



OPEN Sex dependent intergenerational effects of lead in mouse model

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Lead (Pb) exposure negatively impacts fertility in both males and females, pregnancy outcomes, and child brain development. We investigated the reproductive and neurological effects of Pb exposure on male and female mice via Pb-contaminated soil for 4 weeks. Breeding was conducted after completion of exposure, in four groups; group 1 consisted of exposed dams and unexposed sires, group 2 consisted of exposed sires and unexposed dams, group 3 consisted of exposed sires and exposed dams and group 4 was the control. Generally, Pb exposure reduced observed conception rates, with a cumulative decrement observed when both males and females are exposed. Gene expression of the testes revealed oxidative stress as the cause of reduced conception rates. Neurological tests: Morris water maze and rotarod were conducted on F1 generation offspring. Maternally and paternally exposed F1 mice performed poorly in the Morris water maze when compared to the control. The severity of the neurological effects was also parent-dependent and sex-dependent. Paternal Pb exposure effects were more pronounced in female offspring. A comparison of gene expression changes of the hippocampus and prefrontal cortex showed paternal Pb-exposure resulted in more prefrontal cortex changes than in the hippocampus, a trend also recorded in the exposed sires. The pronounced effects in female offspring of paternal Pb exposure may suggest that Pb neurological effects may be X-chromosome-linked.

Keywords Pb, Soil, Infertility, Neurological, Paternal, Maternal

Lead (Pb) toxicity remains endemic in many places of the world with routes of exposure varying from country to country¹. Metals like Pb are concentrated into soil mainly by anthropogenic processes that include manufacturing, smelting and industrial processes². Lead contaminated soil is one of the major sources of exposure, where like other heavy metals Pb does not biodegrade hence persisting in the soil³.

Lead exposure has been associated with impairment of renal, haematopoietic and hepatic systems in children making it a global health concern⁴. It has also been attributed to the cause of reduced fertility, pregnancy loss and neonatal fatalities. In men it is known to reduce libido, sperm viability and quality⁵ as well as reduced levels of testosterone⁶. Effects on the brain can be detrimental, causing neurological impairment such as reduction in IQ, learning deficits, cognitive impairments, and memory loss⁷. This is more pronounced in children as the developing brain is more susceptible to the effects of Pb when it comes to learning than an already developed adult brain⁸.

Although numerous studies^{9–11} have focused on maternal exposure and the consequent neurological defects that occur when exposure is gestational¹², there has been an increase in the investigation of paternal inherited neurological diseases. This is critical as occupational Pb exposure mostly occurs in men¹³. In an earlier study in humans, an association was noted between paternal Pb exposure and low birth weight and length¹⁴. Since paternal exposure affects overall mass development of neonates, information on the possibility that brain development may be affected is lacking. Therefore, in the current study, we investigated if Pb exposure would cause neurological deficits via paternal exposure.

To fully distinguish these effects, we compared the effects of Pb exposure on conception, depending on whether sires or/dams were exposed in F0. Male fecundity was evaluated using Serine inhibitor Kazal type

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2 (*Spink 2*), cysteine-rich secretory protein 2 (*Crisp2*) and *Pou5F1*. To determine how differently the above exposures in F0 individuals would affect the F1 generation, we used neurological tests (water Morris and rotarod tests) and brain plasticity-related biomarkers (brain-derived neurotrophic factor (*Bdnf*), cAMP response element binding protein (*Creb*), N-methyl-D-aspartate receptors (*Grin2A* and *Grin2B*) in hippocampal and prefrontal cortex tissues.

Methodology

Soil preparation

Soil containing lead sulphide at concentration 5000 mg/kg was selected according to environmental concentrations recorded by Nakata et al.¹⁵ and Wade et al.¹⁶. The soil was prepared two weeks before the onset of the exposure experiment. Soil was sieved to 2 mm diameter and Pb was added and left to mature. The control group was kept on Pb-free soil. The soil was changed weekly in both exposed and control groups. All the mice in the current experiment including F1 generation were kept on soil.

Animals

The current study was conducted under the ethical considerations approved by Hokkaido University, approval number (22-0092) which is accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC International). This animal experiment complied with the Guide for the Care and Use of Laboratory Animals and the ARRIVE guidelines (Animal Research: Reporting of In Vivo Experiments). ARRIVE check list is attached in the Supporting Information. Twenty-one male and 42 female C57BL/67 mice aged 4 weeks old were obtained from Japan SLC Co. Ltd, weighing between 17 and 22 g. Males and females were kept in separate cages and fed standard rodent pellets; water was administered ad libitum. After 1 week of acclimatisation, the mice were randomly divided into Pb-exposed and control groups.

Exposure on lead contaminated soil lasted for 4 weeks, after which mice were kept on clean soil, and breeding was then conducted. Sires and dams were kept together for a week, after which all sires were euthanised. All mice in this experiment were euthanised using a carbon dioxide chamber under the American Veterinary Medical association (AVMA) guidelines. The dams were euthanised after F1 pups were weaned. Fifteen of the F1 pups were kept for neurological tests as representatives of each group. Of these, nine were male and six were female, this number was selected according to the resultant number and sexes present at weaning in each group. Neurological tests were conducted when these F1 mice were 8 weeks old, as shown in Fig. 1.

Fertility study

Breeding was conducted by keeping males and females in cages at the ratio of 1:2, estrus had been induced prior in females using the Whitten effect¹⁷. The mice were divided into four groups. Group 1 consisted of unexposed sires and exposed dams, Group 2 consisted of exposed sires and unexposed dams, Group 3 had exposed sires and exposed dams, and Group 4 was the control with unexposed sires and dams. After euthanising of sires, dams were observed for pregnancy and were kept in pairs for companionship until a week prior to the expected day of partition when single housing was used. Pups were weighed from weaning till 8 weeks old and weights were recorded.

Water Morris test F1

Eight-week-old mice were subjected to the Water Morris test as described by Tian et al.¹⁸ with a few modifications. Mice were trained for four days, and the probe test was performed on day five.

Rotarod test

Rotarod test was conducted on F1 generation using specially manufactured mice Rotarod by Panlab. Mice were taken to the testing room and left to acclimate for one hour before the beginning of the experiment. The mice were put on rotarod while speed was gradually increased, each trail had an endpoint of 300 s. It was noted if the

TIMELINE OF EXPERIMENT

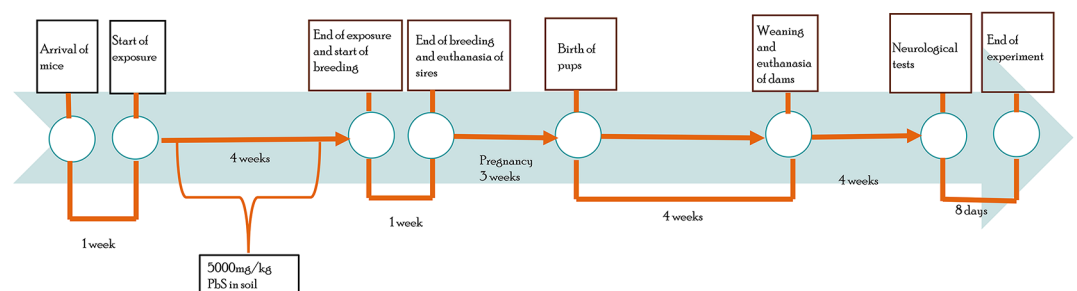


Fig. 1. Shows the timeline of the animal exposure experiment.

mouse jumped off or fell during each trail. After one training trial, each mouse was subjected to three trials with resting intervals of fifteen minutes.

Real-time PCR

Total RNA was isolated from the brain’s prefrontal cortex, hippocampus and testis samples using FastGene® kit (Nippon Genetics). cDNA was made using Toyobo cDNA synthesis kit. Quantitative real-time RT-PCR was used to quantify the expression levels of different genes, using Beta actin mRNA as the normalization standard. Primers used are shown in Supplementary data Table S4.

Lead measurement

Brain and bone samples were weighed and dried for 48 h at 50 °C. Dried samples (20–100 mg) and 0.1 ml of blood were then added to 5 ml of 30% nitric acid (Cica reagent, Specific gravity of 1.38, 60%; Kanto Chemical Corp., Tokyo, Japan) and 1 ml of hydrogen peroxide (Cica reagent, 30%; Kanto Chemical Corp.). These were then digested in a speed wave microwave (Berghof, Eningen, Germany). Solutions from this process were analysed for Pb concentrations using inductively coupled mass spectrometry (ICP-MS, 7700 series; Agilent Technologies, Tokyo, Japan). Analytical quality control was conducted using DOLT-5 (dogfish liver; National Research Council of Canada) and NIST 1400 Bone Ash (Stigma Aldrich; United States of America) as certified reference materials. The recovery rates in replicate measurements yielded were 96–100% for DOLT-5 and 86–90% for Bone Ash.

Statistical analysis

Results were subjected to student’s t-Test for F0 and ANOVA for F1. Assessing differences among group means was done by using Tukey’s test. Data has been presented as mean ± S.E.M. and *p*-values < 0.05 were considered significant. Statistical studies were done using JMP version 16.

Results

Breeding results

The conception rate dropped when either sires or dams were exposed to Pb. The conception rate was lesser when sires were exposed than when dams were exposed, but these rates were not significantly different from control breeding rates. The conception rate was less than 50% (Table 1) when both sires and dams were exposed to Pb, and this value was statistically significant when compared to the pregnancy rate in the control group.

Lead concentrations in F0

Eleven exposed sire mice and 10 control sire mice had blood and bone Pb levels measured at the end of the breeding period. The bone Pb mean value was 15.6 mg/kg and other values ranged from 26.7 to 6.13 mg/kg (Fig. 2A). In the blood, the exposed sires’ mean Pb level was 5.73 µg/dl, levels ranged from 8.19 to 2.96 µg/dl (Fig. 2B). In the Control group, Pb levels in the bone ranged from 0.498 to 0.0905 mg/kg with a mean value of 0.287 mg/kg, while those in the blood ranged from 0.498 to 0.227 µg/dl with a mean value of 0.367 µg/dl. Twenty-one control dams and 20 exposed dam’s bone and blood Pb levels were measured upon weaning of F1 offspring. One exposed dam experienced dystocia during parturition and was then excluded from further analysis. Exposed dams bone Pb levels ranged from 12.4 to 7.25 mg/kg with a of mean of 9.82 mg/kg (Fig. 2C). Blood Pb levels ranged from 1.57 to 13.9 µg/dl with a mean of 2.65 µg/dl (Fig. 2D). In the control group the bone Pb levels ranged from 0.382 to 0.131 mg/kg with a mean value of 0.187 mg/kg. The blood lead levels were ranged from 0.738 to 0.100 µg/dl with a mean of 0.515 µg/dl.

A comparison of Pb levels was made between exposed pregnant dams and non-pregnant exposed dams to determine the effect of pregnancy and lactation on Pb distribution in the bone and blood (Fig. 3). The results did not show any significant differences in bone lead as both pregnant exposed dams and non-pregnant dams had similar means of 9.73 mg/kg and 9.91 mg/kg, respectively (Fig. 3A). The blood Pb levels showed significant differences with pregnant dams having mean Pb levels of 1.94 µg/dl and non-pregnant dams having 3.35 µg/dl (Fig. 3B).

F1 generation mice are described by the exposure status of the parents, such offspring born from only exposed dams are termed the Dam exposed group, and those from control dams and sires are termed Control. The offspring from exposed sires are Sire exposed and those from exposed sires and dams are referred to as Both exposed group.

F1 generation weights, litter sex ratios and blood Pb levels are presented in supplementary materials.

Group	1	2	3	4	Total
	EFxUM	UFxEM	EMxEF	UMxUF	
Total females (N)	10	11	11	10	42
Pregnant (N)	8	8	5	10	35
Pregnancy rate (%)	80	72	45*	100	83

Table 1. Shows breeding results where EF represents exposed female, EM represents exposed male, UM is an unexposed male and UF is unexposed female. N indicates sample size. **p* = 0.01.

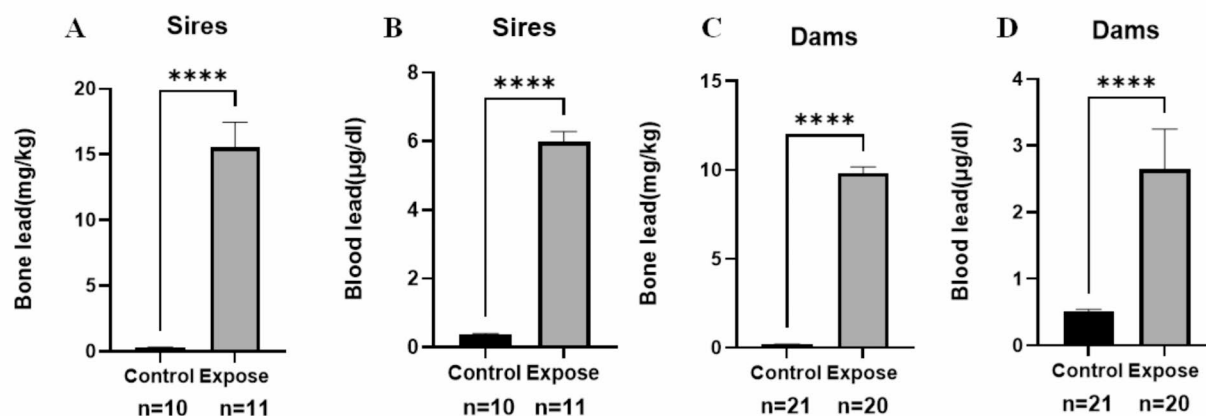


Fig. 2. Shows lead levels in the bone and blood of F0. (A) Shows blood Pb levels and (B) shows bone Pb levels in F0 sires. (C) Shows blood Pb levels and (D) shows bone Pb levels in F0 dams. *Indicates $p < 0.01$.

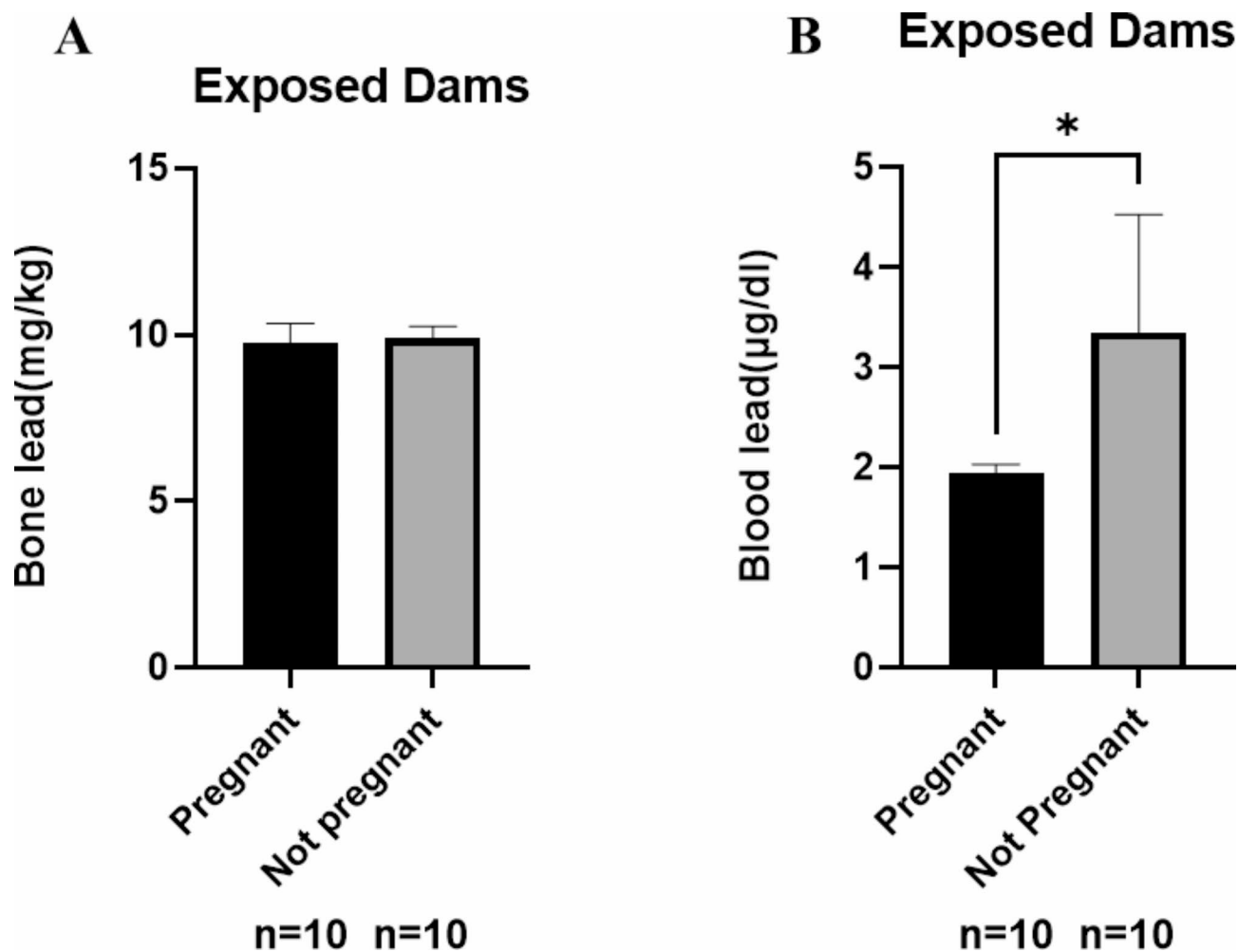


Fig. 3. Shows lead levels in the bone and blood of pregnant and non-pregnant dams of the exposed group. (A) Shows the bone lead levels and (B) shows blood lead levels in exposed dams that were able to get pregnant and those that did not at weaning. *P-value = 0.04.

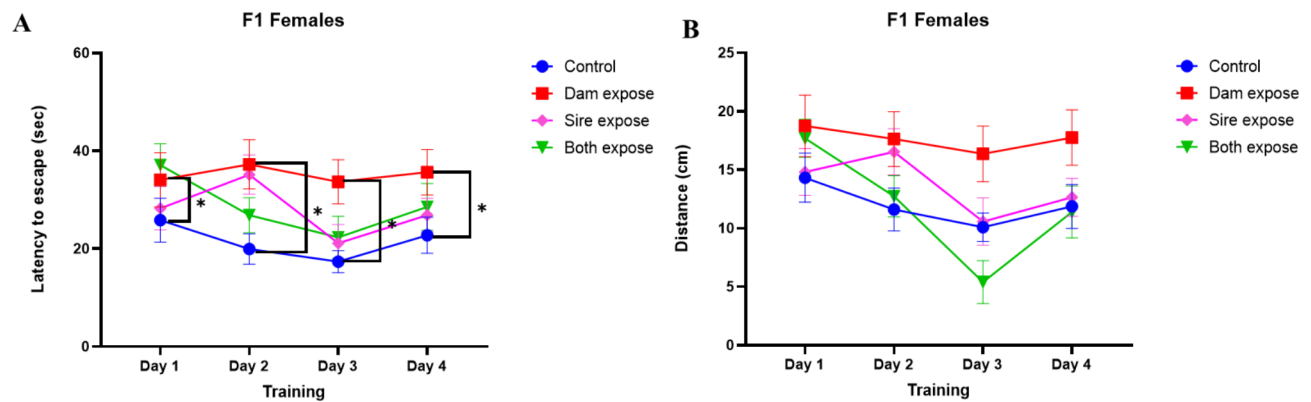


Fig. 4. Shows latency to escape and distance covered in the Morris Water Maze of F1 females. (A) Shows average latency to escape during training of Water Morris Test and (B) shows distance during latency to escape during training of Water Morris Test in F1 females. ($n=6$ in each group).

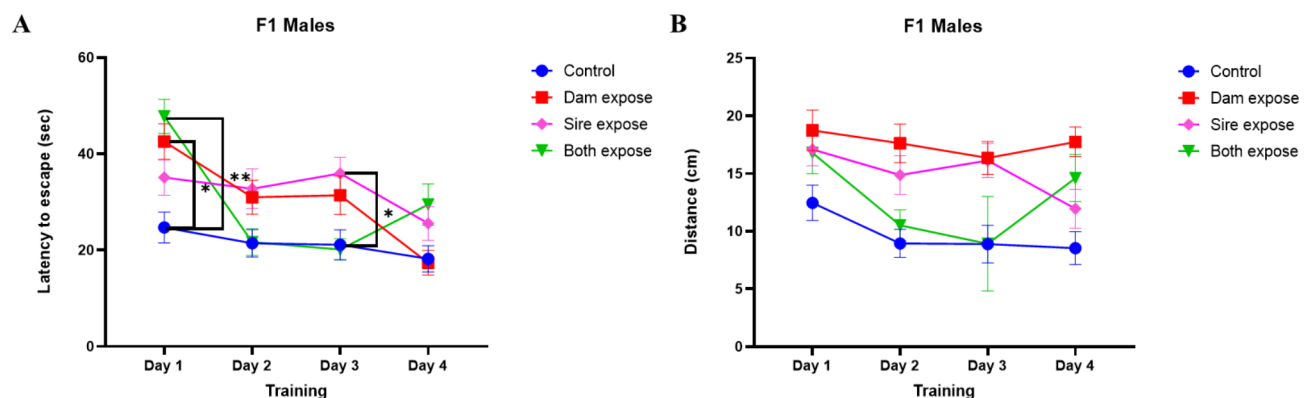


Fig. 5. shows latency to escape, and distance covered in F1 males during the water Morris maze test. (A) Shows latency to escape during training period. (B) Shows distance travelled during training period in F1 males. ($n=9$ in each group).

Water Morris test

The water Morris Test Latency to escape showed a steady decrease in the times in all groups of F1 female offspring (Fig. 4A), there was an increase in time recorded on Day 4 in all groups when compared to Day 3. In Sires exposed group offspring, the learning trend differed from the other groups in that it had an inconsistent pattern. On all training days, significant differences occurred between the Control group and Dam exposed group.

In F1 females distance covered during latency to escape was measured (Fig. 4B) this was the distance the mice swam around until they found the escape platform. There were no significant differences among the groups. It is expected during the test for distance to gradually reduce during the training. There was a downward trend in the distance until Day 3 and a sharp increase in the distance was observed on Day 4 which is also seen in the latency to escape analysis.

During the training of the water Morris maze test the F1 males (Fig. 5A) showed a constant steady decrease in latency to escape time, except for the Both exposed group whose time increased on Day 4. There were no significant differences among the timings in the groups. The distance travelled during latency to escape was measured in F1 males (Fig. 5B), the Dam exposed and Both exposed groups had timings increase on Day 4 similar to the trend observed in F1 females, while Sire exposed, and Control groups showed the downward expected trend in the distance covered.

The probe test (Fig. 6) was conducted to fully understand the spatial learning ability. F1 females showed no significant differences in probe entries and time spent in the target zone (Fig. 6B,C). There was a significant difference observed between Sire exposed females and Control with regards to the latency to escape (Fig. 6A). In F1 males the probe test did not yield any notable or significant differences among the groups (Fig. 6D–F). The test statistics were comparable among all the groups.

Rotarod test

The F1 females' rotarod results showed significant differences in the highest speed attained and time spent on the rod (Fig. 7A, B) among the groups. With regards to speed, the Control group and the Dam exposed group had attained the highest speed. There were significant differences between the Control group and Sire exposed

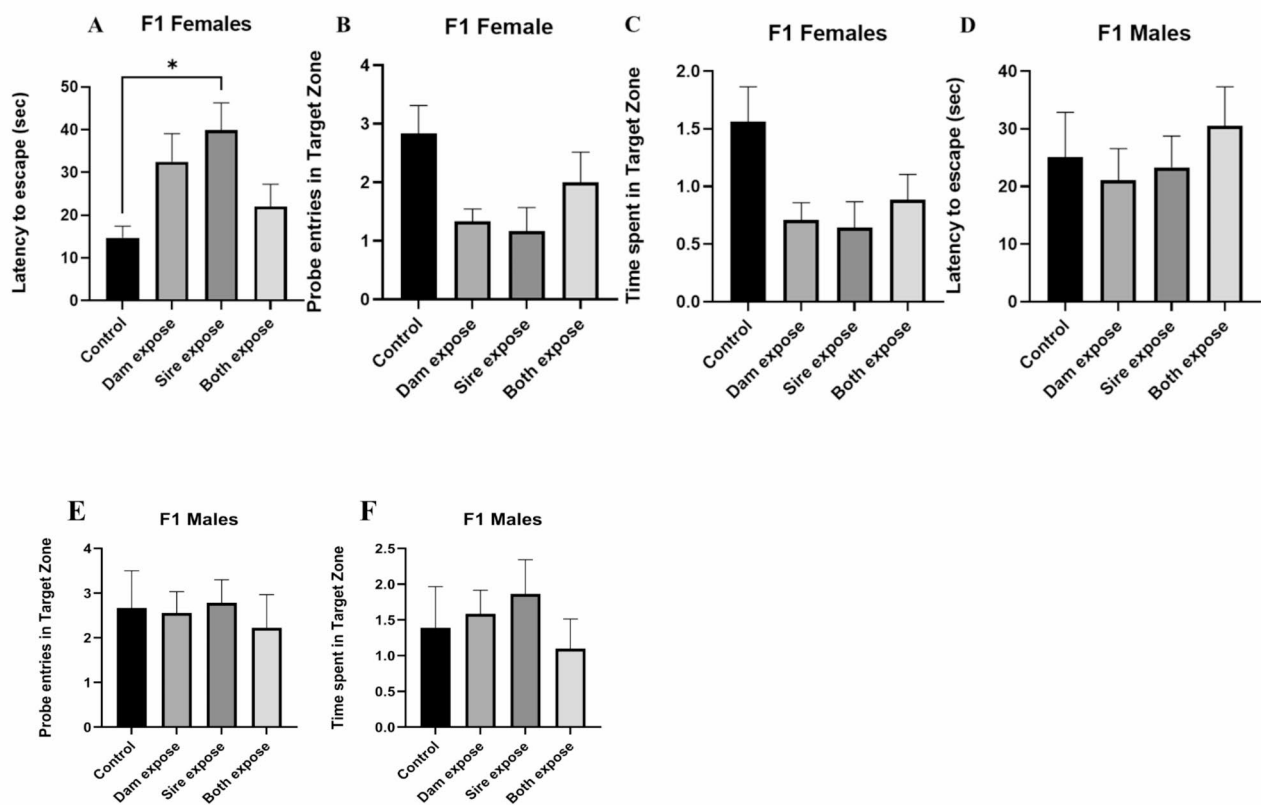


Fig. 6. Shows the results of the probe test for F1 females and F1 males. (A,D) Shows latency to escape during probe test (* p -value=0.01), (B,E) shows entries into target zone and (C,F) shows time spent in target zone in F1 females and F1 males, respectively. (n =6 in each group in F1 females and n =9 in each group in F1 males).

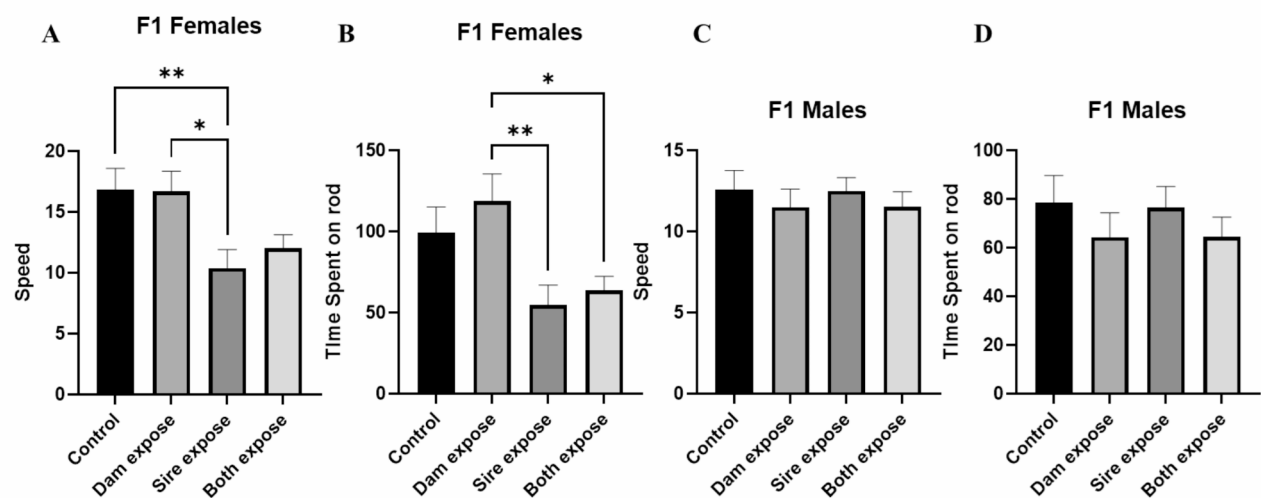


Fig. 7. Shows rotarod test results for F1 females and F1 males. (A,C) Shows speed attained during rotarod test, (B,D) shows total time spent on rotarod test by F1 females and F1 males respectively. (* P -value < 0.05). (n =6 in each group in females and n =9 in each group in males).

group. The Sires exposed group attained the least speed and spent the lowest amount of time on the rotarod. The Dam exposed group female mice spent the most time on the rotarod. This was comparable to Control, however, there were significant differences when compared with those of the Sire exposed and Both exposed groups on the total time spent on the rotarod.

The rotarod test in F1 males did not show any differences among the groups (Fig. 7C,D), the male offspring from Pb exposed groups performed comparably to those of the control group. The F1 males when compared to the F1 females spent less time on the rotarod and had attained a lesser speed.

Lead concentrations in F1

Lead concentrations were measured in F1 generation at four weeks old when excess mice were culled and at 9 weeks old after completion of neurological tests. The Both exposed group had only 7 females at weaning and hence none were culled at 4 weeks old and Pb concentrations are not presented.

In Female F1 mice there were no significant differences in the Pb concentrations among the groups at both 4 weeks old and 9 weeks old (Fig. 8A,C). At 4 weeks the bone Pb levels ranged from 0.106 to 0.0921 mg/kg for the Control and the Sire exposed groups with means of 0.112 and 0.0992 mg/kg respectively. While the Dam exposed group had a range of 0.643 to 0.0542 mg/kg with mean of 0.32 mg/kg. At 9 weeks Control, Dam exposed, and Sire exposed groups had range Pb levels from 0.272 to 0.0903 mg/kg with means of 0.166 mg/kg, 0.180 mg/kg, and 0.140 mg/kg, respectively. While Both exposed had a with mean value of 0.23 mg/kg with a range from 0.338 to 0.148 mg/kg.

In the F1 males bone lead levels at 4 weeks old (Fig. 8B) were significantly different between Control and Both exposed groups, as well as the Sire exposed and Both exposed groups. Lead levels at 4 weeks ranged from 0.119 to 0.0970 mg/kg for both the Sire exposed and Control groups with means of 0.111 to 0.100 mg/kg respectively. The Both exposed group had Pb levels ranging from 0.573 to 0.26 mg/kg, with a mean of 0.385 mg/kg. The Dam exposed group values ranged from 0.248 to 0.393 mg/kg. At 9 weeks old (Fig. 8D) Pb concentration levels ranged from 0.115 to 0.358 mg/kg for all groups. The mean for the Control group was 0.164 mg/kg while the mean for the Sire exposed group was 0.132 mg/kg. The Dam exposed and Both exposed had bone Pb mean levels of 0.235 mg/kg and 0.268 mg/kg respectively.

Gene expression

Testicular genes

Gene expression levels of *Pou5F1*, *Crisp2*, *Spink 2*, and *Nrf2* were analysed from testicular tissue of F0 sires (Fig. 9). *Pou5F1*, *Crisp2*, and *Spink 2* were non-significantly downregulated in the exposed sire, while *Nrf2* was significantly upregulated. This implies that gene expression levels of genes related to sperm production did not differ from sires of the control group, however, upregulation of *Nrf2* showed the presence of oxidative stress.

Brain genes

Female F1 mice brain prefrontal cortical gene expression (Fig. 10) revealed upregulation of *Bdnf* significantly in Dam exposed, the Sire and Both exposed groups. *Creb* appeared to be upregulated non-significantly in the Sire exposed and Both exposed groups. NMDA *Grin2A* gene was upregulated significantly in the Dam exposed female offspring and non-significantly in Both exposed group. There was a corresponding significant downregulation of NMDA *Grin2B* in the Dam exposed and Both exposed groups.

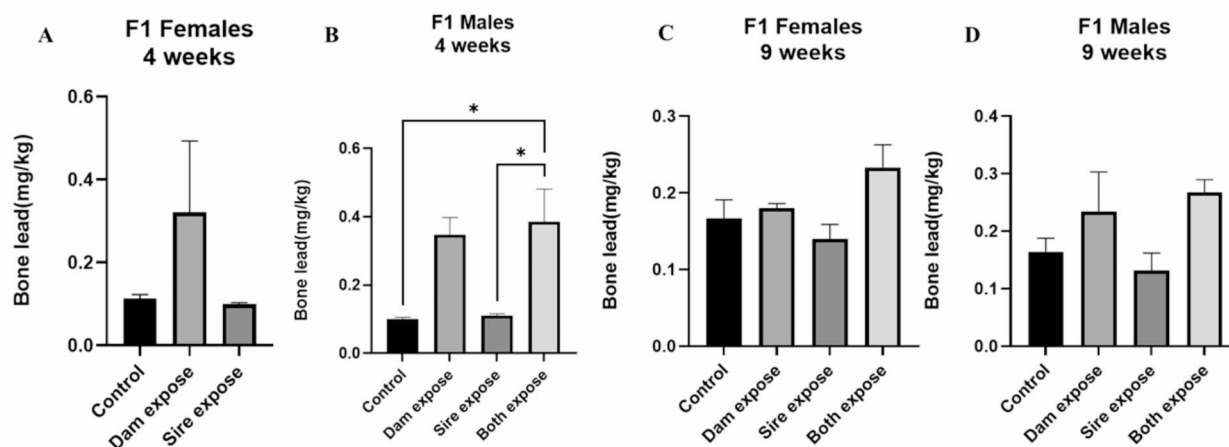


Fig. 8. shows lead levels in the bone and blood of F1 Females and F1 males. (A,B) Represent bone lead levels at 4 weeks old in F1 females and males respectively, while (C,D) are the bone lead levels at 9 weeks old. ($n=3$ in each group at 4 weeks in both F1 females and F1 males), (At 9 weeks $n=6$ in each group in females and $n=9$ in each group in males). * p -value = 0.03. The Both exposed group had only 7 females at weaning and hence none were culled at 4 weeks old and Pb concentrations are not presented.

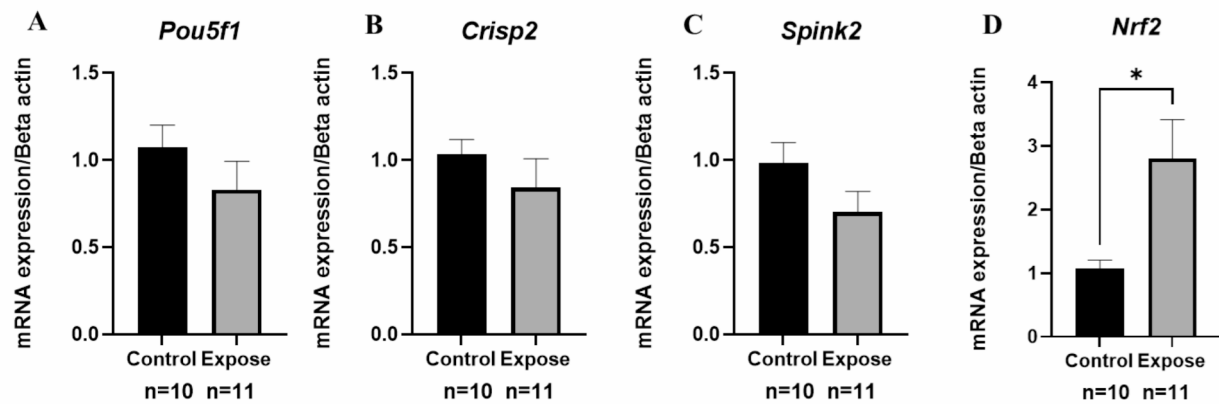


Fig. 9. shows fertility and oxidative stress associated genes expression levels in sire testicular tissue. (A) Shows *Pou5f1* gene expression, (B) shows *Crisp2* expression, (C) shows *Spink2*, (D) shows *Nrf2* expression in the testicular tissue of F0 sires. *p-value = 0.02. Sires' brain expression genes and *Keap1* are shown in the supplementary material.

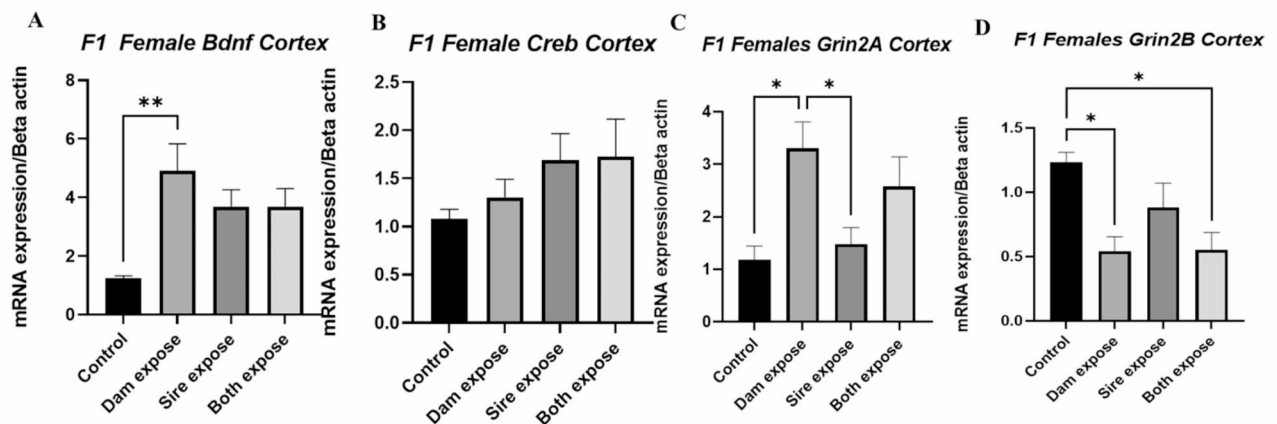


Fig. 10. shows expression levels of brain plasticity related genes in F1 females. (A) *Bdnf* expression, (B) shows *Creb* expression, (C) shows *Grin2A* expression and (C) shows *Grin2B* expression in F1 females. *Indicates p-value = 0.03, **indicates p-value = 0.001. (n = 6 in each group).

In the hippocampus (Fig. 11) *Grin2A* was significantly upregulated in the Sire exposed and Both exposed groups while *Grin2B* was normally regulated in among the groups. *Bdnf* was normally regulated among all groups, however, *creb* expression levels were significantly downregulated in the Dam exposed and Both exposed groups.

In F1 males prefrontal cortex, (Fig. 12) *Bdnf* and *Creb* expression levels from offspring of Pb-exposed individuals were comparable to Control. *Grin2A* was non-significantly upregulated in the Sire exposed group, in the Dam exposed group and the Both exposed group *Grin2A* was significantly upregulated. There was normal regulation of *Grin2B* gene in Dam exposed, Sire exposed, and Both exposed groups.

In the hippocampus (Fig. 13), *Creb* expression levels were significantly downregulated in the Dam exposed males so was *Grin2A*. The other groups had normally regulated gene expression levels for *Grin2A*, *Grin2B*, and *Creb*.

Discussion

We conducted a Pb exposure experiment using soil as a medium of exposure to mimic environmental exposure through inhalation and ingestion. We investigated whether lead effects are sex-dependent concerning conception in the F0 generation and neurological deficits in the F1 generation.

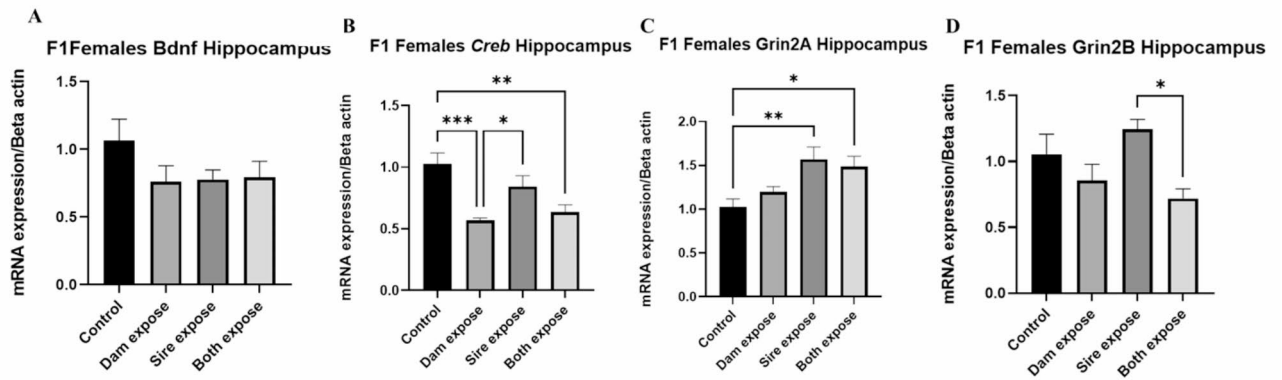


Fig. 11. shows expression levels of plasticity associated genes in F1 females in the hippocampus. (A) Shows *Bdnf* expression levels, (B) shows *Creb* expression, (C) shows *Grin2A* expression and (D) shows *Grin2B* expression in F1 males. ($n = 6$ in each group).



Fig. 12. shows expression levels of plasticity associated genes in F1 males. (A) Shows *Bdnf* expression levels, (B) shows *Creb* expression, (C) shows *Grin2A* expression and (D) shows *Grin2B* expression in F1 males. ($n = 6$ in each group).

F0 generation

Lead exposure lowered the conception rate in our study, this was seen when either or both males and females were exposed to Pb pre-breeding. This reduction was by 20% when only dams were exposed in our study, and by 55% when both sires and dams were exposed. Infertility caused by Pb is well studied and is known to be caused by various mechanisms. In females, a study by Gerald et al.¹⁹ reported that a 10% increase in Pb blood levels led to a 6.75% decrease in concentration of oestradiol. A study by He et al.²⁰ showed that Pb-exposed female quails showed fewer and smaller follicles than the control group of that study. Thus, Pb can lead to the reduction of the overall number of ovulations occurring. Due to our experimental design, we were unable to conduct any fertility tests on the dams. However, we presume these may have been the causes for the reduced fertility rates when females were exposed.

Results from the current study showed that male Pb exposure lowered the conception rate more than female exposure. This could be because testicular tissue is more vulnerable to Pb damage than other body tissue²¹. Lead damages spermatozoa, and damaged sperm DNA negatively affects embryo quality in early development, this may affect implantation and pregnancy outcomes²². Therefore, in our study even if fertilisation had occurred successfully, implantation could have failed, or the resultant embryo could have not been able to survive when the fertilising sperm had undergone DNA damage. Because Pb male exposure has an impact on early embryonic development, it has a higher factor than females in resultant of pregnancy.

Additionally, in our study like that of Dolati et al.²³ *Crisp2* mRNA expression levels did not significantly differ between our sire control and sire exposed groups. This was observed for *Pou5F1* gene as well. *Spink2* gene expression was also non-significantly downregulated in our results. This implies that the cause of the reduction in conception in this experiment could have not been mainly due to impaired sperm production as these genes are closely related to maintaining normal sperm production. However, the significant upregulation of Nuclear

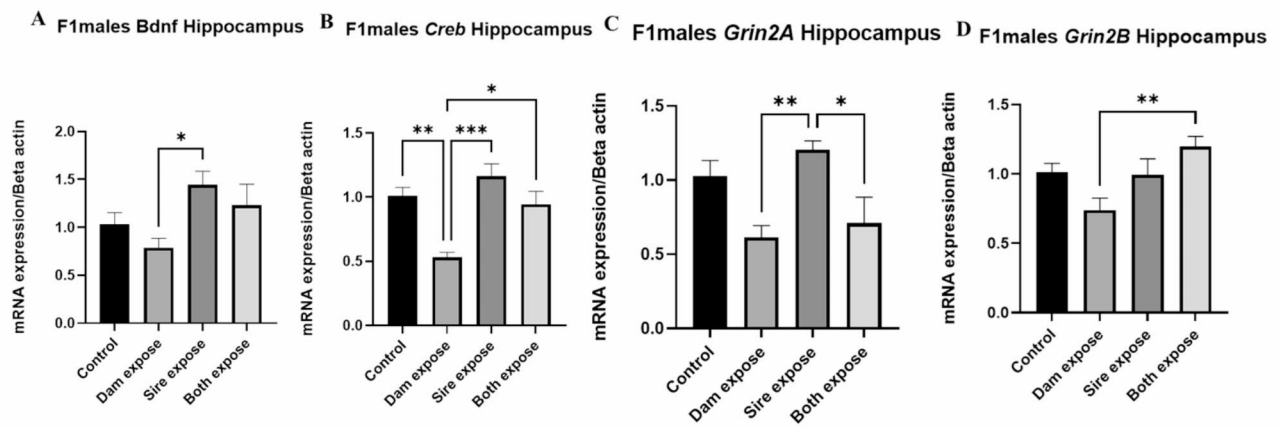


Fig. 13. shows expression levels of plasticity associated genes in F1 females in the hippocampus. (A) Shows *Bdnf* expression levels, (B) shows *Creb* expression, (C) shows *Grin2A* expression and (D) shows *Grin2B* expression in F1 males. ($n = 6$ in each group).

factor erythroid 2-related factor 2 (*Nrf2*) in the testicular tissue in our experiment indicates the presence of oxidative stress, which was comparable to the findings of Wang et al.²⁴. Increased expression of the *Nrf2* is well known for its cellular xenobiotic and oxidative stress response^{25–27}.

Therefore, oxidative damage could have damaged sperm in the sire exposed groups, where an increase in sperm reactive oxygen species that overwhelmed the antioxidant response in Pb exposure could have caused the sperm damage²⁸.

Damage to sperm DNA also prevents the affected sperm from being able to go through normal demethylation required in early mammalian development²⁹. This demethylation is a protective mechanism that prevents certain parental epigenetic changes from being transferred to the next generation. Thus, failure of this mechanism provides a means through which exposed sire could cause deficits in the next generation. Secondly, it has been shown by Yamazaki et al.³⁰ that Pb exposure causes genomic-wide DNA methylation changes in a study conducted in dogs. Takahashi et al.³¹ went further to demonstrate how such epigenetic changes could be transmitted via generations, where artificially generated epigenetic changes that were inserted into the genome were inherited up to two generations down from the initial mutant group. This change was able to elude the process of global demethylation that occurs in mammals after fertilisation. Consequently, demonstrating how paternal epigenetic changes could be passed on to the next generation who are not exposed to the original trigger.

Lead levels in the blood of pregnant mice were significantly lower than those that did not get pregnant. Similarly, a study done in postmenopausal women found that nulliparous women tended to have higher lead in their blood than women who had been pregnant³². This may be due to the transplacental transfer of Pb to the developing foetus during gestation. However, the Pb levels in the bone, which represents Pb storage, did not show any significant difference between the pregnant exposed and the non-pregnant exposed groups at weaning of F1 pups. Lead is known to mobilise from the bone during pregnancy and lactation, evidence of Pb metabolism changes during pregnancy, with effects being more severe when calcium deficiency occurs. Lead mobilisation from the bone is more extensive during the postpartum period when more calcium is needed for lactation³³. Therefore, we expected to record a significant difference in the bone Pb levels. However, the short exposure time in our study could have affected lead deposition levels. In addition, in the maternal blood, 99% of Pb is bound to red blood cells and only 1% is available for transplacental transfer³⁴, therefore the Pb transfer to the foetus is dependent on this free Pb. Which in relation to the amount of Pb circulating in the mother's body is a small amount. Furthermore, in the kidneys, Pb is absorbed in the proximal tubules and very little is excreted in urine³⁵. The body then tends to retain more Pb than it excretes therefore even though Pb is mobilised in pregnancy it is not exclusively excreted. We assume that mobilisation of Pb from the bone does not equal to its elimination, as a result, there was no significant difference in the bone Pb levels between Pb-exposed pregnant dams and non-pregnant dams.

F1 generation

In our study, a litter sex ratio differentiation was seen in the offspring of Pb-exposed individuals. The number of female offspring reduced with Pb exposure when compared to control. We also observed stunted offspring occurring exclusively in the Dam exposed group and only in males. Literature on Pb exposure sex ratio differentiation is contradictory, but there is a strong association between Maternal health status and stunting in offspring³⁶. Stunting effects were not prevalent when sire exposure was involved as expected. These stunted mice's body condition improved immediately after weaning summing up the association between Pb exposure and stunting as confirmed by previous studies³⁷. The severity of the effects that offspring develop from parental exposure is dependent on the sex of that parent.

Neurological tests

Maternally gestationally exposed F1 mice showed learning impairment during the water maze test, which was similar to a study done by Guilarte et al.³⁸. The rats in that study showed similar levels of Pb to control when maze tests were being conducted. This was observed in our study as well, we deduce that most of the Pb had been excreted by the time of neurological tests. In fact, Pb levels in F1 generation in our experiment range below the acceptable levels of 3.5 µg/dl³⁹ for both 4 weeks and 9 weeks old. Therefore, once Pb has caused some neurological injury, those insults remain even after the Pb has been excreted and Pb levels can be considered normal.

Interestingly, our study also showed learning impairment in mice born from exposed sires, with female offspring of exposed sires showing significant differences with control during the probe test. This was not seen in the males of the same group. The males of the Sire exposed group and the Dam exposed group performed better in the water Morris test than the females. These results were similar to what was reported by⁴⁰ where the males outperformed the females.

Rotarod results revealed that male mice from exposed parents did not show any significant differences when compared to control, whereas the females overall did better than the males in motor coordination. Nevertheless, there were some significant differences among the female groups, astoundingly Dam exposed offspring were able to spend the most time on the rotarod. This may be due to the significant upregulation of *Bdnf* in the prefrontal cortex seen in this group, which plays an important role in transmission and plasticity in the motor cortex, subcortical motor structures and motor skill learning⁴¹. Motor skill learning requires the secretion of *Bdnf* to augment synaptic plasticity. These may explain why the Dam expose group was able to perform better in the rotarod as compared to the control. The levels of *Bdnf* were also significantly upregulated in females of Sire exposed and Both exposed groups, we speculate this may be due to the protective role of oestrogen from Pb and positive feedback of oestrogen on *Bdnf*⁴². This is observed mostly in the prefrontal cortex, oestrogen has a huge effect during sexual maturation, which is within the period when neurological tests were conducted, and samples taken.

Gene expression

In our study, expression levels of genes of interest *Grin2A*, *Bdnf*, and *Creb* (Fig. 14) showed more of an upregulation trend in the prefrontal cortex which is similar to study by Sánchez-Martín et al.⁴³. *Grin2A* was upregulated significantly in Dam exposed group in both F1 males and Females. It was non-significantly upregulated in the Both and Sire exposed groups in F1 females. This upregulation agrees with the findings of Sánchez-Martín et al.⁴³. Lead reduces expression levels of Glu2A protein in chronic exposure⁴⁴ thereby leading to the overexpression of *Grin2A* as a compensatory mechanism. This may explain why *Grin2A* was upregulated, which was also seen in our exposed sires where it is non-significantly upregulated. Increasing expression levels of *Grin2A* have been associated with neurological abnormalities while upregulation of *Grin2B* has been shown to improve performance in spatial memory tests in mice⁴⁵. In gestationally Pb-exposed F1 females, *Grin2B* was downregulated this was not observed in the F1 males of the same group. The downregulation of *Grin2B* may have led to the poor performance seen in females. The upregulation of *Bdnf* in females may probably have led to be downregulation of *Grin2B* seen in our experiment. This is because, *Bdnf* ensures maturation of NMDA receptors from Glu2B subunits to Glu2A by increasing expression of *Grin2A* (Fig. 15) after birth^{46,47}. The upregulation of *Bdnf* may have indirectly led to the downregulation of *Grin2B* due to this mechanism.

Downregulation of *Creb* in F1 gestationally exposed males and females in the hippocampus and paternal prefrontal cortex⁴⁸ was observed in this experiment.

The binding affinity and downstream effects on NMDA receptors are sexually dimorphic, this makes interpretation of NMDA-dependent effects on synaptic plasticity complicated⁴⁹. Therefore, the gene expression of NMDA subunits in the males and females is not expected to be comparable. Additionally, Pb exposure during development alters gene expression in many transcripts and regulatory genes, these effects are modified by sex. The differences that occur are dependent on how much regulatory networks are distinct between males and females⁵⁰.

The relationship between the hippocampus and the prefrontal cortex is intricate. For new memories to be formed, the hippocampus is required however for the replay sequence to occur the prefrontal cortex needs to coordinate. The prefrontal cortex creates a context for representations that link related memories. This implies that even gene expression may be normally regulated in the hippocampus, if the prefrontal cortex is not functioning normally some memory deficits will occur as seen in our F1 females from exposed sires^{51,52}.

In our experiment, the exposed sires had more prefrontal cortex gene expression changes than hippocampal due to postnatal exposure. Postnatal lead exposure leads to prefrontal cortex changes rather than hippocampal changes as the prefrontal cortex is more sensitive to modifications postnatally⁵³. Female offspring of these sires also showed a similar trend in gene expression. From this result, we assume that Pb neurotoxic developmental effects may be X chromosome linked. Furthermore, the brain expresses more X chromosome linked genes than other organs⁵⁴. Other studies^{55–58} also explain how inheritance of certain paternal origin occurs only in female offspring. Wells⁵⁹, then described how paternally inherited genes become expressed in F2 as they become maternal genes in their female offspring and can be inherited by both F2 female and male (Fig. 16).

Conclusion

Lead exposure in paternal sires affected the neurological development of the offspring as their behaviour and gene expressions mimic those of maternal exposure without showing higher Pb levels than control. We also established that Pb exposure had caused oxidative stress in testes which we presume subsequently caused defects in embryos through the damaged sperm DNA. This damage to sperm could have caused failure of global demethylation mechanism which provides a means through which exposed sires could cause deficits in the next

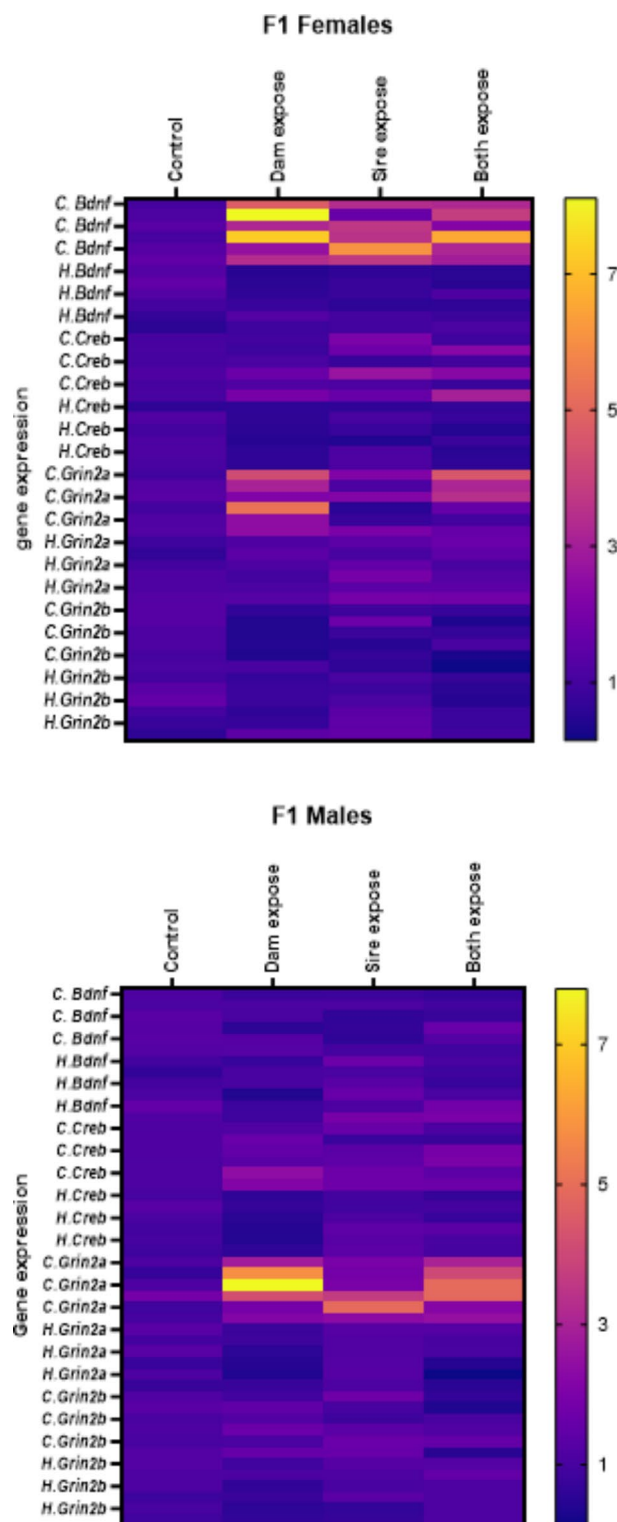


Fig. 14. (A) Shows a heatmap of gene expression comparison between hippocampus and prefrontal cortex in F1 females, where C represents prefrontal cortex and H for hippocampus. (B) Shows a heatmap of gene expression comparison between hippocampus and prefrontal cortex in F1 males, where C represents prefrontal cortex and H for hippocampus.

generation by transfer of Pb-related epigenetic changes. These hypotheses, however, need to be further studied, to fully understand how this occurs in Pb exposure.

Finally, although differences were observed in learning during the training period of the water Morris Test, probe test revealed no significant differences among the F1 male groups. In F1 females' differences were seen, but

In males and females lead inhibits **Glu2A** → ↑ Upregulation of **Grin2A** **Females**

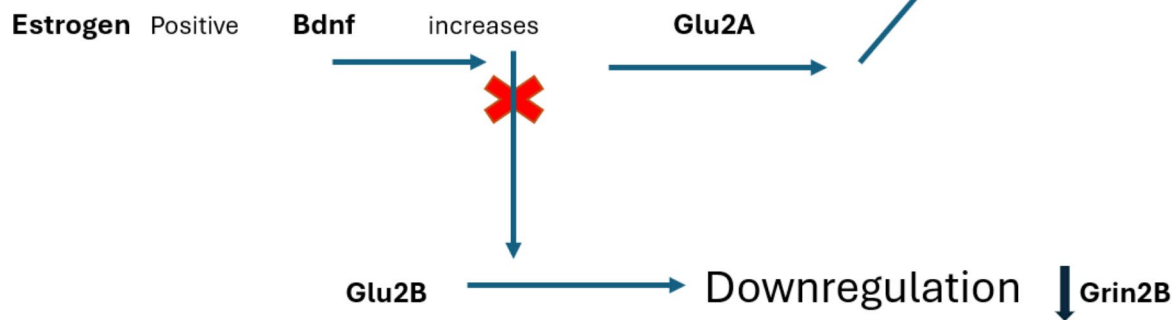


Fig. 15. Shows a schematic diagram that describes relationship between Bdnf, Glu2B, Glu2A and their corresponding genes.

these were not statically significant in all the groups. Gene expression differed between the prefrontal cortex and the hippocampus with prefrontal showing more upregulation trend. Cessation of exposure pre-breeding showed improvements seen mostly in F1 males. Inter-generational effects of Pb are parent-dependent, X-chromosome-linked, and the severity of the resultant neurological impairments are sex-dependent.

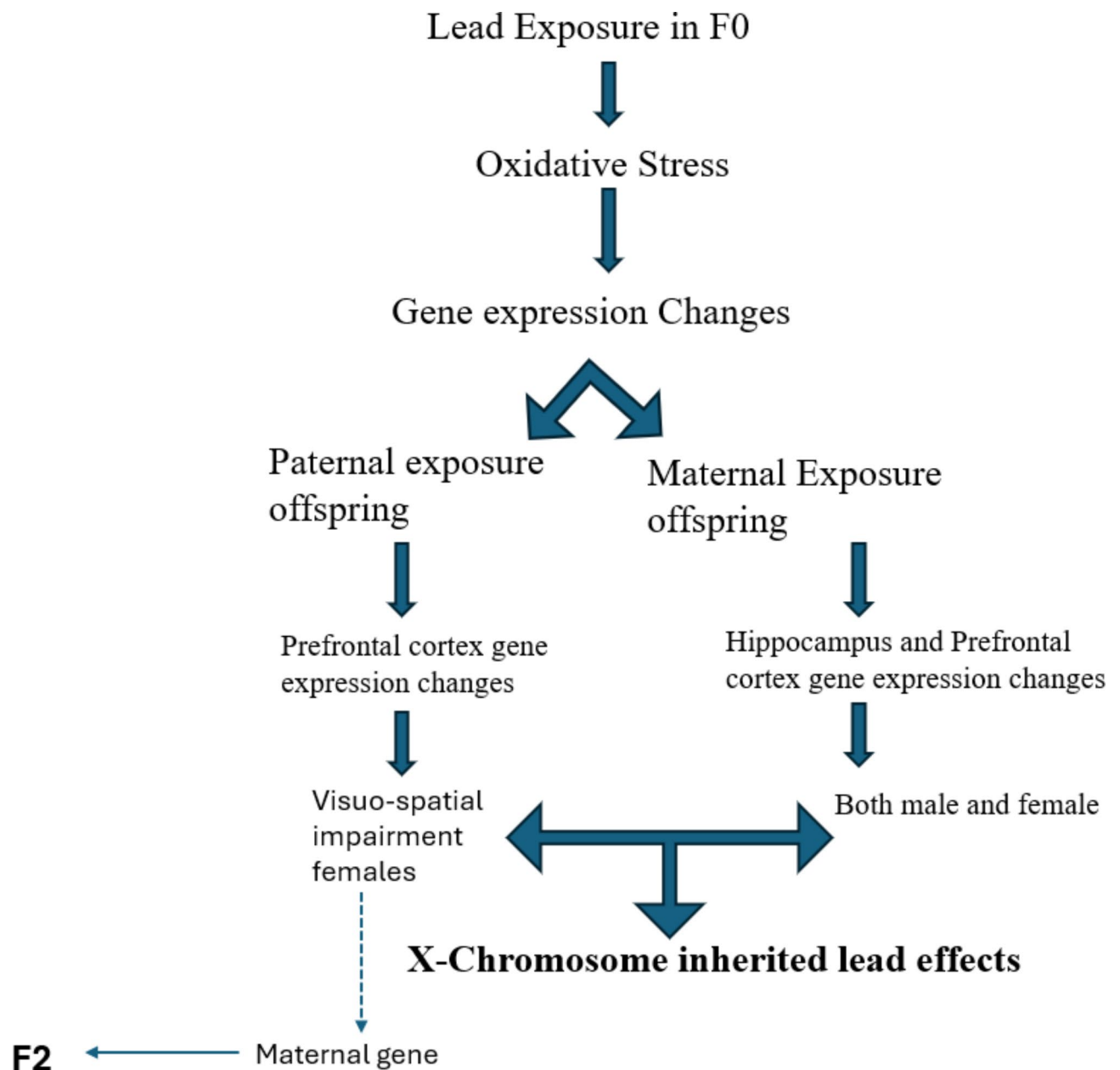


Fig. 16. Demonstrates a flow chart of how intergenerational lead exposure leads to sex-linked neurological deficits depending on which parent is exposed.

Data availability

The datasets used and analysed during the current study are available from the corresponding author on reasonable request.

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Author contributions

Conceptualization; investigation; methodology; data curation; formal analysis; writing—original draft; writing—review and editing, N.B.; investigation; methodology; resources; validation; visualization; writing—review and editing, N.C.S., J.Y., D.R., Y.B.Y., I.Y.; investigation, data curation, resources, validation, software, N.B. and N.C.S.; funding acquisition; project administration; supervision; validation; visualization; writing—review and editing, M.I. and S.M.M.N. All authors have read and agreed to the published version of the manuscript.

Declarations

Competing interests

The authors declare no competing interests.

Additional information

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