ORIGINAL ARTICLE

Partial recovery of suppressed reproduction by *Withania somnifera* Dunal. in female rats following perinatal lead exposure

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Lead (Pb) is a non-biodegradable, ubiquitous, environmental contaminant, and is well known for neurotoxicity. Recent studies indicate that Pb poisoning also affects male reproduction in rats. However, little information is available on the effect of Pb in female reproduction. The present study is aimed at determining the changes in female reproduction in rats exposed to Pb during the perinatal period. Pregnant rats were exposed to 0.05 and 0.15% Pb through drinking water from gestation day 1. Rats were allowed to deliver pups and Pb exposure was continued lactationally till weaning. Female pups were separated and fed on a normal or reformulated experimental diet (containing *Withania somnifera* leaf powder at 500 mg/1 kg normal pellet diet) for 45 days and then the rats were analysed for fertility studies during adulthood after mating with normal adult rats (3 months old). Significant increase in the duration of the estrus cycle was observed in rats exposed to Pb during the perinatal period. The number of implantations also decreased with an increase in the pre- and post-implantation loss. Treatment with *Withania somnifera* partially reversed the Pb-induced suppressed female reproduction. From the results, it can be concluded that exposure to Pb during the perinatal period suppresses the female fertility and the reformulated diet with supplementation of *Withania somnifera* ameliorates Pb-induced female reproductive toxicity.

Key words: Estrus cycle, female reproduction, implantation loss, lead toxicity, *Withania somnifera*

INTRODUCTION

Lead (Pb) is a heavy metal naturally found in the earth crust, and is a well known reproductive toxicant. Pb exposure has been shown to result in a higher prevalence of menstrual disturbances and spontaneous abortion.[1] In both humans and experimental animals, Pb readily crosses the placental–foetal barrier,[2] causing a direct relation between the Pb-exposed mother and the possibility for irreversible developmental damage to the offspring.[3] Earlier studies in humans have shown that not only does Pb accumulate in the foetus from the second trimester onward,[4] but Pb is also excreted into the milk during lactation and thereby influences the offspring.[5,6] Consequently, children are potentially at risk of Pb exposure during all phases of development.[7] The treatments available for controlling Pb poisoning are restricted to certain sulphydryls containing chelating agents such as calcium salt of ethylene diamine tetra acetic acid, 2, 3-dimercaptopropanol, 2, 3-dimercapto-1 propane sulfonic acid, meso – 2, 3 dimercaptosuccinic acid, etc.[8] However, the use of chelating agents is limited due to their numerous side-effects.[9] Keeping in view the problems associated with the therapeutic aspects of Pb poisoning, this work was undertaken with the aim of finding a suitable treatment for Pb poisoning, which can be (i) easy to administer, (ii) inexpensive, (iii) readily available and (iv) has no toxic effects of its own. Although several medicinal plants were reported to provide protection against heavy metal-induced toxicity, *Withania somnifera is* known for its therapeutic efficacy against Pb poisoning.[10] It has been mentioned in ancient Ayurvedic medicine for its properties to treat many disorders, such as arthritis, gastric problems, stress, inflammatory diseases, cancer and neurological disorders in all age groups, in both sexes and even during pregnancy, without any side-effects.[11] An improved reproductive capability was reported in both sexes after the intake of *Withania somnifera*.[12] Despite the fact that Withania has myriad medicinal properties, its efficacy in relation to protecting animals from the toxic effects of metals or metalloids has not been studied thoroughly. Therefore, this study was carried out to examine the efficacy of *Withania somnifera* as a diet supplement against Pb-induced alterations in female reproductive cycles.

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MATERIALS AND METHODS

Chemicals

Lead acetate used in this study was purchased from Sigma Chemical Company, St Louis, MO, USA, and all other chemicals were purchased from Merck, Mumbai, India.

Animal Exposure

Pregnant rats were housed in polypropylene cages (47cm×34cm×20cm) containing sterile paddy husk (procured locally) as bedding material and maintained in the animal facility at 28±2°C and relative humidity 60±10%, with a 12-h light/day cycle. Experimental rats were provided with 0.05 or 0.15% Pb from gestation day 1 and were allowed to deliver pups. Pb exposure was continued up to completion of the lactation period. Female pups were separated and fed with normal feed or experimental feed and water *ad libitum* and then rats were analyzed for fertility studies during adulthood after mating with normal adult rats (3 months). The experimental design and protocols for usage of animals were approved by the Institutional Animal Ethical Committee of Sri Venkateswara University, Tirupati, India.

Collection of Plant Material and Diet Preparation

Withania somnifera used in this study was obtained from the herbal gardens of S.V. Ayurvedic Pharmacy, Srinivasa Mangapuram, Tirupati. The taxonomic identification was done with the help of the herbarium keeper, Department of Botany, S.V. University, Tirupati. The whole plant was washed with sterile distilled water, cleaned and shade-dried at room temperature in sterile conditions. The whole plant was pulverized to a fine powder using a mechanical grinder.

Five hundred milligrams of the shade-dried leaf powder was mixed with 1 kg normal pellet diet and reformulated as experimental diet. The rats exposed to Pb were maintained on the reformulated diet, experimental diet (Withania-supplemented diet) for 45 days.

Experimental Design

The animals were divided into the following five groups:

Group 1 : Control rats with normal pellet diet (NC)
Group 2 : 0.05% Pb + normal pellet diet (LD)
Group 3 : 0.05% Pb + reformulated diet (LD + W)
Group 4 : 0.15% Pb + normal pellet diet (HD)
Group 5 : 0.15% Pb + reformulated diet (HD + W)

Rats were maintained with these diets (normal and reformulated) for 45 days and then analysed from day 80 for estrus cycle variations.

Vaginal Smear Preparation

Vaginal smear cytology is the most practical approach to monitor the estrus cycle normally. The vaginal smear was examined by the method described by Zarrow *et al.*,[13] as reviewed by Copper *et al.*[14] Few drops of 0.9% saline water were introduced carefully into the vagina by means of a specially made Pasteur pipette. The saline water was sucked quickly back into the pipette by releasing the pressure of the rubber bulb of the pipette. The smear was carefully taken on a clean glass slide as a thin layer and observed under a microscope for various stages of the estrus cycle. Three successive estrus cycles were examined.

Fertility Test

After completion of the checking of the estrus cycles, the treated (feed containing Withania leaf) and control females were cohabited with normal males (obtained from the Biotechnology Department, S.V. University, Tirupati) in the ratio of 1:1. The rats were then checked for presence of vaginal plugs or spermatozoa in the vaginal orifice, as evidence of mating. After mating, the males were removed from the cages. The day when the vaginal plugs or spermatozoa were observed was considered as gestation day (GD_O).

To assess the implantation loss, some of the animals in each group were sacrificed on GD_6 to observe the number of corpora lutea and pre-implantation sites. The remaining animals were sacrificed on GD_{18} to assess the post-implantation loss, status of embryos (normal and dead) and the weight of the embryos.

$$\text{Pre-implantation loss} = \frac{(\text{No. of corpora lutea} - \text{No. of implantations})}{\text{No. of corpora lutea}} \times 100$$

$$\text{Post-implantation loss} = \frac{(\text{No. of implantations} - \text{No. of live foetuses})}{\text{No. of implantations}} \times 100$$

Statistical Analysis of Data

Difference between control and treated groups were evaluated statistically by using the Statistical Package for Social Sciences (SPSS) version 16.0 (SPSS Inc., Chicago, IL, USA). The data are expressed as mean±SD. Significance was set at $P<0.05$.

RESULTS

No mortality was observed during the experimental period both in the control and in the treated groups. All the animals were active and no clinical signs of toxicity were noticed in the animals.

There were no significant changes in the durations of proestrus, estrus and metaestrus phases of the estrus cycle

among the different groups of rats. However, significant changes were observed in the duration of the diestrus phase. In the control rats, the mean length of the diestrus phase of three successive estrus cycles was found to be 2.001±0.125. But, there was a significant increase (P<0.001) in the mean length of the diestrus phase in perinatal Pb-exposed rats over the control rats. The mean lengths were found to be 2.9±0.173 and 3.5±0.173, respectively, in 0.05 and 0.15% Pb-exposed rats. In case of rats fed with the Withania-mixed diet, a significant decrease (P<0.001) was observed in the mean length of the diestrus phase (2.4±0.264 and 2.33±0.208) when compared with the rats exposed to low and high doses of Pb [Figure 1].

The number of implantations in perinatal Pb-exposed rats was significantly decreased when compared with controls. NC-10.83±0.408 vs LD-9.66±0.577, P<0.01; NC-10.83±0.408 vs HD-8.5±0.707, P<0.01. However, treatment with Withania increased the number of implantations in both the groups of Pb-exposed rats. LD-9.66±0.577 vs LD+W-11.33±0.577, P<0.001; HD-8.5±0.707 vs HD+W-10.33±0.57, P<0.001 [Figure 2].

Dose-dependent alterations were observed in case of number of corpora lutea. In case of rats exposed to a high dose of Pb, the number of corpora lutea was found to be decreased significantly when compared with the control rats. In case of rats exposed to a low dose of Pb, the number of corpora lutea was not significantly altered. NC-11.6±0.516 vs LD-11.66±0.152; NC-11.6±0.516 vs HD-10.33±0.115, P<0.01. However, Withania-fed rats showed a significant increase in the number of implantations when compared with perinatal Pb-exposed rats. LD-11.66±0.152 vs LD+W-12.66±0.115, P<0.001; HD-10.33±0.115 vs HD+W-12.33±0.577, P<0.001 [Figure 2].

Substantial reduction in the numbers of live foetuses was observed in case of rats exposed to low and high dose of Pb when compared with control rats. NC-10.5±0.83 vs LD-9.33±0.115, P<0.001; NC-10.5±0.83 vs HD-6.66±0.577, P<0.001. In case of rats fed on the Withania diet, the number of live foetuses was increased significantly, implicating the restoration ability of Withania. LD-9.33±0.115 vs LD+W-10.28±0.129, P<0.001; HD-6.66±0.577 vs HD+W-9.5±0.129, P<0.001 [Figure 3].

Pre- and post-implantation losses were increased in perinatal Pb-exposed rats (16.55% and 15.23%, respectively, with 0.05% Pb-exposure; 22.72 and 22.5%, respectively, with 0.15%) when compared with the control rats (7.2%) [Figures 2 and 3], indicating more damage in the high-dose group. Administration of Withania reduced both pre- and post-implantation losses, suggesting its protective effect against Pb-induced reproductive toxicity in females. The

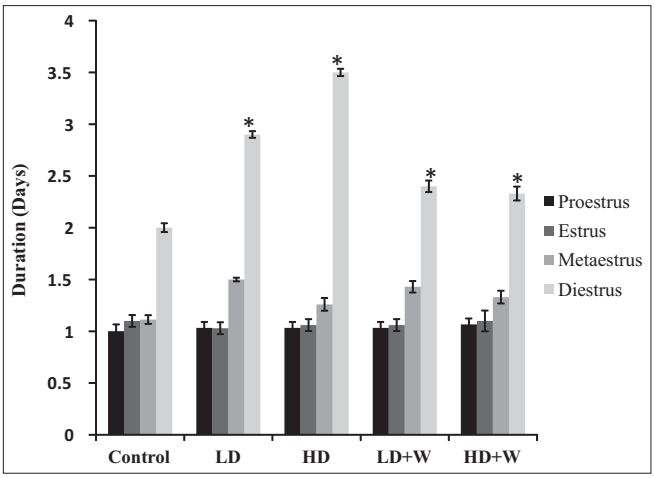

Figure 1: Effect of *Withania somnifera* on the estrus cycle of female rats following perinatal Pb exposure. HD, high dose Pb; LD, low dose Pb; LD+W, low dose Pb+Withania; HD+W, high dose Pb+Withania. Values are mean±SD (n=7). *P<0.001 vs control rats

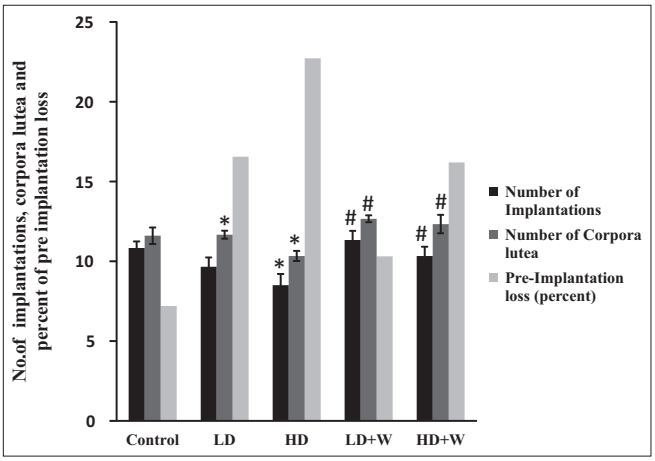

Figure 2: Effect of *Withania somnifera* on the number of implantations, corpora lutea and pre-implantation sites of female rats following perinatal Pb exposure. HD, high dose Pb; LD, low dose Pb; LD+W, low dose Pb+Withania; HD+W, high dose Pb+Withania. Values are mean±SD (n=7). *P<0.001 vs control rats; #P<0.001 vs LD and HD

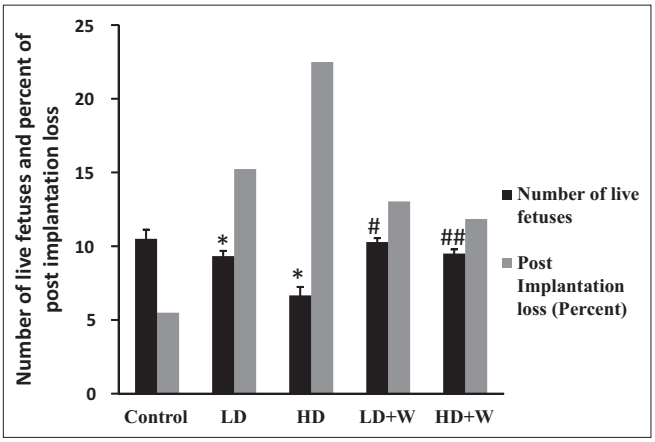

Figure 3: Effect of *Withania somnifera* on the number of live foetuses and post-implantation sites of female rats following perinatal Pb exposure. HD, high dose Pb; LD, low dose Pb; LD+W, low dose Pb+Withania; HD+W, high dose Pb+Withania. Values are mean±SD (n=7). *P<0.001 vs control rats; #P<0.01 vs LD; ##P<0.001 vs HD

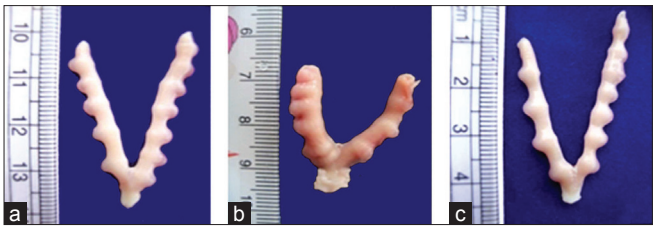

Figure 4: Effect of *Withania somnifera* on pre-implantation sites of female rats following perinatal Pb exposure (0.15%). (a) Controls, (b) decrease in the length of uterine horn and implantations in Pb-exposed rats and (c) increase in the length of uterine horn and implantations in Withania-fed rats

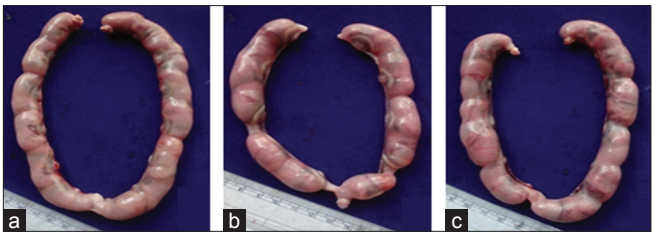

Figure 5: Effect of *Withania somnifera* on the post-implantation sites of female rats following perinatal Pb exposure (0.15%). (a) Controls, (b) decrease in the number of live foetuses in Pb-exposed rats showing post-implantation loss and (c) increase in the number of live foetuses in Withania-fed rats with the restored post-implantation loss

reduction in pre- and post-implantation losses when compared with the Pb-exposed rats was 10.31 and 16.19%, and 13.04 and 11.85%, respectively [Figures 4 and 5].

DISCUSSION

There has been an increasing concern that environmental contaminants cause reproductive problems in women by altering the release of gonadotrophins and, thereby, affecting reproduction at multiple levels of the hypothalamus–pituitary–gonadial axis.[15] Pb is one such contaminant that causes deleterious effects to the reproductive system. In case of male rats, it was shown that Pb crosses the blood–testis barrier[16] and alters testicular functions like production of sperms and testosterone. In case of female rats, earlier studies demonstrated that Pb causes a significant delay in the onset of puberty as it could suppress the serum levels of important puberty-related hormones such as IGF-1, LH and E_2.[7] In the present study, there were no alterations in the body weights of all groups, indicating the absence of overt general toxicity. Significant increase in the duration of the estrus cycle, particularly the prolongation of the diestrus phase observed in Pb-exposed rats, indicates the altered hormonal secretions induced by Pb affecting the LHRH/LH releasing system.[17] The results of the present study also showed that the reproductive end points, such as number of implantations, corpora lutea and live foetuses, were significantly decreased, with an increase in the pre- and post-implantation loss following Pb exposure. This may be due to developmental toxicity induced by the altered LHRH/LH releasing system in the presence of Pb.

Rats fed on a diet with the *Withania somnifera* leaf powder significantly decreased the duration of the estrus cycle and partially restored all the reproductive end points. *Withania somnifera* has been mentioned in Ayurveda for its antioxidant property[18] and protection against pregnancy problems[19] and musculoskeletal conditions.[20] Several earlier studies indicated that it possesses anti-inflammatory, anti-stress and antioxidant properties[21] besides positively influencing the endocrine system.[22] Previous reports inferred that the administration of the aqueous extract of Withania was able to decrease the serum levels of FSH and increase the LH in male rats, suggesting that, possibly, *Withania somnifera* has inhibitory and elevatory effects on the FSH and LH gonadotrophins, respectively.[23] Considering the fact that ovulation and gestation, at least in the early phases in women, are controlled by the LH/progesterone system,[24] it can be concluded that *Withania somnifera* has a positive effect on reproduction by increasing LH and progesterone. Follicle growth and maturation of the ova and folliculogenensis are very sensitive to pro-oxidant/antioxidant balance.[25] The principle constituents of *Withania somnifera* are Withanolides, mainly localized in leaves, which have a potent antioxidant activity.[26,27] Withaferin A, a steroidal lactone, was the first member of this group of compounds to be isolated,[28] also reported for its anti-cancerous and antioxidant properties.[29] From this study, it is evident that *Withania* is capable of restoring the Pb-induced reproductive alterations in female rats. The possible mechanism by which it could restore the changes may be due to the presence of Withanolides, mimicking the steroidal hormones and their antioxidant protection. Although Withanolides are found to be major contributors to the therapeutic efficacy of Withania, further studies are required to understand the composition of the *Withania somnifera* leaf and to establish the molecular basis of the mechanisms involved in reversing the Pb-induced reproductive changes in female rats.

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