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Co-consumption of selenium and vitamin E altered the reproductive and developmental toxicity of methylmercury in rats

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Abstract

Methylmercury (MeHg), an environmental contaminant primarily found in fish and seafood, may pose long-term health risks to pregnant women and their developing children. The objective of this study was to determine whether co-consumption of nutritional supplements would alter the effects of MeHg on reproductive and developmental toxicity using a rodent model. Adult female rats were fed a diet containing additional selenium (1 ppm), additional vitamin E (225 IU/kg) or a combination of the two for 4 weeks before oral dosing of MeHg (1.25 mg/kg/day). Treatment with MeHg and dietary supplementation continued throughout pregnancy after which the dams were allowed to deliver their offspring. In addition to routine evaluations including periodic body weight measurements and daily clinical signs observations, dams and pups were evaluated for auditory startle habituation and pups were evaluated for developmental landmarks and reflexology. The dams and offspring were euthanized approximately 4 weeks after birth of the offspring. Results indicated that treatment with MeHg caused adverse effects on both reproduction of the dams and decreased progeny survival. However, the dams showed significant improvement in body weight gain during lactation and average auditory startle response time when the diet was enriched with both selenium and vitamin E. The combination of both vitamin E and Se also resulted in a significant increase in post-natal survival when compared to MeHg-treated group. There was no nutrient effect on the MeHg toxicity shown in offspring physical landmarks, performance in reflex tests and assessment of simple auricular startle response. Also, accelerated development as indicated by earlier opening in the pups of the supplemental diet groups was observed. These results suggest that antioxidant nutrients in the diet may alter MeHg reproductive and developmental toxicity. The underlying and human health implications warrant further investigations.

Keywords: Methylmercury; Reproductive and developmental toxicity; Selenium; Vitamin E

1. Introduction

Methylmercury (MeHg) is a contaminant that is found in many ecosystems. MeHg has a propensity to accumulate in fish tissue as well as other aquatic life through its ingestion and absorption [27]. The consumption of these contaminated fish poses a universal health risk to humans [33] and the consequential effects of high exposure are well documented. The U.S. Food and Drug Administration (US FDA) issued fish consumption advisories for pregnant women regarding their intake of certain fish species due to high mercury levels [29]. According to a report for U.S. congress, the consumption of fish and seafood by Americans has increased almost 30% per capita since 1970 [28]. Recently, the U.S. FDA issued a directive warning women of childbearing age to eat no more than two meals or 12 ounces of seafood, including canned tuna, weekly [30]. That directive was based in part on results of a study conducted by the U.S. Environmental Protection Agency that one in six pregnant women in the United States had blood mercury high enough to damage her child, meaning approximately 630,000 U.S. newborns are at risk [12].

Numerous experiments using laboratory animals have confirmed the toxic effects of MeHg on reproduction and offspring neurobehavioral function. Most of these animal

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studies have examined the sensitivity of the developing rodent fetus to maternal MeHg exposure [1,2,6–9,17,20,26,31,36]. Developmental anatomical indices and auditory startle measurements have been suggested [2,9,26,31] to be appropriate endpoints for evaluation of MeHg-induced toxicity.

The effectiveness of different agents and nutrients to prevent or reverse MeHg toxicity has been investigated in the past. More than three decades ago, it was found that selenium and mercury accumulated together in certain fish species, and it was postulated that the additional accumulation of selenium might represent a natural mechanism to protect against heavy metal toxicity [10]. Animal models have been used to evaluate interactions between mercury and selenium in adult and developing systems [4,9,10,19,24,25,32], and have illustrated the benefits of selenium. In comparison, relatively little evidence is available on the role of the other major dietary antioxidants on MeHg toxicity. Some earlier studies have shown that vitamin E may also protect against mercury toxicity [5,34].

The World Health Organization (WHO) recommended that nutrients that alter toxicity associated with environmental contaminants, such as MeHg, be investigated [35]. The effects of antioxidant nutrients on MeHg toxicity are not known. So far, there are no animal studies investigating the impact of maternal consumption of supplemental selenium and vitamin E on reproduction and offspring development during MeHg exposure. Therefore, the objective of this study was two-fold: (1) evaluate whether the ingestion of selenium and vitamin E would alter MeHg toxicity in pregnant rats and (2) investigate whether the development of offspring exposed to MeHg in-utero would be affected by supplementation of vitamin E and/or selenium. The general hypothesis is that co-administration of selenium and/or vitamin E will decrease the toxicity of MeHg in the dams and offspring.

2. Materials and methods

2.1. Animals

The protocol was approved in advance by the Institutional Animal Care and Use Committee (IACUC) and the care and use of animals was conducted in accordance with guidelines of the USA National Research Council and the Canadian Council on Animal Care. Female Sprague–Dawley rats [CD®(SD) IGS BR] were obtained from Charles River Canada (St. Constant, Quebec) and identified individually by tail tattoo. Environmental conditions in the animal room were set at 22±3 °C, 50±20% relative humidity and 12 h light/12 h dark. All animals had ad libitum access to a standard powdered rodent chow diet and purified water. Fifteen females were randomly assigned, based on body weight, to each of five groups as described in Table 1 and received the appropriate treatment starting at approximately 7.5 weeks of age. These animals formed the F_0 generation and were housed individually, except during mating to naïve males (same strain and source), initially in stainless-steel cages and later during littering in plastic solid-bottom cages. Mating was confirmed by positive identification of spermatozoa in a vaginal lavage smear and was designated as gestation day 0. The day females completed littering was defined as lactation or post-natal day 0.

Table 1
Assignment of F_0 generation females to treatment groups

Group	No. of animals	Selenium-enriched diet[a]	Vitamin E-enriched diet[b]	MeHg treatment[c]
Control	15	No	No	No
MeHg	15	No	No	Yes
MeHg+Se	15	Yes	No	Yes
MeHg+Vit E	15	No	Yes	Yes
MeHg+Se+Vit E	15	Yes	Yes	Yes

[a] Animals received an additional 1 ppm selenium in the diet for at least 4 weeks prior to mating and during pregnancy.
[b] Animals received an additional 225 IU vitamin E/kg in the diet for at least 4 weeks prior to mating and during pregnancy.
[c] Following at least 4 weeks of enriched diet, the animals were treated daily with methylmercury (MeHg), orally by gavage, for at least 28 days. MeHg treatment continued until gestation day 20 or approximately 3 weeks after the end of the mating period for animals not confirmed as having mated. Control animals received the vehicle only for same period.

2.2. Preparation of MeHg dose formulations and fortified diets

Methyl mercury chloride (purity>95%; adjusted for chloride salt) was obtained from Alfa Aesar (Ward Hill, MA, U.S.A.) and was dissolved using a sonicator in nitrogen-purged deionized water. The dose formulations were stored in glass vials under a nitrogen blanket in a refrigerator until required. Dose formulations of MeHg were prepared on a weekly basis and administered by oral gavage at a constant volume of 5 mL/kg body weight/day (1.25 mg of MeHg/kg body weight/day). The choice of dose was based on a preliminary experiment showing that relative few toxic endpoints were affected at 1.0 mg MeHg/kg body weight/day under the same dosing regime. Control animals received only nitrogen-purged water (vehicle) at the same dose volume. Selenium (>99% pure), in the form of seleno-L-methionine, was obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Vitamin E (purity≥98%), in the form of DL-α-tocopherol acetate, was obtained from Aldrich Chemical Co. (Milwaukee, WI, U.S.A.). The powdered rodent diet (Certified 5002) was obtained from PMI Nutrition International (Richmond, IN, U.S.A.). Analyses by the manufacturer indicated that the unfortified powder rodent diet used in this study contained basal levels of 0.3 ppm selenium and 79.6 IU/kg vitamin E. Fortified diets were prepared by adding the appropriate amounts of selenium (dissolved in water) and/or vitamin E (dissolved in corn oil) to a small amount of the powder rodent diet in a mortar and pulverizing using a pestle until the mixture appeared homogeneous. Incremental amounts of additional powder rodent diet were added to the mixture, mixed by hand and then by a Hobart blender with whisk attachment. The diets of all groups included the addition of corn oil (0.5% w/w of total diet weight) in order to balance any nutritional advantage this may have provided to groups receiving additional dietary vitamin E. The fortified diets were prepared on a monthly basis and were stored frozen at −20 °C until required.

2.3. Evaluations of F_0 generation

The females were observed daily for abnormal clinical signs including during parturition. Body weight and food intake were measured prior to mating and throughout gestation and lactation (food intake data for lactation not presented). Auditory startle habituation was measured on lactation day 23 in sound attenuated chambers (San Diego Instruments®). After a 4-min acclimation period in the chambers to background noise (65 dBA), the animals were subjected to a brief sound pulse of 120 dBA on 50 identical trials. Each trial was separated by 8 s where only background sound was presented. Measurements of animal "startle" for each trial included average and maximum startle amplitude across the recording window of 100 ms as well as the time to reach the maximum startle amplitude. At study completion, the animals were given a necropsy; surviving dams were euthanized on lactation day 30. The number of uterine implantation site scars was visually counted and recorded for pregnant animals. The pregnancy status of animals that did not deliver was confirmed by staining the uterus with 10% (v/v) ammonium sulfide solution and checking for implantation site scars [21].

2.4. Evaluations of F_1 generation

Following birth, the pups were examined, sexed and counted (dead and alive). Their general condition was evaluated each subsequent day and body weights were recorded periodically. Each litter was randomly culled to eight pups (four per sex), when necessary, and the selected pups were identified by injection of India ink into their paws 4 days after birth. The pups were weaned at 3 weeks of age. Developmental landmarks (pinna unfolding, tooth eruption, eye opening) and reflex tests (surface righting reflex, negative geotaxis, simple auricular startle) were conducted on the offspring. Negative geotaxis was performed by placing the pup on an inclined surface (23°) with head facing down. Up to 60 s was allowed for the pup to turn 180° and crawl up the surface. For surface righting test, the pup was placed on its back on a flat surface and allowed up to 30 s to turn over and regain a normal position on all four paws. The auricular startle was performed by made by tapping two steel bars together and the presence of a startle movement was recorded. Auditory startle habituation testing was performed on one male and one female (randomly selected from the cage) weanling per litter (all surviving weanlings in MeHg and MeHg+Se group) on post-natal day 23 using the same procedure as described for adults. All surviving pups were euthanized on post-natal day 30.

2.5. Mercury analysis

Mercury was measured in the brain, kidney and liver of selected dams and offspring. The tissue (0.5 g) was digested in concentrated nitric acid and the total Hg was measured by cold vapor atomic absorption spectrophotometry. For statistical purposes, values below the limit of detection (0.001 μg/g) were reported as zero.

2.6. Statistical analysis

All analyses were performed using SAS version 6.12 software [22]. Means and standard deviations, frequencies or incidences, depending on the data type, were calculated for each dose group. For F_1 offspring, the litter was used as the statistical unit and each gender was analyzed separately. Survival data was evaluated with genders combined as there was no difference in gender (Student's t-test result). Quantitative data was first tested for homogeneity of variances using Levene's test. Where variances were homogeneous, the data was analyzed by SAS general linear models (GLM) procedure using an analysis of variance (ANOVA) followed by Bonferroni multiple comparison tests. Where variances were heterogeneous, the data were log transformed and re-analyzed as above, or were compared by computation of Wilcoxon scores (rank sums) and analyzed using a Kruskal–Wallis test with chi-square approximation followed by Wilcoxon two-sample test with normal approximation and continuity correction for pairwise comparisons. Incidence data was subjected to dose group comparisons using a Fisher's exact test (two-tailed). Frequency data was subjected to Kruskal–Wallis and Wilcoxon tests as described above. For all post-hoc comparisons to the control and MeHg group, differences were declared significant when a $p<0.05$ was reached. In order to control for type I (experiment-wise) errors, the α-level was adjusted (i.e. divided by the square root of the number of comparisons).

3. Results

3.1. Mercury concentrations

Mercury concentrations in the tissue of dams and offspring 30 days after pup birth are shown in Table 2. Treatment of MeHg increased the mercury body burden of the animals. Dams exhibited higher levels of mercury (13- to 57-fold) compared to the offspring in all tissues evaluated. The highest concentrations in dams and offspring were found in the kidney,

Table 2
Tissue mercury concentrations in F_0 and F_1 generations on day 30 post-partum

Group	Brain	Kidney	Liver
F_0 generation			
Control	0.01±0.02 [6]	0.09±0.01 [6]	0.01±0.01 [6]
MeHg	3.39±0.76 [6]	99.16±17.51 [6]	11.23±1.51 [6]
MeHg+Se	4.29±1.55 [6]	132.98±23.34 [6]	16.07±5.79 [6]
MeHg+Vit E	3.18±1.08 [6]	84.95±12.92 [6]	11.85±5.25 [6]
MeHg+Se+Vit E	3.15±1.16 [6]	106.42±20.69 [6]	13.78±9.53 [6]
F_1 generation			
Control	0.08±0.19 [6]	0.03±0.03 [6]	0.01±0.02 [6]
MeHg	0.24±0.13 [5]	2.07±0.60 [5]	0.45±0.23 [5]
MeHg+Se	0.25±0.09 [5]	2.35±0.80 [5]	0.54±0.21 [5]
MeHg+Vit E	0.24±0.09 [6]	2.23±0.95 [6]	0.46±0.16 [6]
MeHg+Se+Vit E	0.25±0.05 [6]	2.34±0.34 [6]	0.50±0.10 [6]

Data are mean±standard deviation in μg/g tissue. Sample size is in brackets.
All MeHg treatment group had significant higher Hg concentrations in all three tissues.
There was no significant difference between MeHg treatment groups.

followed by the liver and brain. There was no statistical difference in mean tissue mercury concentrations between the MeHg group and those groups receiving additional selenium (MeHg+Se), vitamin E (MeHg+Vit E) or both nutrients (MeHg+Se+Vit E).

3.2. F_0 generation

3.2.1. Mortality and clinical signs

No animals displayed signs of MeHg toxicity prior to the mating period. During late gestation, early lactation or approximately 3 weeks following the completion of the mating period, signs of MeHg-related intoxication began to appear. At this time, some animals in the MeHg, MeHg+Se and MeHg+Vit E groups began to show an abnormal gait and a lack of coordination. Shortly after 3 weeks, additional animals in these groups displayed similar findings. A total of five animals were affected in each of the MeHg, MeHg+Se and MeHg+Vit E groups. In some cases, an abnormal gait was accompanied by a sporadic, exaggerated steppage that sometimes included trailing of the hindlimbs. In addition, muscle flaccidity and hyper-reactivity to handling were also observed at about the same time in select animals. Interestingly, hyper-reactivity to handling was limited to a single animal in the MeHg+Se+Vit E group.

One animal in the MeHg+Se+Vit E group died on lactation day 2 after delivering a litter of eight viable pups. Prior to its death, it showed red vaginal discharge and pallor of the paws. A gross pathological examination failed to indicate the cause for the mortality as findings were limited to multiple dark areas exteriorly on the kidney and renal medulla discoloration.

One of the first animals to display severe signs of intoxication was in the MeHg+Vit E group and was euthanized on lactation day 7 due to poor condition. On the day of euthanasia, the animal showed splayed hindlimbs, decreased activity, emaciation, weak and dehydrated appearance, reduced body temperature, and had failed to deliver a viable litter. Necropsy findings included visible dark areas in the glandular portion of the stomach and a small thymus.

One animal in the MeHg group was euthanized on lactation day 6, also due to poor condition. On the day of euthanasia, this animal had an abnormal gait, dragged its hindlimbs, was difficult to handle and had delivered no live pups. However, there were no abnormal necropsy findings.

Table 4
Summary of food consumption for F_0 generation

	Pre-mating/pre-MeHg treatment period	Pre-mating/MeHg treatment period	Gestation/MeHg treatment period
	Study days 1 to 29	Study days 29 to 57	Days 0 to 20
Control	462.3±47.7 [14]	466.7±46.7 [15]	429.5±34.7 [13]
MeHg	448.4±32.0 [14]	433.6±36.8 [14]	380.2±39.6* [13]
MeHg+Se	482.2±39.0 [15]	455.4±26.1 [14]	386.0±19.8* [13]
MeHg+Vit E	452.3±29.6 [14]	411.9±35.3* [14]	351.6±55.3* [12]
MeHg+Se+Vit E	459.2±27.9 [14]	443.1±44.0 [15]	376.5±32.5* [13]

Data are mean±standard deviation in grams. Sample size is in brackets.
* Denotes statistically significant ($p<0.05$) difference from control group.

3.2.2. Body weights

A significant ($F_{4,70}=3.89$, $p\leq0.01$) group effect was observed for body weight gain during the period of feeding of supplemental diets before initiation of MeHg administration, study days 1 to 29 (Table 3). Post-hoc comparisons to the control and MeHg groups indicated significantly ($p\leq0.02$) higher values for the MeHg+Se group. During the MeHg treatment period prior to mating (study days 29 to 57), a significant ($F_{4,70}=8.58$, $p<0.01$) difference was observed between the groups for body weight gain. Significantly ($p\leq0.01$) lower body weight gains occurred for the MeHg+Se, MeHg+Vit E and MeHg+Se+Vit E groups compared to control. In comparison to the MeHg group, the MeHg+Vit E group indicated a significantly ($p\leq0.01$) lower body weight gain.

The body weight gain of pregnant females from gestation days 0 to 20 was significantly ($F_{4,65}=4.82$, $p<0.01$) lower ($p\leq0.02$) for the MeHg, MeHg+Se and MeHg+Vit E groups than for the control.

From lactation days 0 to 21, there was a significant ($F_{4,63}=3.87$, $p<0.01$) treatment-effect with body weight gain. Values of the MeHg+Se+Vit E group were significantly ($p<0.01$) higher than the MeHg group.

3.2.3. Food consumption

There were no significant differences in food consumption from days 1 to 29 (Table 4). However, the largest mean food intake did occur for the MeHg+Se group, which corresponded with higher body weight gain during this period. Significant group effects were observed for total food intake ($F_{4,67}=4.24$, $p<0.01$) from study days 29 to 56. Post-hoc comparisons

Table 3
Summary of body weight gain for F_0 generation

	Pre-mating/pre-MeHg treatment period	Pre-mating/MeHg treatment period	Gestation/MeHg treatment period	Lactation period
	Study days 1 to 29	Study days 29 to 57	Days 0 to 20	Days 0 to 21
Control	86.7±14.2 [15]	43.6±9.5 [15]	128.0±14.9 [15]	19.3±14.7 [15]
MeHg	81.5±12.3 [15]	36.5±8.9 [15]	104.5±22.0* [15]	4.7±21.6 [14]
MeHg+Se	99.1±15.5*,# [15]	30.9±6.7* [15]	97.3±26.0* [12]	2.3±22.6 [12]
MeHg+Vit E	84.2±12.0 [15]	26.8±8.1*,# [15]	87.9±39.4* [15]	9.2±29.5 [14]
MeHg+Se+Vit E	87.1±11.9 [15]	33.9±8.3* [15]	105.8±20.4 [13]	30.9±17.5# [13]

Data are mean±standard deviation in grams. Sample size is in brackets included all animals within the treatment group (with or without live litters).
* Denotes statistically significant ($p<0.05$) difference from control group.
# Denotes statistically significant ($p<0.05$) difference from MeHg group.

Table 5
Summary of pregnancy data for F_0 generation

Group	No. of mated females	No. of days to mating	Length of gestation (days)	No. of pregnant females	No. of pregnant females with live litters on post-natal day 0	No. of implantation sites per pregnant female	No. of live pups per litter on post-natal day 0	Post-implantation loss (%)
Control	15	2.4±1.2	21.6±0.5	15	15	16.1±1.8	14.9±1.6	7.3±5.9
MeHg	15	3.5±2.7	21.9±0.3	15	13	15.5±1.6	11.1±5.0	39.3±35.9*
MeHg+Se	13	2.4±1.2	22.3±0.7*	12	11	14.6±4.5	10.5±3.5*	38.2±28.6*
MeHg+Vit E	15	3.0±3.3	22.1±0.3*	15	13	14.3±2.6	10.2±5.0*	38.1±38.7
MeHg+Se+Vit E	14	3.0±1.8	22.1±0.3*	14	13	14.8±3.0	11.9±4.2	24.6±28.7

Data are mean±standard deviation, where applicable. Sample size is in brackets.
* Denotes statistically significant ($p<0.05$) difference from control group.

indicated a significantly ($p<0.01$) lower food consumption for the MeHg+Vit E group as compared to the control. During gestation, MeHg treatment significantly ($F_{4,59}=7.04$, $p<0.01$) affected food intake and, compared to the control, significant ($p<0.01$) reductions in food consumption were observed for all groups treated with MeHg.

3.2.4. Maternal performance and litter size

There was no effect on mating behavior or on the pregnancy rate (Table 5). A significant ($\chi^2=19.3$, $p<0.01$) difference between groups was noted for the length of gestation. Pair-wise comparisons to the control group indicated significant ($p<0.01$) increases in gestational length for the MeHg+Se, MeHg+Vit E and MeHg+Se+Vit E groups. The number of implantation sites was not statistically different between the groups. However, there was a significant ($\chi^2=12.7$, $p<0.01$) treatment effect for a number of live pups on post-natal day 0, with significantly ($p<0.01$) fewer viable pups in the MeHg+Se and MeHg+Vit E groups compared to control. As a result, the post-implantation loss was significantly ($\chi^2=16.7$, $p<0.01$) affected for these groups, although all groups treated with MeHg had increased post-implantation losses, only the MeHg and MeHg+Se groups had significant ($p<0.01$) increases in post-implantation loss as compared to the control.

No total litter loss occurred in the control group, whereas the MeHg+Se+Vit E group had 11 viable litters, the MeHg+Vit E group had 8, and the MeHg and MeHg+Se groups followed at 4 and 3, respectively (Fig. 1). The marked total litter loss of the MeHg and MeHg+Se groups took place approximately within the first 3 days after birth and stabilized thereafter. In the MeHg+Vit E and MeHg+Se+Vit E groups, some litter loss occurred during the first post-natal days. Afterwards, the number of viable litters remained stable to the weaning stage.

3.2.5. Auditory startle habituation

There was a significant ($F_{4,63}=4.76$, $p<0.01$) treatment effect for the maximum startle amplitude (Table 6). The MeHg group was significantly ($p<0.01$) above that of the control and represented the only group significantly affected. The time to reach the maximum startle was significantly ($F_{4,63}=2.97$, $p<0.05$) affected by MeHg treatment, resulting in a significantly ($p<0.01$) shorter time for the MeHg group. There was a significant ($F_{4,63}=4.68$, $p<0.01$) treatment effect for average startle, with the MeHg and MeHg+Vit E groups having significantly ($p<0.02$) increased amplitude. In contrast, the average startle amplitude of the MeHg+Se+Vit E group was significantly (<0.02) lower than the MeHg group and closer to the control.

3.3. F_1 generation

3.3.1. Mortality, clinical signs and survival

Abnormal clinical signs of some pups were noted during the early post-natal stage for groups treated with MeHg. These signs included weak, thin and/or dehydrated appearance. A small number of pups in these same groups had dark areas in the periorbital region (behind closed eyelids). Some pups were observed with empty stomachs and an absence of observable

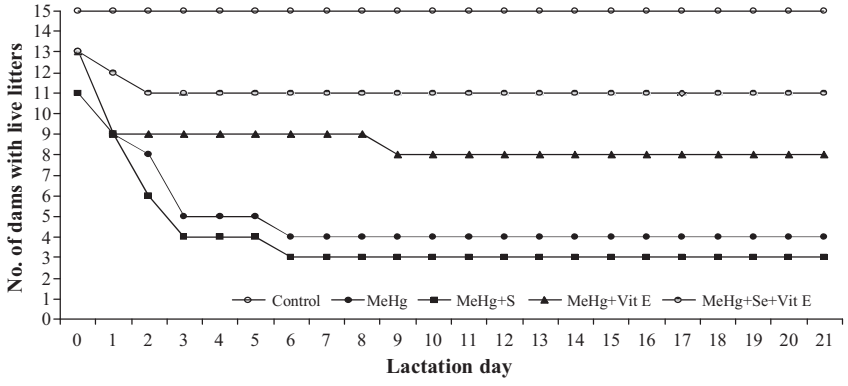

Fig. 1. Number of dams with live litters during lactation.

Table 6
Summary of auditory startle habituation on lactation day 23 for F_0 generation

	Maximum startle	Time to reach maximum startle	Average startle
Control	382.5±278.3 [15]	29.3±3.8 [15]	76.4±53.5 [15]
MeHg	940.2±371.0* [14]	25.3±2.4* [14]	176.9±67.3* [14]
MeHg+Se	677.7±382.1 [12]	27.2±2.2 [12]	124.4±63.1 [12]
MeHg+Vit E	688.8±303.4 [14]	26.9±3.0 [14]	133.4±62.2* [14]
MeHg+Se+Vit E	634.5±393.0 [13]	26.7±4.0 [13]	115.5±71.1# [13]

Data are mean±standard deviation of 50 trials in millivolts (maximum and average startle) or milliseconds (time to reach maximum startle). Sample size is in brackets.
* Denotes statistically significant ($p<0.05$) difference from control group.
# Denotes statistically significant ($p<0.05$) difference from MeHg group.

milk bands, thus indicating respective dams were not nursing adequately. For some of the litters, particularly those in the MeHg group, nursing was apparently reduced and pups maintained their thin appearance beyond the first few days following delivery.

The survival of the pups (sexes combined) was significantly ($\chi^2=29.1$, $p=0.0001$) different between groups from post-natal days 0 to 4 (Fig. 2). Post-hoc comparisons to control indicated significantly ($p<0.01$) increased mortality rates for all groups treated with MeHg. The percentages of pups surviving from post-natal days 0 to 4 were 99, 30, 27, 53 and 63, for the control, MeHg, MeHg+Se, MeHg+Vit E and MeHg+Se+Vit E groups, respectively. The highest survival rate out of the MeHg-treated groups was amongst pups in the MeHg+Se+Vit E group. Males and females showed similar survival patterns.

From post-natal days 4 to 21, a significant ($\chi^2=14.6$, $p<0.01$) group effect was observed regarding the survival of total pups (sexes combined). In comparison to the control group that had no mortality, MeHg and MeHg+Se groups indicated significant ($p<0.01$) decreases in survival. The survival percentages were 100, 52, 66, 82 and 94, for the control, MeHg, MeHg+Se, MeHg+Vit E and MeHg+Se+Vit E groups, respectively. Again, the viability of pups was highest in the MeHg+Se+Vit E group. When the data were analyzed for sexes separately, significant ($\chi^2=13.9$, $p=0.0076$ for males; $\chi^2=16.6$, $p=0.0023$ for females) inter-group differences were observed, and males in the MeHg and MeHg+Vit E groups and females in the MeHg and MeHg+Se groups showed significant ($p<0.01$) increases in mortality when compared to controls. When compared to the MeHg group, females in the MeHg+Se+Vit E group showed a significantly ($p<0.01$) lower rate of mortality.

3.3.2. Body weights

There were no statistically significant differences in pup body weight on post-natal day 0 (Table 7). On post-natal day 4, there was a significant ($F_{4,37}=8.14$, $p=0.0001$ for males; $F_{4,38}=7.40$, $p=0.0002$ for females) group effect. The body weights of male pups were significantly ($p<0.01$) lower for the MeHg, MeHg+Vit E and MeHg+Se+Vit E groups compared to the control. Female pups in all groups given MeHg had significantly ($p<0.01$) lower body weights than controls. On post-natal day 7 and until study completion, the body weights of male and female offspring in all groups exposed to MeHg were significantly ($p<0.02$) lower than control values. All groups appeared to show similar body weight progressions.

3.3.3. Developmental and reflexological tests

The data for developmental measurement of pinna unfolding and the outcome of the surface righting reflex test did not suggest a clear effect associated with MeHg exposure (Tables 8a and 8b).

A significant ($\chi^2=22.6$, $p=0.0002$ for males; $F_{4,35}=4.85$, $p=0.0032$ for females) treatment effect was noted for negative geotaxis resulting in a significant ($p<0.02$) delay for males in

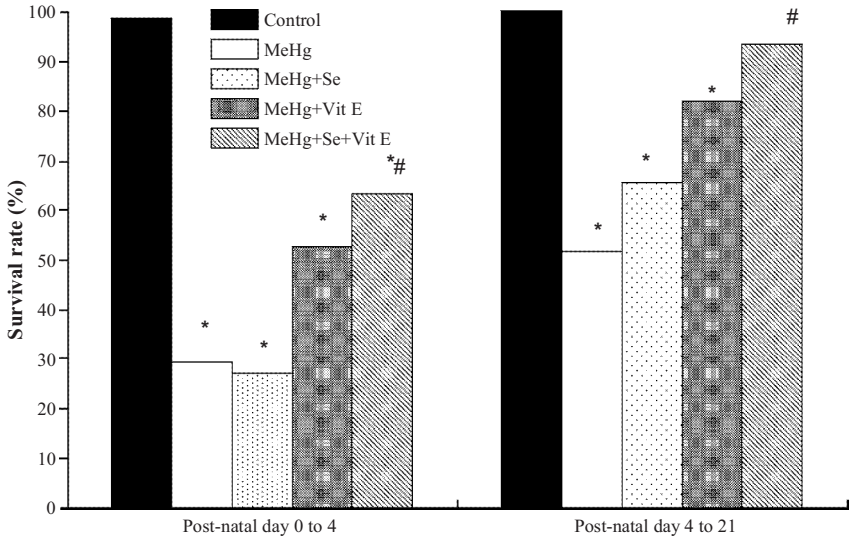

Fig. 2. Survival of F_1 generation during post-natal period. Data are means. *Denotes statistically significant ($p<0.05$) difference from control group. #Denotes statistically significant ($p<0.05$) difference from MeHg group.

Table 7
Summary of body weight (g) for F_1 generation

	Post-natal day						
	0	4	7	14	21	24	30
Males							
Control	6.1±0.6 [15]	9.7±0.9 [15]	15.8±1.6 [15]	32.8±3.1 [15]	51.5±5.0 [15]	66.7±4.6 [15]	102.6±7.5 [15]
MeHg	5.7±0.5 [13]	7.8±1.2* [5]	11.5±1.9* [3]	22.7±5.9* [3]	35.2±11.3* [3]	43.1±14.9* [3]	69.5±21.7* [3]
MeHg+Se	5.6±0.5 [9]	8.6±0.5 [3]	12.4±1.1* [3]	26.0±2.1* [3]	38.3±3.8* [3]	51.9±2.6* [3]	78.5±6.2* [3]
MeHg+Vit E	5.6±0.7 [13]	8.4±1.0* [8]	11.8±2.7* [8]	25.6±2.9* [7]	38.1±3.1* [7]	52.1±4.5* [7]	81.8±7.3* [7]
MeHg+Se+Vit E	5.7±0.6 [13]	8.3±0.9* [11]	11.8±2.2* [11]	24.4±5.6* [11]	36.6±7.6* [11]	47.1±9.6* [11]	74.5±13.8* [11]
Females							
Control	5.8±0.4 [15]	9.4±0.6 [15]	15.3±1.1 [15]	31.9±2.6 [15]	49.8±3.6 [15]	63.3±3.3 [15]	96.3±5.7 [15]
MeHg	5.4±0.5 [12]	7.5±1.0* [5]	10.3±2.2* [4]	23.2±5.4* [4]	36.5±8.9* [4]	43.4±10.4* [4]	66.7±13.0* [4]
MeHg+Se	5.2±0.7 [11]	7.2*±1.4* [4]	11.1±1.3* [3]	22.8±4.5* [3]	34.0±3.7* [3]	45.3±4.0* [3]	69.3±6.2* [3]
MeHg+Vit E	5.3±0.8 [13]	7.9±1.3* [9]	10.9±3.1* [9]	24.4±4.5* [8]	35.0±6.0* [8]	45.0±6.8* [8]	70.1±9.8* [8]
MeHg+Se+Vit E	5.4±0.6 [13]	8.0±0.7* [10]	11.6±1.4* [10]	23.9±4.6* [10]	36.1±6.6* [10]	45.5±7.3* [10]	69.4±9.6* [10]

Data are mean±standard deviation in grams. Sample size (litters) is in brackets.
* Denotes statistically significant ($p<0.05$) difference from control group.

the MeHg, MeHg+Se and MeHg+Se+Vit E groups, and for females in the MeHg+Vit E and MeHg+Se+Vit E groups compared to the control. The day of development for tooth eruption was significantly ($F_{4,35}=2.92$, $p=0.0351$ for males; $F_{4,36}=8.22$, $p=0.0001$ for females) different amongst the groups. Significant ($p\leq 0.02$) acceleration in development compared to control occurred for males and females in the MeHg+Vit E and MeHg+Se+Vit E groups and for females in the MeHg group. Females in the MeHg+Se group showed a significantly ($p<0.01$) later development for tooth eruption compared to the MeHg group, a result more similar to the control group.

A significant ($F_{4,34}=6.80$, $p=0.0004$ for males; $F_{4,35}=4.99$, $p=0.0027$ for females) group effect was noted for the day of auricular startle development. Post-hoc comparisons to the control group indicated significant ($p<0.01$) delays in the onset of this reflex for males and females in the MeHg, MeHg+Vit E and MeHg+Se+Vit E groups. Eye opening was significantly ($F_{4,34}=8.95$, $p=0.0001$ for males; $F_{4,35}=9.26$, $p=0.0001$ for females) affected by MeHg treatment with significantly ($p<0.01$) earlier development occurring for males and females in the MeHg+Se, MeHg+Vit E and MeHg+Se+Vit E groups. In addition, males in the MeHg+Se, MeHg+Vit E and MeHg+Se+Vit E groups and females in the MeHg+Vit E and MeHg+Se+Vit E groups showed eye opening significantly ($p<0.01$) sooner than the MeHg group.

3.3.4. Auditory startle habituation

The was no statistical difference between control and treatment groups in maximum startle and average startle amplitudes(Table 9). The time to reach the maximum startle amplitude was significantly ($F_{4,33}=10.22$, $p=0.0001$ for males; $F_{4,34}=5.09$, $p=0.0025$ for females) increased with MeHg treatment. Significant ($p<0.02$) increases in maximum startle response times were noted for males and females in the MeHg+Vit E and MeHg+Se+Vit E groups and for males in

Table 8a
Summary of developmental landmarks for F_1 generation

	Pinna unfolding[a]				Tooth eruption[b]	Eye opening[b]
	1	2	3	4		
Males						
Control	0.0±0.0 [14]	23.5±33.7 [15]	82.2±32.4 [15]	100.0±0.0 [15]	10.4 1.0 [15]	14.6±0.7 [15]
MeHg	0.0±0.0 [8]	9.6±25.3 [7]	100.0±0.0 [5]	100.0±0.0 [5]	9.0±1.7 [3]	14.8±0.3 [3]
MeHg+Se	0.0±0.0 [9]	30.5±47.6 [6]	100.0±0.0 [4]	100.0±0.0 [4]	9.0±0.9 [3]	12.92±0.1*,# [3]
MeHg+Vit E	0.0±0.0 [9]	23.1±39.6 [8]	100.0±0.0 [8]	100.0±0.0 [8]	8.9±1.2* [8]	13.4±0.7*# [7]
MeHg+Se+Vit E	0.0±0.0 [11]	15.1±30.8 [11]	90.9±30.2 [11]	100.0±0.0 [11]	9.0±1.5* [11]	13.7±0.7*# [11]
Females						
Control	0.0±0.0 [14]	29.9±33.7 [15]	86.6±28.5 [15]	100.0±0.0 [15]	10.6±0.9 [15]	14.5±0.5 [15]
MeHg	0.0±0.0 [8]	14.1±22.4 [7]	100.0±0.0 [5]	100.0±0.0 [5]	7.8±0.6* [4]	14.3±0.6 [4]
MeHg+Se	3.3±10.0 [9]	50.0±54.8 [6]	100.0±0.0 [5]	100.0±0.0 [5]	10.5±2.1# [3]	13.0±0.7* [3]
MeHg+Vit E	0.0±0.0 [9]	41.2±45.5 [9]	100.0±0.0 [9]	100.0±0.0 [9]	8.5±1.3* [9]	13.0±1.0*,# [8]
MeHg+Se+Vit E	0.0±0.0 [12]	16.4±33.6 [11]	95.5±9.6 [10]	100.0±0.0 [10]	8.6±1.4* [10]	13.2±0.8*# [10]

Sample size (litters) is in brackets.
[a] Group litter mean±standard deviation for percentage of pups showing positive response on post-natal days 1 to 4.
[b] Group litter mean±standard deviation for post-natal day of development.
* Denotes statistically significant ($p<0.05$) difference from control group.
# Denotes statistically significant ($p<0.05$) difference from MeHg group.

Table 8b
Summary of reflex tests for F_1 generation

	Surface righting reflex[a]				Negative geotaxis[b]	Simple auricular startle[b]
	1	2	3	4		
Males						
Control	81.8±17.4 [14]	79.1±22.2 [14]	78.7±26.6 [13]	88.0±25.7 [10]	8.68±1.20 [15]	12.13±0.63 [15]
MeHg	63.3±34.7 [8]	87.7±18.8 [7]	97.3±5.5 [4]	89.0±15.6 [2]	11.17±0.72* [3]	13.19±0.17* [3]
MeHg+Se	78.9±32.0 [9]	68.6±41.7 [5]	12.3±10.8* [3]	55.0±7.1 [2]	11.25±0.43* [3]	12.67±0.38 [3]
MeHg+Vit E	80.2±32.8 [9]	75.1±22.0 [7]	96.2±5.9 [6]	95.4±6.3 [5]	11.12±2.11 [7]	13.04±0.83* [7]
MeHg+Se+Vit E	84.9±19.6 [11]	76.1±26.3 [9]	89.8±9.3 [8]	87.0±12.6 [6]	12.24±1.14* [11]	13.27±0.48* [11]
Females						
Control	74.3±18.5 [14]	67.3±22.6 [14]	71.2±27.6 [13]	79.4±13.3 [10]	8.82±1.20 [15]	11.90±0.69 [15]
MeHg	50.0±28.9 [8]	76.5±39.3 [6]	78.0±37.6 [4]	73.5±9.2 [2]	9.75±1.90 [4]	13.21±0.74* [4]
MeHg+Se	70.3±30.6 [9]	54.0±35.8 [5]	56.7±5.8 [3]	66.7±28.9 [3]	11.39±1.13 [3]	12.50±0.25 [3]
MeHg+Vit E	79.9±18.6 [9]	48.4±40.0 [7]	72.7±26.8 [6]	66.6±47.2 [5]	11.06±1.50* [8]	12.69±0.80* [8]
MeHg+Se+Vit E	61.9±37.1 [12]	46.3±37.6 [9]	85.0±16.3 [7]	93.3±16.3 [6]	11.38±2.28* [10]	12.79±0.45* [10]

Sample size (litters) is in brackets.
[a] Group litter mean±standard deviation for percentage of pups showing positive response on post-natal days 1 to 4.
[b] Group litter mean±standard deviation for post-natal day of development.
* Denotes statistically significant ($p<0.05$) difference from control group.

the MeHg+Se, MeHg+Vit E and MeHg+Se+Vit E groups as compared to the control.

4. Discussion

This study investigated the potential benefits of the dietary supplements, selenium and vitamin E, on adult female rats administered MeHg prior to and during pregnancy. The co-administration of both these nutrients mitigated the signs of MeHg impairment in the average startle response an improved weight gain during lactation of adult animals. This corroborated the work by others [34] who found that when selenium and vitamin E were given together MeHg toxicity in adult rats was reduced, and improved growth, fewer clinical signs of toxicity and longer survival time occurred. However, the adult females exposed to MeHg with selenium supplements alone did not show any protection against MeHg toxicity. In one study [16], selenium administered in the drinking water to adult rats delayed the onset of MeHg toxicity. In another study [4], adult rats fed selenium showed improved growth when compared to animals given MeHg alone. Further studies [5,3,11] have shown diminished clinical signs and improved body weight progression normally associated with MeHg treatment in adult rats and hamsters using parenteral routes of selenium administration. Supplementation of either vitamin E or selenium or both also increased gestation time (Table 5).

Table 9
Summary of auditory startle habituation for F_1 generation

	Maximum startle	Time to reach maximum startle	Average startle
Males			
Control	282.0±74.9 [15]	20.8±1.5 [15]	51.1±11.4 [15]
MeHg	198.9±117.7 [3]	24.4±1.5 [3]	37.6±21.1 [3]
MeHg+Se	411.9±108.6 [3]	27.1±4.2*	75.0±15.5 [3]
MeHg+Vit E	243.3±112.8 [7]	24.4±2.7* [7]	46.6±19.8 [7]
MeHg+Se+Vit E	278.8±145.1 [10]	27.1±3.5* [10]	54.6±25.4 [10]
Females			
Control	316.9±112.3 [14]	21.1±1.1 [14]	56.0±20.4 [14]
MeHg	224.4±103.7 [4]	24.1±1.0 [4]	43.4±18.5 [4]
MeHg+Se	254.4±67.9 [3]	24.8±1.6 [3]	46.6±14.2 [3]
MeHg+Vit E	235.9±129.2 [8]	24.1±3.2* [8]	44.0±22.3 [8]
MeHg+Se+Vit E	279.9±105.7 [10]	25.8±4.1* [10]	52.1±18.7 [10]

Data are mean±standard deviation for 50 trials in millivolts (maximum and average startle) or milliseconds (time to reach maximum startle). Sample size (litters) is in brackets.
* Denotes statistically significant ($p<0.05$) difference from control group.

There were no apparent benefits to pre-natal offspring survival by administering selenium alone in our study. However, one study [24] showed that MeHg-induced mortality of mouse embryos and fetuses was decreased by administration of selenium.

The most remarkable effects of vitamin E and selenium supplement were observed in the survivability of the pups. MeHg treatment resulted in high post-natal pup mortality. For the first few days following birth, 70% or more of the pups died in the MeHg alone and selenium supplemented groups. This is in contrast to death rates for vitamin E and selenium/vitamin E supplemented groups of 47% or less. Observations of the dams suggested that this may have, at least in part, been due to inadequate nesting behavior and poor nursing. For the remainder of the pre-weaning period, the pup mortality was 48% in the nonsupplemented group and 34%, 18% and 6% in the selenium, vitamin E and selenium/vitamin E supplemented groups, respectively. These data indicate that the addition of both vitamin E and selenium significantly attenuated the MeHg-related survival rate, while the individual nutrients alone did not show a significant effect on survival numbers.

The pup body weights on the day of birth were approximately 7–10% lower in all groups given MeHg compared to controls. This is comparable to a study [25] where mice were administered MeHg or co-administered MeHg and selenium (sodium selenite) on gestation day 9. Average pup weights at birth were found to be 11–13% lower in both groups as compared to the controls. A similar observation was reported

by Fredriksson et al. [9] where rats were treated with MeHg from gestation days 6 to 9 and selenium was added to the diet (sodium selenite) before mating and throughout pregnancy and lactation. Lower birth weights aside, pups that survived until the completion of that study had growth patterns that appeared to be generally similar for all groups treated with MeHg although the body weights were always lower than controls.

MeHg treatment affected the development of offspring physical landmarks, performance in reflex tests and assessment of simple auricular startle response (Table 8). There were delays in the development of negative geotaxis and auricular startle, as well as accelerated development of tooth eruption for female pups. Additional selenium and vitamin E in the diet of the dams did not improve pup development. However, eye opening in the pups of the supplemental diet group appeared to develop earlier than those in the control or MeHg groups. Other researchers were able to show an improvement in righting reflex and walking activity of mice offspring [25] and antagonism to hypoactivity of rat pups [9] associated with selenium administration to the dam.

In several cases, dietary supplementation affected performance while MeHg did not show any effects. For example, the auditory startle response test indicated that while the MeHg had no effect on habituation, the nutrient supplementation delayed response time longer than control groups. The pups in the nutrient supplement groups showed advanced development as indicated by shorter post-natal days when landmarks for development such as tooth eruption and eye opening occurred, while MeHg showed no effects. It is not clear whether this is a statistical or biological significant effect. The low survival rate of the pups in the MeHg only treatment reduced the sample size to $N=3$.

Contrary to the benefits of selenium in adult animals reported in the literature, these benefits were not observed in this study. There are at least four possible explanations for why this may have occurred: (1) form of selenium, (2) route of administration and (3) quantity of selenium.

The form of selenium used in our study differed from inorganic selenium or sodium selenite used in most other animal studies. Seleno-methionine is believed to more closely resemble the biological form of selenium found in human diets. The availability of biological selenium for reaction with MeHg has been shown to be lower than that of selenite [15]. It was found that the organic form of selenium increased mercury levels in blood and liver, thus confirming an interaction with mercury. Increased levels of mercury in the blood of 2-day-old pups exposed in-utero to MeHg and selenium as sodium selenite indicated that MeHg blood levels in the progeny were altered in association with mercury–selenium interactions [9].

A second possible explanation for these differences may be attributed to the exposure route, which can influence deposition rates of mercury in offspring. Selenium administered by injection during gestation increased mercury concentrations in the neonatal brain [23], whereas oral administration had reduced brain levels [9].

A third explanation is the possibility that an insufficient quantity of selenium was given to the dams. Magos [13,14] has indicated that MeHg increases the elimination of selenite, which becomes more pronounced as the amount of selenite increases in the body. As a result, MeHg decreases the bioavailability of selenite, which in turn could reduce the ability of selenite to prevent intoxication. In this study, high doses of MeHg were administered, as well as organic forms of selenium like seleno-methionine.

Vitamin E showed a marked capability to reduce offspring mortality after birth and augment the efficacy of selenium in post-natal survival. It is possible that vitamin E has more roles than what is currently known or is more available to the offspring than selenium. These findings could be attributed to the similar functions that both nutrients share albeit using different mechanisms.

The National Academy of Sciences in the United States [18] has recommended that women of child bearing age have a dietary intake that includes 8 mg α-tocopherol equivalents and 55 μg selenium/day or around 0.1 mg α-tocopherol equivalents/kg body weight/day and 0.9 μg selenium/kg body weight/day. During pregnancy, the recommendations for these two nutrients are higher. In the current study, the adult female rats in the vitamin E and selenium fortified diet groups had a dietary intake equal to on average approximately 24 mg α-tocopherol acetate and 0.1 mg selenium/kg body weight/day during the supplementation period. These numbers represent more than 200- and 100-fold increases of vitamin E and selenium, respectively, when compared to the NAS minimal recommendations. However, supplemental vitamin E formulations of α-tocopherol acetate sold in stores for human consumption are typically in 100, 200, 400 or 800 IU capsules. This is equivalent to around 1 to 10 mg α-tocopherol acetate/kg body weight/day, which is only about 2- to 20-fold lower in magnitude than what the rats received on the current study.

The benefits to the pregnant animal and developing offspring from maternal selenium and vitamin E intake in association with MeHg toxicity have been only sparsely studied thus far. This is surprising given the evidence from adult animal studies that implicate these nutrients in reversing, or at least delaying, the neurotoxicity associated with MeHg. The data from this study have demonstrated a significant improvement in the survivability of the pups exposed to high dose of MeHg during gestation when selenium and vitamin E are administered in tandem prior to and during pregnancy. Further dose–response and toxicokinetic studies are needed to translate this result into public health recommendations.

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