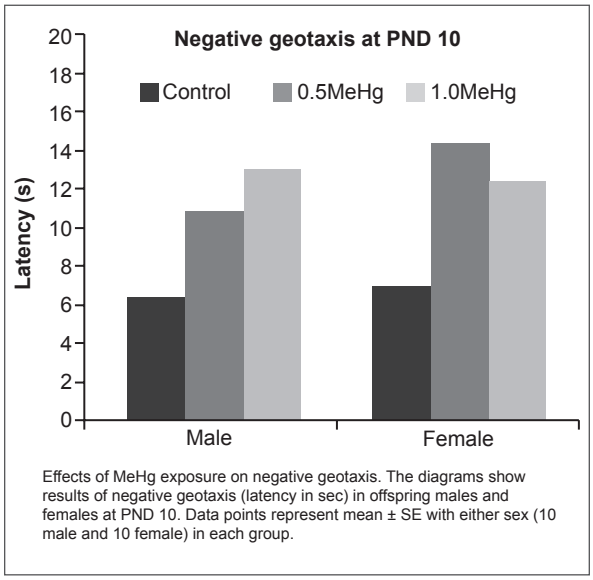
Mid-air righting reflex on PND 13 of the male offspring was significantly depressed [F (2,30) = 5. P < 0.013; Tukey *post-hoc* test: control *vs* 1.0 mg/kg MeHg, p < 0.05] in the 1.0-mg/kg/day MeHg exposure group *(Figure 5)*. Pivoting was observed in either sex on PND 7, 9 and 11. Pivoting either on PND 7 of male [F (2,30)= 58.66. P < 0.0001; Tukey *post-hoc* test: control *vs* 0.5 and 1.0 mg/kg MeHg, p < 0.01] and female [F (2,30) = 72.75. P < 0.0001; Tukey *post-hoc* test: control *vs* 0.5 and 1.0mg/kg MeHg, p < 0.01] or on PND 9 of male [F (2,30) = 75.35. P < 0.0001; Tukey *post-hoc* test: control *vs* 0.5 and 1.0 mg/kg MeHg, P < 0.01] and female [F (2,30) = 77.18. P < 0.0001; Tukey *post-hoc* test: control *vs* 0.5 and 1.0 mg/kg MeHg, P < 0.01] were significantly altered in all the exposure groups. Thus, one-way ANOVAs and *post hoc* comparison suggested that both highest and lowest exposure groups as well as PND 7 and PND 9 had significantly shortened the pivoting time in either sex as compare with control *(Figure 6)*.

Males and females of each dose exposure groups performed better in the negative geotaxis test than their counterparts from the control group. Our results indicated non-significant, dose-dependent increases in either male offspring [F (2,30) = 1.74. P < 0.193] or female offspring [F (2,30) = 1.71. P < 0.198] in negative geotaxis in MeHg exposed groups *(Figure 7)*. However,



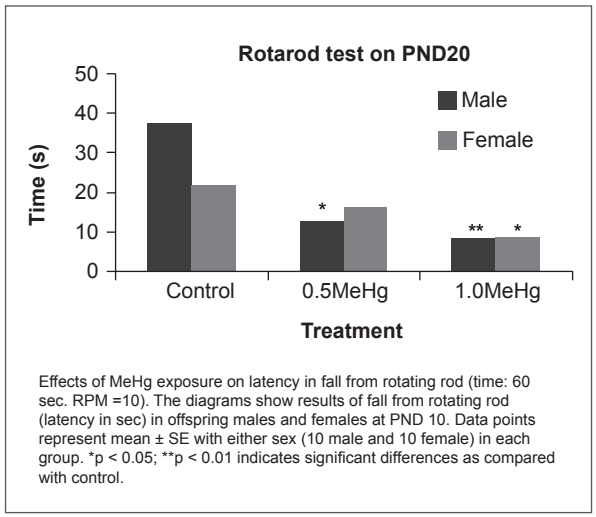
Effect of MeHg exposure on pivoting. The diagrams show results of pivoting (sec) in offspring males and females from PND 7, 9 and 11. Data points represent mean ± SE with either sex (10 male and 10 female) in each group. **p < 0.01 indicates significant differences as compared with control.

Figure 6
Effect on pivoting in the offspring of rat exposed to methylmercury (MeHg) on gestational day GD 5 till parturition at PND 7, 8 and 11.



Effects of MeHg exposure on negative geotaxis. The diagrams show results of negative geotaxis (latency in sec) in offspring males and females at PND 10. Data points represent mean ± SE with either sex (10 male and 10 female) in each group.

Figure 7
Effect on negative geotaxis in the offspring of rat exposed to methylmercury (MeHg) on gestational day GD 5 till parturition at PND 10.



Effects of MeHg exposure on latency in fall from rotating rod (time: 60 sec. RPM =10). The diagrams show results of fall from rotating rod (latency in sec) in offspring males and females at PND 10. Data points represent mean ± SE with either sex (10 male and 10 female) in each group. *p < 0.05; **p < 0.01 indicates significant differences as compared with control.

Figure 8
Effect of rotarod test in the offspring of rat exposed to methylmercury (MeHg) on gestational day GD 5 till parturition at PND 20.

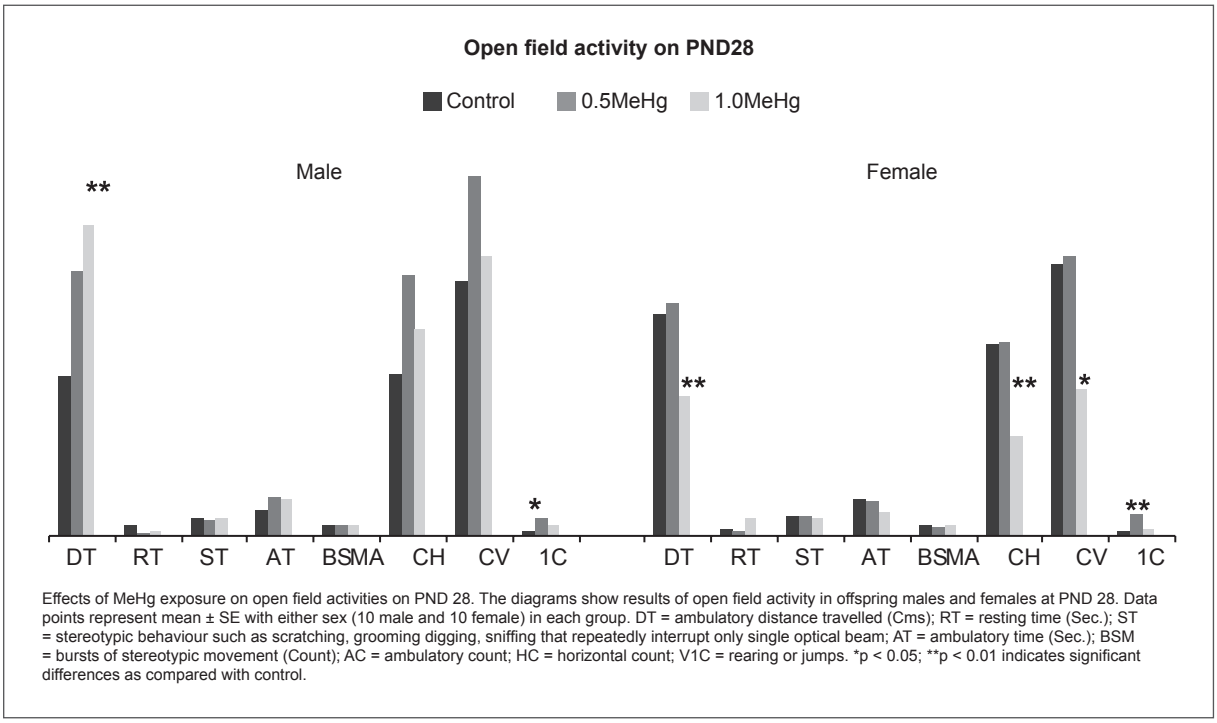
the latency to complete the negative geotaxis response of 10-day old rats were non-significant increased by 0.5 mg/kg (10.87 ± 2.48s in male; 14.30 ± 3.54s in female) and 1.0 mg/kg MeHg (12.95 ± 3.05s in male; 12.37 ± 2.29s in female) when compared with control rats (6.32 ± 1.38s in male; 6.96 ± 2.08s in female) (Data Not Shown). The offspring's motor ability was investigated using rota rod on PND 20. Male offspring [F (2,30) = 6.39. P < 0.005; Tukey *post-hoc* test: control *vs* 0.5mg/kg MeHg, p < 0.05 and control *vs* 1.0 mg/kg MeHg, p < 0.01] at dose levels 0.5 and 1.0 mg/kg/day MeHg exposed groups, spent shorter time on rotating rod than control offspring whereas similar result were found in female offspring [F (2,30) = 3.98. P < 0.05] with 1.0 mg/kg/day MeHg exposed group *(Figure 8)*.

Activity and emotional reactivity

On PND 28 offspring were tested for exploratory activity, measured by animal activity monitoring system. Following, specific parameters considered to be indicative of spontaneous locomotion were evaluated: distance travels (DT), immobility or resting time (RT) and rearing (V_1C). There was a significantly increase in ambulatory distance travel in male offspring (F (2,30) = 5011, p < 0.0213; Tukey *post-hoc* test: control *vs* 0.5 and 1.0 mg/kg MeHg, p < 0.05] as compared to control group with 0.5 mg/kg/day and 1.0 mg/kg/day MeHg, whereas in female [F (2,30) = 4.67. P < 0.017; Tukey *post-hoc* test: control *vs* 1.0mg/kg MeHg, p < 0.05]; resting time in female offspring with 1.0 mg/kg/day MeHg [F (2,30) = 5.54. P < 0.009; Tukey *post-hoc* test: control *vs* 1.0 mg/kg MeHg, p < 0.05 and increase in intensity of rearing in male with 0.5 mg/kg/day MeHg dose group [F (2,30) = 3.45. P < 0.045; Tukey *post-hoc* test: control *vs* 0.5 mg/kg MeHg, p < 0.05] , in female [F (2,30) = 10. P < 0.005; Tukey *post-hoc* test: control *vs* 0.5mg/kg MeHg, p < 0.01 and 0.5 mg/kg MeHg *vs* 1.0 mg/kg MeHg, P < 0.01]. Stereotypic behaviors remained similar in all treatment groups. Pups from the MeHg treatment groups displayed significant hyperactivity in open field-testing, as indicated by increase in ambulatory distance compared to control pups *(Figure 9)*.

Functional observational battery (FOB)

There were no significant differences between the dosage groups in the large majority of the FOB measures during the 4th weeks of exposure. Statistically significant (p < 0.01) differences in the 0.5 and 1.0 mg/kg/day MeHg dose groups compared to control were found by measuring CNS activity and excitability measurements such as rearing; neuromuscular function/measures such as forelimb grip strength, hindlimb grip strength, and hindlimb splay were affected *(Table 2)*.



Effects of MeHg exposure on open field activities on PND 28. The diagrams show results of open field activity in offspring males and females at PND 28. Data points represent mean ± SE with either sex (10 male and 10 female) in each group. DT = ambulatory distance travelled (Cms); RT = resting time (Sec.); ST = stereotypic behaviour such as scratching, grooming digging, sniffing that repeatedly interrupt only single optical beam; AT = ambulatory time (Sec.); BSM = bursts of stereotypic movement (Count); AC = ambulatory count; HC = horizontal count; V1C = rearing or jumps. *p < 0.05; **p < 0.01 indicates significant differences as compared with control.

Figure 9
Open field activity in the offspring of rat exposed to methylmercury (MeHg) on gestational day GD 5 till parturition on PND 28.

DISCUSSION

Very few animal studies have been examined the potential adverse effects of MeHg on the developing offspring taking into account the human exposure scenario of chronic ingestion of MeHg through the consumption of contaminated fish. Numerous experiments using laboratory animals have confirmed, especially earlier ones [7, 8, 18-20], the toxic effect of MeHg on reproduction and offspring neurobehavioral functions, for only a brief period during gestation. In addition, the endpoints evaluated were often limited in scope. The selection of dose levels, manner of administration and duration of exposure will directly impact on the outcomes being measured. The dose levels in the present study were selected so as to obtain a continuum of effects ranging from little or no toxicity to significant toxicity in both dams and progeny. A gavage dose administered on a daily basis was meant to mimic the consumption of a fishmeal in humans. The present treatment period was gestational day (GD) 5 till parturition. In the present study, the highest dose of 2.0 mg/kg/day MeHg group affected mating behaviour or pregnancy rate, implants per litter, live foetus per litter, dead foetus per litter, total percentage of resorbed per litter, percentages of postimplantation and sex ratio along with obvious signs of maternal toxicity were reported [21]. One of the most common abnormal findings observed for the rats was ataxia and gait impairment that corresponds well documented with signs observed in human accidental poisonings cases of MeHg in Japan and Iraq [22].

Rats treated with MeHg by gavage at 6 mg/kg/day from GD 6 to 9 and reported a comparable extension of gestation length, reduced embryonic implantations in the uterus and the number of dams bearing live litters was markedly diminished. Failure to deliver or sustain live pups illustrated the extreme toxicity of this dose [18]. In contrast, the present study with MeHg by gavage at 1.0 and 2.0 mg/kg/day from GD 5 till parturition, showed a significantly extension of gestational length, reduced embryonic implantations in the uterus and the number of dams bearing live litters were markedly diminished, failure to deliver or sustain live pups illustrated the extreme toxicity at 2.0 mg/kg/day MeHg from GD5 to till parturition [21]. Similar findings reported that pregnant mothers exposed to MeHg at high doses from contaminated fish in Japan experienced miscarriages, or had children stillborn or dying shortly after birth [23].

The body weights of the pups were unaffected at birth and continued to be unaffected throughout the pre-weaning as well as post-weaning period. Vorhees, 1985 [18], reported that MeHg administration to pregnant rats at 6 mg/kg/day from GD 6 to 9 did not affect the body weight of pups at birth but deviations from controls occurred as the animals, especially males, aged and a reduced body weight was noted for males at PND 60. There were no significant differences among the 0.5 and 1.0 mg/kg/day MeHg treatment groups in the fetal body length, tail length and body weights at PND 1, 4, 7, 21 in either sex. The numbers of total resorption and dead fetuses, as well as the percentage of post implantation loss were only significantly affected with 2.0 mg/kg/day MeHg treatment group.

Most developmental landmarks in either sex on the age at first appearance of pinna detechment, eye opening, auditory startle reflex, incisor eruption, development of fur, testes descent or vaginal opening were unaffected by MeHg in the current study, however, earlier

Table 1
Summary of statistical tests

Analysis	End points
ANOVA (1)	F_0 maternal body weight gain GDs 0-20; gestation length; live litter size
ANOVA (2)	F_1 morphometric measurements (malformations dependent on outcome of the whole body length, tail length analysis)
ANOVA (3)	F_1 physical features development- pinnadetechment; eye opening; auditory startle reflex; testes descent and vaginal opening.
ANOVA (4)	F_1 pups body weights; F_1 mid air righting reflex; F_1 surface righting reflex; F_1 pivoting; F_1 negative geotaxis
ANOVA (5)	F_1 motor activity; F_1 forelimb grip strength; F_1 hind limb grip strength; F_1 rotarod test.
ANOVA (6)	F_1 Adult open-field test; F_1 Adult FOB tests- forelimb grip strength; hindlimb grip strength; hindlimb splay and rearing
K-W (7) Kruskal-Wallis test	Pup viability; males per litter

Factors in the models included treatment group (TRT: 1-7), sex (2, 4-6), time (1, 4, 5), and litter (random effect:3, 4-6). Interaction terms included TRT X Sex (2, 4-6), TRT X Time (1, 4, 5), and TRT X Sex X Time (5, 6). Individual group comparisons with the control were made by One-way ANOVA, followed by Tukey's HSD posthoc test (1-6). We used K-W (7) test for comparing pup viability: males per litter. MeHg 0.05 was considered significant.

Table 2
FOB (CNS excitability and neuro-muscular function/measures) in the offspring of rat exposed to MeHg on gestational day GD5 till parturition on PND28

Treatment groups	Rearing (n.)	Forelimb grip (g)	Hindlimb grip (g)	Hindlimb splay (cm)
CONTROL (n = 20)	4.55 ± 0.15	183.40 ± 2.04	63.80 ± 0.80	4.75 ± 0.19
MeHg (0.5 mg/kg/day) (n = 20)	6.15 ± 0.18**	196.40 ± 1.51**	68.95 ± 0.87**	4.60 ± 0.15
MeHg (1.0 mg/kg/day) (n = 20)	6.25 ± 0.12**	186.60 ± 0.94	58.25 ± 0.81	4.20 ± 0.17

*Data are presented in mean ± SE, n =20 rats in each exposed groups in either sex. Significantly different from the control group: ** $p < 0.01$ significant.*

studies, resulted in an earlier opening of the pups eyes [19, 24], development for incisor eruption [18, 19, 24], delayed development of righting reflex [18, 24, 25], as well as alterations for negative geotaxis [21, 24], and swimming ability [18, 24, 25].

There were no significant adverse effects of MeHg exposure on cliff avoidance, surface righting reflex, startle reflex, swimming ontogeny, forelimb and hindlimb grip strength. However, mid-air righting reflex on PND 13 of the male offspring was significantly depressed in the 1.0 mg/kg/day MeHg exposure group as compared to control. The appearance of the startle reflex coincided with ear channel opening, and hearing neuronal circuitry is developed at this age [26, 27], suggesting that earlier ear channel opening is a possible explanation for earlier occurrence of the startle reflex. Buelke-Sam *et al*., 1985 [19], dosed pregnant rats from GD 6 to 9 at 6 mg/kg and showed that offspring of both pre and post-weaning age had increased auditory startle habituation response amplitude. Other investigators [24, 25] have also observed similar effects on auditory startle reflex in rats. Unfortunately, some studies [28-30] have failed to show significant change in auditory startle reflex in rats exposed from GD 6 to 15 at 4 mg/kg/day, even in the presence of reproductive toxicity. Disruptions in vision and hearing of adult human populations exposed to MeHg have been documented [31].

Males and females of each dose exposure groups performed better in the negative geotaxis test than their counterparts from the control group. Our results indicated non-significant dose-dependent increases in negative geotaxis in MeHg exposed groups, suggesting no alteration in motor performance of exposed rats. In contrast, the earlier studies on motor performance (latency to complete a negative geotaxis response) of rats reported that decreased in negative geotaxis scores [18] whereas increased in negative geotaxis scores [32] in MeHg-intoxicated animals, showed impaired performance in negative geotaxis test. These differences among results may be attributed to different metals, onset, and duration of exposure and method of imposing heavy metal intoxication.

Motor function, and in locomotor activity, has been investigated frequently in rodents. The results have been somewhat inconsistent with certain studies finding no effect on motor activity, while others have found decreases, increases or even sometimes both [7, 8, 18, 24, 25, 33-35]. The offspring's motor ability was investigated using rota-rod on PND 20. Male offspring, spent shorter time on rotating rod than control offspring whereas similar result was found in female offspring with 1.0 mg/kg/day MeHg exposed group. The results revealed the prenatal exposure of MeHg affected the motor development of offspring. These findings are consistent with results from high-exposure human studies, which revealed significant delays in aspects of mo-

tor development such as crawling, standing, and walking. For neurological reflexes, dynamic tests, such as negative geotaxis, cliff avoidance and righting reflexes, were used to evaluate the sensorimotor development [36]. The results of the present study revealed no delay in surface righting on PND 4, 6, 8 indicating no impairment in the coordinating movement of the offspring in either sex. Meanwhile, the decrease in forelimb grip strength time measuring on rotating rod on PND 20 suggests that MeHg could be delay neuromuscular development in the exposed groups. Most of the measured variables, including air righting reflex, negative geotaxis and cliff avoidance, did not seem to be impaired. It seems that the dose levels (0.5 mg/kg and 1.0 mg/kg) of MeHg probably had minor and/or slight effects on sensorimotor development. There was no significant adverse effect of MeHg exposure on swimming ontogeny (PND 6). Elsner, 1991 [37], examined impairment of motor function as well as swimming ability in rats exposed with MeHg at 1.5 or 5 ppm during gestation. In the present study, motor skills evaluated by way of swimming performance on PND 6 in water were unaffected, and agility, coordination and finer motor function, were evaluated quantitatively using the rota-rod test on PND 20.

Exploratory activity in rats exposed to MeHg, measured by animal activity monitoring system on PND 20, was significantly higher in male offspring as compared to control group in respect to ambulatory distance travel with 0.5 mg/kg/day and 1.0 mg/kg/day MeHg in either sex; resting time in female offspring with 1.0 mg/kg/day MeHg and increase in intensity of rearing in either sex with 0.5 mg/kg/day MeHg dose group. However, stereotypic behaviors remained similar in all treatment groups. Pups from the MeHg treatment groups displayed significant hyperactivity in open field-testing, as indicated by increase in ambulatory distance compared to control pups. This hyperactivity was also accompanied by a significant loss of thigmotaxis (preference for the periphery) in all treatment groups and also increase in the number of vertical rearing in either sex with 0.5 mg/kg/day MeHg exposed group. Thus, prenatal exposure to MeHg resulted in hyperactivity and loss of thigmotaxis in open field-testing. There were no significant differences between the dosage groups in the large majority of the FOB measures during the 4th weeks of exposure. Statistically significant ($p < 0.01$) differences with 0.5 and 1.0 mg/kg/day MeHg dose group compared to controls were found by measuring CNS activity and excitability measurements such as rearing; neuromuscular function/measures such as forelimb grip strength, hindlimb grip strength, hindlimb splay were affected.

Based on the earlier reports [7, 8, 13, 18-20, 24, 38] cited above, the results at different dose levels, which have been presented in the present experimental scenario (GD 5 till parturition), significantly impaired pup growth and survival, decreased grip strength (by rotating-rod method), and caused hyper activity and loss of central aversion in open field-testing, as well as results obtained by other authors [25, 32, 36, 38-40] have concluded that the effectiveness of gestational exposure to MeHg affect early morphological but not physical development as well as some neurobehavioral functions in the progeny to this neurotoxicant.

CONCLUSION

In conclusion, at dose-level 2.0 mg/kg/day gestational exposure to MeHg significantly showed signs of toxicity such as gait alterations and hyperactivity resulting in the failure to deliver sustainable viable pups or resorption. Additionally, behavioral data suggest possible gender-related differences with respect to MeHg neurotoxicity, whereas many of the behavioral tests were not significantly altered. This conclusion, however, cannot be generalised; it cannot be excluded (or it is almost certain) that in other conditions (different gestational exposure period, different concentrations, different route and with different experimental endpoints) the results (and the conclusions) would be different. In this situation, exact determination of the dose-response relationship(s), future investigations should focus on the precise determination of the conditions (gestational exposure period, manner of administration and selection of dose levels) in which MeHg decreases the risk of adverse developmental as well as neurobehavioral effects.

Acknowledgements

The author (Dinesh N. Gandhi) acknowledges the Indian Council of Medical Research (ICMR), New Delhi, India for funding the *ad-hoc* Research Project grant No. 2010-04390 and awarding Senior Research fellow to the author (Dinesh K. Dhull) under this project.

Conflict of interests statement

There is no conflict of interest.

Received on 10 July 2013.
Accepted on 25 November 2013.

REFERENCES

1. Grandjean P, Weihe P, White RF, Debes F, Araki S, Yokoyama K, Murata K., Sorensen N, Dahl R, Jorgensen PJ. Cognitive deficit in 7-year-old children with prenatal exposure to methylmercury. *Neurotoxicology and Teratology* 1997;19(6): 417-28. DOI: 10.1016/S0892-0362(97)00097-4
2. Hoskins BB, Hupp EW. Methylmercury effects in rat, hamster and squirrel monkey. *Environ Res* 1978;15:5-19. DOI: 10.1016/0013-9351(78)90073-7
3. Khera KS. Teratigenic effects of methylmercury in the cat: Note on the use of this species as a model for teratogenicity studies. *Teratology* 1973;8:293-304. DOI: 10.1002/tera.1420080309
4. Fuyuta, M, Fujimoto T, Hirata S. Embryotoxic effects of methylmercuric chloride administrated to mice and rats during organogenesis. *Teratology* 1978;18:353-66. DOI: 10.1002/tera.1420180310
5. Ornaghi F, Ferrini S, Prati M, Giavini E. The protective effects of *N*-acetyl-L-cysteine against methylmercury embryotoxicity in mice. *Funda Appl Toxicol* 1993;20:437-45.
6. Gomez M, Sanchez DJ, Colomina MT, Domingo JL,

Corbella J. Evaluation of the protective activity of 2,3-dimercaptopropanol and sodium 2,3-dimercaptopropane-1-sulfonate on methylmercury-induced developmental toxicity in mice. *Arch Environ Contam and Toxicol* 1994;26:64-8. DOI: 10.1007/BF00212795
7. Eccles CU, Annau Z. Prenatal methylmercury exposure: I. Alterations in neonatal activity. *Neurobehav Toxicol Teratol* 1982a;4:371-6.
8. Eccles CU, Annau Z. Prenatal methylmercury exposure: II. Alterations in learning and psychotropic drug sensitivity in adult offspring. *Neurobehav Toxicol Teratol* 1982b;4:377-82.
9. Domingo JL. Metal-induced developmental toxicity in mammals: A review. *J Toxicolol Environ Helth* 1994;42:227-34. DOI: 10.1080/15287399409531868
10. Gray DG. A physiologically based pharmacokinetic model for methylmercury in the pregnant rat and fetus. *Toxicol Appl Pharmacol* 1995;132:91-102.
11. Cagiano R, De Salvia MA, Renna G, Tortella E, Braghiroli D, Parenti C, Zanoli P, Baraldi M, Annau Z, Cuomo V. Evidence that exposure to methylmercury during gestation induces behavioural and neurochemical changes in offspring of rats. *Neurotoxicology and Teratology* 1990;12:23-8. DOI: 10.1016/0892-0362(90)90108-O
12. Moser VC. Neurobehavioral screening methods. *Neurotoxicology* 1997;18(4):925-1079.
13. Lapointe G, Nosal G. A rat model of neurobehavioral development. *Experientia* 1979;35:205-7. DOI: 10.1007/BF01920618
14. Vorhees CV, Butcher RE, Brunner RL, Sobotka TJ. A developmental test battery for neurobehavioral toxicity in rats: a preliminary analysis using monosodium glutamate, calcium carrageenan, and hydroxyurea. *Toxicol Appl Pharmacol* 1979b;50:267-82. DOI: 10.1016/0041-008X(79)90152-2
15. Kandel ER. *Cellular basis of behavior.* San Francosco: WH Freeman; 1976.
16. Moser VC. Screening approaches to neurotoxicology: a functional observational battery. *J Am Colle Toxicol* 1989;8:85-93. DOI: 10.3109/10915818909009095
17. Tilson HA, Moser VC. Comparison of screening approaches. *Neurotoxicology* 1992;13:1-14.
18. Vorhees CV. Behavioral effects of prenatal methylmercury in rats: A parallel trial to the collaboration behavioural teratology study. *Behav Toxicol Teratol* 1985;7:717-25.
19. Buelke-Sam J, Kimmel C, Adams J, Nelson C, Vorhees C, Wright D, Omer V, Korol B, Butcher R, Geyer M, Holson J, Kutscher C, Wayner M. Collaborative behavioural teratology study: Results. *Neurobehav Toxicol Teratol* 1985;7:591-624.
20. Fredriksson A, Gardlund A, Bergman K, Oskarsson A, Ohlin B, Danielsson B, Archer T. Effects of maternal dietary supplimentation with selenite on the postnatal development of rat offspring exposed to methylmercury *in-utero*. *Pharmacol Toxicol* 1993;72:377-82. DOI: 10.1111/j.1600-0773.1993.tb01348.x
21. Gandhi DN, Panchal GM, Dhull DK. Influence of gestational exposure on the effects of prenatal exposure to methylmercury on postnatal development in rats: A preliminary evaluation. *Cen Eur J Pub Helth* 2013;21(1):30-5.
22. World Health Organization. *Environmental health criteria 101: Methylmercury*. Published under the joint sponsorship of the United Nations Environmental Programme, the International Labour Organization, and the World Health Organisation. Geneva: World Health Organisation; 1990.
23. Harada M. Minamata diseases: Methylmercury poisoning in japan caused by environmental pollution. *Crit Rev Toxicol* 1995;25(1):1-24. DOI: 10.3109/10408449509089885
24. Geyer M, Butcher R, Fite k. A study of startle and locomotor activity in rats exposed prenatally to methylmercury. *Neurobehav Toxicol Teratol* 1985;7:759-65.
25. Elsner J, Suter KE, Ulbrich B, Schreiner G. Testing strategies in behavioural teratology. IV. Review and general conclusions. *Neurobehav Toxicol Teratol* 1986;8:585-90.
26. Rodier PM. Chronology of neuron development: animal studies & their clinical implications. *Develop Medi and Child Neurol* 1980;22(4):525-45. DOI: 10.1111/j.1469-8749.1980.tb04363.x
27. Swerdlow NR, Caine SB, Geyer MA. Regionally selective effects of intracerebral dopamine infusion on sensorimotor gating of the startle reflex in rats. *Psychopharmacol* 1992;108(1-2):189-95.
28. Magos L, Butler W. The kinetics of methylmercury administered repeatedly to rats. *Ach Toxicol* 1976;35:25-39. DOI: 10.1007/BF00333983
29. Magos L. The absorption, distribution and excretion of methyl mercury. In: Eccles CV, Annau Z (eds.). *The toxicity of Methylmercury.* Baltimore: Johns Hopkins University Press; 1987. pp. 24-44.
30. Burbacher TM, Sackett GP, Mottet NK. Methylmercury effects on social behavior of *macaca fasicularis* infants. *Neurotoxicol Teratol* 1990;12:65-71. DOI: 10.1016/0892-0362(90)90114-R
31. Lebel J, Mergler D, Branches F, Lucotte M, Amorim M, Larribe F, Dolbec J. Neurotoxic effects of low-level mercury contamination in Amazonian basin. *Environ Res* 1998;79(1):20-32.
32. Rocha JBT, Freitas AJ, Marques MB, Pereira ME, Emanuelli T, Souza DO. Effects of methylmercury exposed during the second stage of rapid postnatal brain growth on negative geotaxis and on delta-aminolevulinate dehydrgenate in rats. *Braz J Med Biol Res*1993;26:1077-83.
33. Chang L, Dudley A, Dudley M, Ganter H, Sunde M. Modification of the neurotoxic effects of methylmercury by selenium. In: Roizon L, Hiraki HS, Groevic N. (Eds.). *Neurotoxicology*. New York: Raven Press; 1997. pp. 428-36.
34. Kim CY, Nakai K, Kasanuma Y, Satoh H. Comparison of neurobehavioral changes in three inbred strains of mice prenatally exposed to methylmercury. *Neurotoxicol Teratol* 2000;22:397-403. DOI: 10.1016/S0892-0362(99)00077-X
35. Gimenez-Llort L, Ahlbom E, Dare E, Vahter M, Ogren S, Ceccatelli S. Prenatal exposure to methylmercury changes dopamine-modulated motor activity during early ontogeny: Age and gender –dependent effects. *Environ Toxicol Pharmacol* 2001;9:61-70.
36. Khan Z, Carey J, Park Hj, Lehar M, Lasker D, Jinnah HA. Abnormal motor and vestibular dysfunction in the stargazer mouse mutant. *Neurosci* 2004;127:785-96. DOI: 10.1016/j.neuroscience.2004.05.052
37. Elsner L. Tactile-kinesthetic system of rats as an animal model for minimal brain dysfunction. *Ach Toxicol* 1991; 65 :465-73. DOI: 10.1007/BF01977358
38. Beyrouty P, Chan HM. Co-consumption of selenium and vitamin E altered the reproductive and developmental toxicity of methylmercury in rats. *Neurotoxicol Teratol* 2006;28:49-58. DOI: 10.1016/j.ntt.2005.11.002
39. Elsner J, Hodel B, Suter K, Oelke D, Ulbrich B, Schreiner G, Cuomo V, Cagiano R, Rosengren L, Karlsson J, Haglid K. Detection limits of different approaches in behavioural teratology, and correlation of effects with neurochemical parameters. *Neurotoxicol Teratol* 1988;10:155-67. DOI: 10.1016/0892-0362(88)90080-3
40. 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. DOI: 10.1007/s002210050237