Research Article

Reproductive Toxicity Analysis of Potential Toxic Element Lead in Mice and their Regenerative Potential

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ABSTRACT

World Health Organization has highlighted the increase in rates of infertility and poor reproductive health globally and potential toxic elements are among the major culprits for increase in infertility. The present study was conducted to evaluate the hypothesis that "the germ cells of mice have regenerative potential to recover from toxicity induced because of heavy metal exposure". For this, experiments were conducted to analyze *in vivo* reproductive toxicity of lead acetate in male mice (*Mus musculus)* and the self-regenerative potential of their germ cells. A total of 27 male mice (*Mus musculus)* were grouped into 3 groups containing 9 animals each. Group 1 animals were given oral treatment of lead acetate (10 mg/kg b.w.) on daily basis; while Group 2 animals were administered 50 mg/kg b.w. on weekly basis; and Control group were administered distilled water. Three animals from each group were selected randomly after 24 hr and 40 days of treatment. The remaining animals were given normal diet for next 40 days to check the regenerative potential of animal's germ cells. Animals from each group after 24 hr, 40 and 80 days were sacrificed by cervical dislocation and sperm abnormality assay was performed. The results revealed that lead acetate induced significantly higher sperm abnormalities in mice than the control group. The study also indicated that mice can show significant self-regenerative potential of germ cells after period of recovery.

Key words: Potential toxic elements, Lead acetate, *Mus musculus*, Reproductive health, Sperm abnormality assay.

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INTRODUCTION

Reproduction is the essence for the continuation of life on earth. Healthy reproductive system is important for survival of human race.[1] However, recent reports documented that the infertility rate which was 7% to 8% in the 1960s has increased to 20 to 35%.[2,3] Worldwide, infertility affects 15% of reproductive aged couples.[4] The information available on national health portal of Government of India reported that infertility rate in India is between 3.9 to 16.8%. Agarwal and Mulgund[5] postulated that the percentage of male infertility is primarily due to poor quality of semen. This poor quality of semen can be because of physiological, genetical and environmental factors. Various lifestyle factors such as alcohol consumption, smoking, poor diet, lack of exercise also contributes to the poor reproductive health.[6]

Several studies have pointed out different environmental factors for inductions of sperm abnormalities in mammalian test systems which include factors such as air pollutants, pesticides, solid waste, radiofrequency radiations and potential toxic elements.[7-12] Among various potential toxic elements lead (Pb) is one of the

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prominent toxicants in the environment because of its potential hazards to living organisms and is being used in many industries including mining and refining.[11] Different compounds of lead like, lead arsenate, lead chromate, lead dioxide, lead fluoborate, lead sulfide and lead sulfate are listed in the EPA list of Hazardous Substances.[13]

Lead has significant effects on the reproductive system which includes toxic pathological changes in the testes such as atrophy of the organ.[11] Seminal cytology of lead treated animals normally depicts the abnormalities in the sperms like asthenospermia, hypospermia, teratospermia, changes in sperm count and changes in the morphology of sperms.[14] Workers employed in industries using lead are more exposed to lead than common people. Many studies revealed that 15-30% of lead exposure in humans occurs through inhalation and 70-85% with food and drinks.[15] Lead-induced hypofertility, especially due to occupational factors, is thought to be due to the direct toxic effects of lead on male gonads.[16]

Scientific fraternity around the globe have relied on various plant and bacterial bioassays for testing mutagenic activity of chemicals/agents, but many assumptions are required to extrapolate the results obtained from these bioassays to human beings, so there is a need of rapid bioassays which can be used for screening the mutagenic, teratogenic and carcinogenic effects of chemicals *in vivo* in mammals.[17,18] The sperm abnormality assay is a simple and effective measure to determine the toxic effect of chemicals on reproductive system.[19] Sperm abnormality assay is widely used bioassay for the assessment of genotoxic effects on exposed mice. This bioassay is based on visual scoring of sperms having abnormal head forms and shapes.[18,20]

There are sufficient scientific reports about the regenerative potential of various medicinal plants, nutraceuticals, and other micronutrients which ameliorate the reproductive toxicity of various heavy metals.[21] But there is lack of information on the self-regenerative potential of germ cells in mice following exposures to heavy metals. So, the present study was planned to evaluate the hypothesis that "the germ cells of mice have regenerative potential to recover from toxicity induced because of heavy metal exposure". The main objectives of the present study were to evaluate the reproductive toxicity of lead acetate in germ cells of mice by using sperm abnormality assay and to analyze the regenerative capacity of the germ cells. The present study is an extension of our previous studies where we have analyzed the genotoxic effect of lead acetate by using *Allium sativum* root chromosomal aberration assay[22] and its tumor inducing potential by using crown gall tumor disc bioassay.[23]

MATERIALS AND METHODS

The present study was designed to evaluate the reproductive toxicity of lead acetate and further check the potential of the germ cells of mice to recover from this toxicity on their own.

Procurement of animal

For the present study, male *Mus musculus* mice belonging to Balb c strain, were procured from Central Research Institute, Kasauli, H.P. These were maintained in the animal house of the Department of Biosciences, Himachal Pradesh University under suitable hygienic conditions with 16 h day light. The mice were provided feed (Hindustan Lever Ltd.) and water *ad libitium*. Normal healthy-looking mice (N=27) showing no sign of morbidity were weighed and selected for the present study. Animal care procedures were followed in accordance with the guidelines of Institutional Animal Ethical Committee.

Experimental design

The experiment was performed on 27 male *Mus musculus* mice, weighing 16-20 g. These mice were divided into three groups (Control, Group 1 and Group 2) having 9 animals in each group. To study the reproductive effects of lead acetate, two doses 10 and 500 mg/kg body weight were selected and administrated to the animals orally as follows:

Group I – All mice in group I were given 10 mg/kg b.w./day of lead acetate daily. From this group, 3 mice were taken after 24 hr randomly and sacrificed by cervical dislocation. The remaining 6 mice were given the dose for 40 days. After 40 days, 3 mice were taken randomly and sacrificed. Remaining 3 mice were sacrificed after 80 days (no treatment was given during this period).

Group II – In this group mice were given 500 mg/kg b.w./week dose of lead acetate on weekly basis and 3 mice were sacrificed after 24 hr from this group. After 40 days 3 mice from remaining 6 mice were taken and sacrificed. Remaining 3 mice were sacrificed after 80 days (no treatment was given during this period).

Group III – This group serves as a control group without any treatment and 3 mice were sacrificed along with the group I and II mice at each stage.

Sperm Abnormality Assay

For sperm abnormality assay, the procedure of Wyrobek and Bruce[18] with some modifications was followed. Three animals from each group as described in the experimental design were selected randomly after 24 hr,

40 days and 80 days. These animals were sacrificed by cervical dislocation and the sperm abnormality assay was performed by removing their cauda epididymides. The sperm suspension was made by mincing the cauda epididymides in 3 ml of normal saline. The resulted suspension was filtered through muslin cloth to remove the tissue debris.

Staining and preparation of slides

To 500 µl of the sperm suspension taken in 1 ml eppendorf 50 µl of 1 % aqueous Eosin Y stain was added. This mixture was incubated for 30 min at room temperature. The slides were prepared by putting a drop of stained suspension and making a smear by passing one slide over the other. After the smear dried, the slide was mounted with DPX.

Preparation of 1 % aqueous Eosin Y

Eosin Y was used to stain the sperm suspensions in the present study as mentioned in the procedure of Wyrobek and Bruce.[18] 1 % aqueous solution of Eosin Y was prepared by dissolving 1 g of water-soluble Eosin Y in 100 mL of distilled water and used for staining.

Scoring

From each sperm suspension obtained from a mice approximately 1000 sperms were examined for each replicate (animal) under the microscope at 1000X magnification and sperms were observed for various kinds of abnormalities.

Calculation

$$\% \text{ sperm abnormality} = \frac{\text{Number of abnormal sperms}}{\text{Number of total sperms}} \times 100$$

Statistical Analysis

The analysis of sperm abnormalities induced following different treatments of lead acetate and controls was done in triplicates and the values are represented as Mean±SD. One-way ANOVA followed by Tukey's HSD test as post hoc was used to compare the means of number and percentage of abnormal sperms in case of different treatments.

RESULTS

The frequencies of abnormal sperms following treatment with different concentrations of lead acetate are given in Table 1. Figure 1 shows the representative micrographs of different kinds of sperm abnormalities induced in mice following treatment with different concentrations of lead acetate in the present study.

Table 1: Frequency of abnormal sperms in mice following treatment with different concentrations of lead acetate for different time periods.

Treatment period	Group	Average No. of Abnormal Sperms	% Abnormal Sperms
24 h	C	47.33 ± 4.51 bC	4.58 ± 0.34 bC
	G_1	80.67 ± 7.37 aC	7.86 ± 0.39 aC
	G_2	86.00 ± 7.00 aC	8.47 ± 0.59 aC
40 days	C	60.67 ± 3.06 cB	5.81 ± 0.01 cB
	G_1	229.00 ± 8.54 bA	21.54 ± 0.62 bA
	G_2	315.33 ± 6.51 aA	30.53 ± 1.11 aA
80 days*	C	85.67 ± 6.03 cA	8.02 ± 0.66 cA
	G_1	116.33 ± 13.61 bB	10.67 ± 0.50 bB
	G_2	195.00 ± 17.35 aB	18.44 ± 0.76 aB

C – Control; G_1 – Group 1- lead acetate 10 mg/kg b.w./day ; G_2 – Group 2 – lead acetate 50 mg/kg b.w./weak; 80 days* represent 40 days of recovery after the withdrawal of lead acetate treatment for 40 days.
Mean values of number of abnormal sperms and percentage of abnormal sperms followed by different letters (Lowercase for different groups in same treatment period; Uppercase for different treatment periods in same group) in columns are significantly different (one-way ANOVA; Tukey's test, $p \leq 0.05$).

Folded, coiled tail, hookless, Bend at Cephalocaudal Region (BCR) and amorphous head were dominant kind of sperm abnormalities encountered in the present study. Banana, pinhead and short tail were also observed but in a lesser frequency. The percentage of different kinds of abnormal sperms is shown in Figure 2. The frequency of abnormal sperms was found to be more in mice from Group II, which received 50 mg lead acetate/kg b.w./week for all the time durations i.e. 24 hr, 40 days and 80 days (which represent 40 days of recovery after the withdrawal of lead acetate treatment for 40 days). Group I mice which received 10 mg of lead acetate/kg b.w./day for 40 days exhibited comparatively lesser frequency of abnormal sperms. The frequency of abnormal sperms decreased in both group I and group II mice following recovery period of 40 days. The variation in number and percentage of abnormal sperms was found to be statistically significant among the three groups (control, G1 and G2) for the same treatment period (ANOVA+Tukey's test). Statistically significant variation in number and percentage of abnormal sperms was also observed for the three groups for different durations of treatments i.e. 24 hr, 40 days and 80 days (ANOVA+Tukey's test).

DISCUSSION

At the end of the spermatogenesis sperm cells are stored in the caudal epididymis. The shape of sperms can be influenced by age, diet, drug, temperature and successive ejaculation.[24] The normal sperm of albino

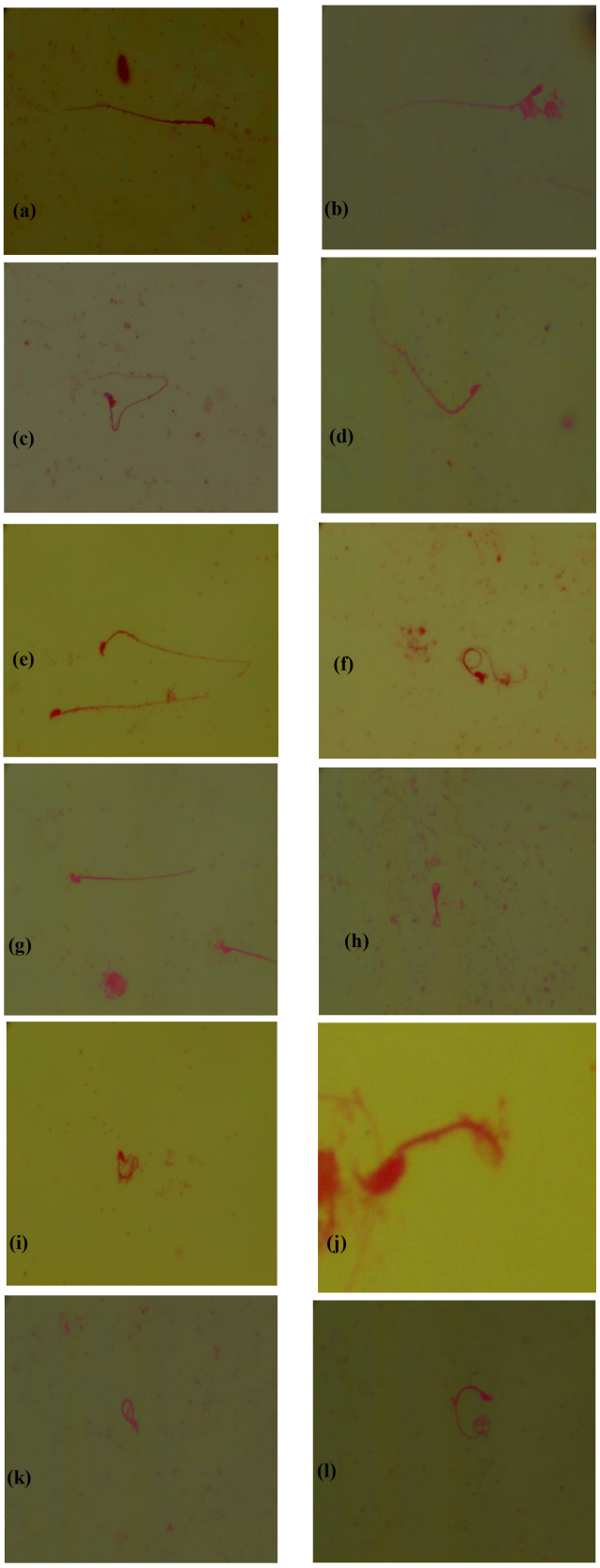

Figure 1: Different types of sperm abnormalities observed (at 100X) in mice treated with lead acetate: (a) normal sperm; (b) amorphous head; (c and d) BCR (Bend at cephalocaudal region); (e) hookless; (f) hookless with coil tail; (g) Banana head; (h and i) folded; (j) short tail; (k and l) coiled tail.

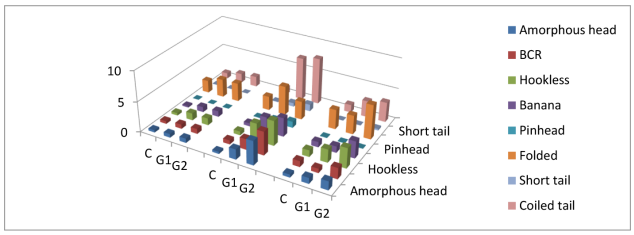

Figure 2: Percentages of different kinds of abnormal sperms in mice following treatment with different concentrations of lead acetate for different time periods
C- Control; G1-Group 1- lead acetate 10 mg/kg b.w./day; G2-Group 2- lead acetate 50 mg/kg b.w./weak; 80 days* represent 40 days of recovery after the withdrawal of lead acetate treatment for 40 days; BCR – Bend at Cephalocaudal Region.

rat constitute a hook shaped head, a thin neck, mid piece (acrosome) and tail for whipping. Presence of hook at the head of spermatozoa is a distinct feature in rats and mice.[25] During spermatogenesis, DNA synthesis occurs only during the pre-meiotic phase and no further creation of DNA occurs during the cell cycle. That is why, the sperm head after its normal development becomes exceptionally stable. Thus, we can conclude that morphological abnormalities induced in sperms of mice may be because of naturally occurring irregularities during the cell cycle or by a chemical/physical environmental mutagen which might increase the frequency of these irregularities during spermatogenesis.[26] These abnormalities can also be attributed to irregularities during the packaging of the genetic material in the sperm head or because of an abnormal chromosome complement.

Sperm abnormality assay is one of the quickest, simplest and sensitive protocols to evaluate mutagens and carcinogens that act upon germ cells.[18] The abnormal shapes of sperms may be because of mistakes made in packaging the genetic material in the sperm head or because of abnormal chromosomal complement.[27]

The results of the present study revealed that number of different types of abnormal sperms *viz*. amorphous head, Bend at Cephalocaudal Region (BCR), hookless, banana, pinhead, folded, short tail and coiled tail were higher in both the groups i.e. group I and group II as compared to control at various periods of treatment. The frequency of abnormal sperms was higher (8.47 %) in group II as compared to control (4.58 %) after 24 hr of treatment.

The increase in the frequency of abnormal sperms was significantly higher in both group I and II as compared to control after 40 days of treatment. The frequency of abnormal sperms was 21.54 % and 30.53 % in group I and II mice, respectively; which were significantly

higher than the control (5.80 %). The significant increase in the frequency of abnormal sperms in case of the two groups treated with lead acetate (G1 and G2) can be attributed to the toxicity of lead acetate on the reproductive system of mice. Acharya *et al*.[28] also found increase in the abnormalities of sperm morphology after administration of lead acetate (200 mg/kg/b.w.) and have postulated that this increase is because of increase in ROS production. Godínez-Solís *et al*.[14] reported increase in the frequencies of abnormal sperms post lead treatment and documented that lead targets the spermiogenesis. Li *et al*.[11] also reported reduction in sperm quality in mice after administration of lead acetate. Apostali *et al*.[29] found a positive correlation between blood lead level and concentration of lead in testis and epididymis, which is related to reduction in sperm quality and induction of abnormalities in sperms. Based upon the earlier studies and possible mechanisms reported in the scientific literature,[11,28,30,31] a representative diagram of how lead is affecting the sperm parameters is given in Figure 3. After 40 days of treatment, mice in both group I and II were administered with normal diet and drinking water as was given to control group for next 40 days to check the autorecovery in the germ cells. It was observed that the frequency of abnormal sperms after 80 days of treatment significantly decreased in both the groups as compared to the frequencies of abnormal sperms after 40 days treatment period, indicating the autorecovery in G1 and G2 mice. However, the frequency of abnormal sperms in both groups after 80 days of treatment was significantly higher than the control, which shows that mice in these two groups have not recovered fully after 40 days of recovery period. An increase was observed in the frequency of abnormal sperms in the control group as the treatment period was increased which may be because of growing age factor.[32]

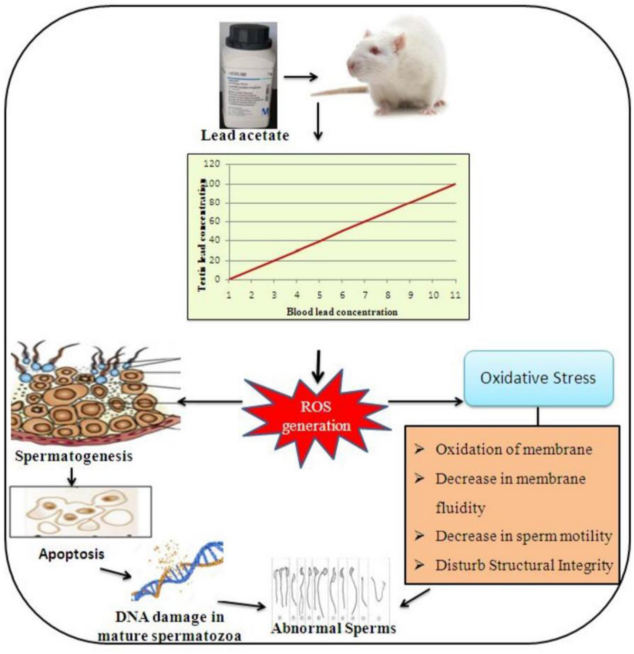

Figure 3: Sperm abnormality induction mechanism in mice.

Therefore, the present study indicated that lead acetate can cause abnormalities in reproductive systems of mice which suggested that it can also damage the reproductive systems of other mammals including human beings. But if the toxicants such as lead are removed from system and organisms are given periods of recovery with healthy diet, the reproductive systems of mammals also have auto recovery capacity. Further research must be carried out to explore the nutraceuticals values of diet (feed) given to animals for auto recovery; checking the correlation with the climatic factors; and exploring the content of MDH, H_2O_2 *etc* during the study period. The study provides scientific basis for evaluations of safety evaluation criteria of lead and other heavy metal products.

CONCLUSION

The present study showed that lead acetate can induce reproductive toxicity in mammalian systems such as mice. Several morphological abnormalities were observed in sperms of mice which were treated with lead acetate. The frequencies of sperm abnormalities in case of mice groups treated with lead acetate were significantly higher than the control group. The changes induced in morphology of sperm suggested that lead acetate can have deleterious effects on testis of mice. The study also showed that if given a period of recovery the reproductive systems of mice can also show significant regenerative potential. Further research must be carried out to explore the nutraceuticals values of diet (feed) given to animals for auto recovery; checking the correlation with the climatic factors; and exploring the content of MDH, H_2O_2 *etc* during the study period. The study provides scientific basis for evaluations of safety evaluation criteria of lead and other heavy metal products.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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