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Reproductive potential of male Portan rats exposed to various levels of lead with regard to zinc status

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The present study was designed to elucidate the mechanisms accounting for disruption of the normal function of the testis exposed to various levels of Pb. Three different doses of Pb (10, 50, 200 mg Pb/kg body weight per d) were given orally to male Portan rats (groups 2, 3, 4). Zn (1 mg Zn/kg body weight per d) was also given with Pb (50 mg Pb/kg body weight per d) in group 5. Treatments continued for 3 months. Plasma luteinizing hormone and follicle-stimulating hormone concentrations were found to be decreased in Pb-exposed rats. This was in turn reflected in the appreciable decline in fertility status. In cell kinetic studies, significant declines in various cell populations (preleptotene, pachytene, young (step 7) spermatids and mature (step 19) spermatids) were seen. However, in group 5 after Zn supplementation, hormone levels, cell numbers and fertility status were found to be close to normal. It is concluded that Pb might act at maturation level to cause conspicuous degenerative changes in the testis; Zn supplementation protected against these effects.

Lead: Zinc: Testis: Luteinizing hormone: Follicle-stimulating hormone: Fertility

Pb is well documented as a testicular toxicant and has been shown to perturb reproductive capability. However, the cellular mechanism regarding the adverse effect of Pb on steroidogenesis is still poorly understood. Reports of a decrease in human sperm quality in the last 50 years (Carlsen *et al.* 1992) have added new impetus to the search for various environmental agents and pollutants containing metals. Boscolo *et al.* (1988) and Murthy *et al.* (1991) found increased numbers and sizes of lysosomes and vacuoles in Sertoli cells in Pb-exposed animals. Others, however (Nathan *et al.* 1992), did not reveal any change in the course of spermatogenesis or in Sertoli cell function in Pb-treated rats. Experimental studies designed by various authors regarding adverse effects of Pb on reproductive functions had differing findings. Johansson & Wilde (1986) observed a reduction in fertility of adult Pb-exposed male mice, whereas Leonard and colleagues (A Leonard, G Linden and GB Gerber, unpublished results) and Pinon-Lataillade *et al.* (1993) did not find any change. Under identical conditions of exposure, however, Piasek & Kostial (1987) observed a significant reduction in female reproductive capability. Chowdhury *et al.* (1986) postulated that Pb may inhibit spermatogenesis in rats at the premeiotic stage because of lack of testosterone. Based on cell kinetic studies, there was a significant decrease in the young spermatids:pachytene spermatocytes and mature spermatids:young spermatids ratios in Pb-treated rats (Kaushal *et al.* 1996).

As regards hormonal status, the adverse effects of Pb exposure on male reproductive functions both in human subjects and animals remain open to debate. Pinon-Lataillade *et al.* (1993) and Nathan *et al.* (1992) investigated the reproductive toxicity of Pb in Sprague–Dawley rats. They found that concentrations of follicle-stimulating hormone (FSH) and luteinizing hormone (LH) remained unchanged. Testosterone concentration, however, was found to be either unchanged (Nathan *et al.* 1992) or reduced (Sokol & Berman, 1991). Zn has been shown to be important for normal testicular development, maintenance of the germinal epithelium and sperm motility (Anderson *et al.* 1993). In human subjects, sperm quality was improved after supplementation of Zn in infertile patients (Favier, 1992).

In the present study, cell kinetics of the seminiferous epithelium of the testis was examined to pinpoint the exact spermatogenesis step(s) inhibited by Pb treatment. LH and FSH concentrations were also studied to assess the functional damage to the testis. Fertility checks were also carried out in all the treatment groups. The present study was extended by the simultaneous supplementation of Zn and Pb.

Materials and methods

Animals

Male rats of the Portan strain (8 weeks old), weighing 125–150 g, were obtained from the Central Animal House (Panjab University, Chandigarh, India). Animals

Abbreviations: FSH, follicle-stimulating hormone; LH, luteinizing hormone.

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were kept in separate cages and received standard pelleted rat feed (Hindustan Lever Ltd, Bombay, India) and drinking water *ad libitum*. The animals were divided into five groups of five or six animals each. The Pb-treated groups (groups 2, 3, 4) were given 10, 50, 200 mg Pb/kg body weight per d respectively orally as lead acetate. The Zn-supplemented group (group 5) was given orally 50 mg Pb as lead acetate/kg body weight per d and 1 mg Zn as zinc sulfate/kg body weight per d separately. All treatments were given in a 1 ml volume (from stock solutions) by gavage daily and continued for 3 months. Control animals (group 1) were administered an equivalent volume of distilled water. Pb and Zn stock solutions were made separately by dissolving lead acetate (12·8 g/l distilled water) and zinc sulfate (4·0 g/l distilled water) respectively. These treatments were selected on the basis of previous determinations of effective doses (Flora *et al.* 1991; Kaushal *et al.* 1996). At the completion of treatments, animals were anaesthetized using diethyl ether and killed; the organ of interest was removed and processed for further studies.

Tissue preparation and staining

Fresh pieces of testis of rats taken randomly (left or right) from all groups were fixed in formalin for 48 h, washed, dehydrated with graded alcohol and embedded in paraffin wax. Sections (5–8 μm thick) were cut and stained with haematoxylin–eosin.

Cell kinetics

Random fields were selected from the stained slides from each animal. Differential cell counts of seminiferous epithelium at stage VII were done at × 400 magnification (Leblond & Clermont, 1952). This stage was selected because it contains two generations of spermatocytes (young resting preleptotene, more mature pachytene spermatocytes), two generations of the spermatids, young (step 7) and fully mature (step 19) spermatids and also the relatively mature spermatogonia, spermatogonia A. Nuclei of Sertoli cells in young spermatids were scored only when nucleoli were visible. Hence spermatogonia A, preleptotene, pachytene, young spermatids and mature spermatids were counted based on the shape of these cells as shown in Russell & Clermont (1976). The number of germinal cells per 100 Sertoli cells was calculated.

Fertility studies

At the end of the treatment period of all the groups, a week before killing, animals were mated with control female rats. Two or three male treated rats from each group were kept with female control rats at a 1:2 ratio. After 1 week, the male rats were separated, killed and used for measuring other variables. Female rats were observed for litter size for 21 d.

Hormone studies

After completion of treatment period in all the groups, blood was drawn into heparinized vials by puncturing the orbital sinuses of rats with the help of a clean glass capillary. Plasma was separated from blood. Plasma FSH and LH were measured in duplicate using RIA kits supplied by Beckman Coulter (Brea, CA, USA). For FSH and LH, the results were expressed as IU/ml. The detection limit for FSH and LH was 0·2 IU/l.

Statistical analyses

Mean values and standard deviations were calculated. ANOVA was used in evaluation of the significance of results.

Results

Cell kinetics

Cell kinetic data are shown in Table 1. Since Sertoli cells were unaffected in animals treated with Pb alone and in the Zn-supplemented groups, the number of germinal cells in different groups was normalized per 100 Sertoli cells. There was no significant decrease in spermatogonia A in the testis in group 2, but there was a significant decrease in preleptotene ($P<0·01$), pachytene ($P<0·001$), young spermatids ($P<0·001$) and mature spermatids ($P<0·001$). At doses of 50 mg Pb and 200 mg Pb, significant decreases in the all cell populations counts were seen. There was 45·7 % decrease in preleptotene, 28·6 % decrease in pachytene, 39·0 % decrease in young spermatids and 43·4 % decrease in mature spermatids in group 3

Table 1. Cell kinetic studies in testis of male Portan rats following lead treatment for 3 months‡§
(Mean values and standard deviations for five to six observations per group)

	Spermatogonia A		Preleptotene		Pachytene		Young spermatids		Mature spermatids	
Groups	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
1	216·4	16·1	163·2	9·3	132·9	12·2	389·0	44·8	405·7	57·2
2	206·6	10·1	148·4**	13·3	106·0***	9·3	323·4***	31·4	347·1**	18·0
3	185·7**	19·9	88·5***	9·8	94·9***	17·5	237·0***	24·5	229·6***	22·8
4	93·9***	8·2	77·0***	6·2	64·8***	7·3	108·4***	7·1	96·3***	8·1
5	211·2††	18·7	158·1†††	12·3	125·0†††	7·4	306·4†††	24·5	291·2†††	20·1

Mean values were significantly different from those of the control group: **$P<0·01$, ***$P<0·001$.
Mean values were significantly different from those of group 3: ††$P<0·01$, †††$P<0·001$.
‡ For details of groups, diets and procedures, see pp. 387–388
§ Cell counts were done per 100 Sertoli cells.

($P<0{\cdot}001$). At maximum dose level (group 4), a dramatic decrease of 51–76% was seen in all the cell types ($P<0{\cdot}001$). When Zn was administered with Pb (group 5), there were no significant changes in the numbers of spermatogonia A, preleptotene, pachytene and young spermatids; however, the number of mature spermatids still decreased compared with group 1, whereas a significant increase in all the cells was observed as compared with group 3. Hence, there was an overall effect of Pb treatment on all the germinal cell types.

On further analysis, it was observed that the preleptotene:spermatogonia A, young spermatids:pachytene and mature spermatids:young spermatids ratios were unaffected in group 2, whereas these ratios were significantly reduced in groups 3 and 4, i.e. at higher dose levels. When Zn was supplemented together with Pb (group 5), these ratios were unaffected as compared with control values; there was an increase in preleptotene:spermatogonia A, pachytene:preleptotene and young spermatids:mature spermatids ratios as compared with group 3 where same dose of Pb was given.

Concentrations of follicle-stimulating hormone and luteinizing hormone

Plasma FSH and LH were measured in Pb-exposed rats in all the groups (Table 2). The concentration of plasma LH diminished by 41·2% ($P<0{\cdot}001$) in group 2, 74·2% ($P<0{\cdot}001$) in group 3 and 82·4% ($P<0{\cdot}001$) in group 4 as compared with the control group. In group 5 (Zn-supplemented), there was a marked improvement in LH concentrations. The decrease in FSH concentrations in groups 2, 3 and 4 were 26·8 ($P<0{\cdot}001$), 37·8 ($P<0{\cdot}001$) and 39·5 ($P<0{\cdot}001$) % respectively. With Zn supplementation, there was a recovery in FSH values.

Fertility check

In Pb-exposed male rats, the fertilizing capacity was significantly affected at higher dose levels (Table 3). At the lowest dose (group 2), no change in number of litters and their litter size was observed as with the control group. At higher doses in group 3 and 4, both the number of litters and their sizes were decreased. The effect was more pronounced in group 4. However, on Zn supplementation together with Pb (group 5), there was a recovery in litter number and sizes.

Table 2. Alterations in plasma hormone levels in male Portan rats following lead treatments for 3 months‡§

(Mean values and standard deviations for five to six observations per group)

Treatment groups	Luteinizing hormone (IU/ml)		Follicle-stimulating hormone (IU/ml)	
	Mean	SD	Mean	SD
1	8·7	0·5	2·3	0·5
2	5·1***	1·1	1·7***	0·3
3	2·2***	0·4	1·4***	0·1
4	1·5***	0·1	1·4***	0·5
5	3·9†††	0·8†††	1·9†††	0·1

Mean values were significantly different from those of the control group, ***$P<0{\cdot}001$.

Mean values were significantly different from those of group 3: †††$P<0{\cdot}001$.

‡ For details of groups, diets and procedures, see pp. 387–388.

§ Cell counts were done per 100 Sertoli cells.

Table 3. Fertility studies in male Portan rats following lead-treatments for 3 months*

Groups	No. of male rats (*n*)	No. of female rats (*n*)	Litters (*n*)	Litter size (*n*)
1	2	4	4	12, 11, 13, 14
2	2	4	4	11, 11, 14, 14
3	3	6	4	2, 2, 3, 3
4	2	4	1	1
5	2	4	4	6, 6, 7, 7

* For details of groups, diets and procedures, see pp. 387–388.

Discussion

The maturation of germinal cells to the release of mature spermatids is divided into nineteen stages, progressing from the spermatogonia to the spermatocytes and finally to the spermatids. The germinal cell counts were expressed per 100 Sertoli cells because of their resistant nature to various physical or chemical insults. In the present study, a marked change in different cell types was seen. Young spermatids were found to be most affected at higher Pb dose levels, suggesting that spermatogenesis was suppressed at maturation level. Significant alterations in the preleptotene:spermatogonia A, young spermatids:pachytene and mature spermatids:young spermatids ratios were also found, showing the impairment of different stages of maturation of germ cells. According to Bansal & Davies, (1986), any alteration or disturbance in these ratios indicates impairment of spermatogenic process. Similarly, Corpas *et al.* (2002) also found a reduction in the number of spermatogonia and spermatocytes as a consequence of Pb exposure. The actions of the precise mechanism of changes brought out by Pb treatment may be at the level of the hypothalamic–pituitary axis and/or a combined effect involving the gonads (Sokol *et al.* 1985). Significant reductions in LH and FSH concentrations were found, suggesting that the hypothalamic–pituitary–testicular hormonal axis was adversely affected by the present Pb exposure. Pb could exert its influence through metal-dependent regulatory DNA sequences and/or receptors common to hormonal genes. Zn finger receptors, which are known to mediate steroid hormone–DNA interactions (Freedman, 1992), could be competitive targets for Pb binding and hence altered transcriptional rates. Atypical divalent metal ions such as Pb may compete for the Zn-binding site in hormone receptors. It is possible that Pb can competitively and more effectively replace Zn in its structural functions within these receptor proteins. In the present studies, decrease in plasma LH concentrations in Pb-exposed rats compared with control concentrations might reflect impairment of a negative feedback control of testosterone by pituitary LH synthesis and/or of hypothalamic LH releasing hormone secretion. (Rotten, 1991). Hence, inhibition of circulating LH concentrations and pituitary LH β mRNA by Pb suggest that the growth effects of Pb may due to a delay in the development of sex-specific pituitary growth.

Reduction of FSH could be due to impairment of some Sertoli cell protein secretion (Maguire *et al.* 1993). It might indicate that Pb has a slight effect on production of few proteins secreted by the Sertoli cells. Thus, the reproductive and growth effects of Pb are complex, sex-dependent and appear to involve multiple sites on the hypothalamic–pituitary–gonadal axis.

The arrest of spermatogenesis at the maturation level was also cross-checked by the fertility check. The fertilizing capacity of male rats was appreciably affected. A complete spermatogenic arrest was noticed at the highest dose of Pb. This is in accordance with the results of Piasek & Kostial (1987), who found a decline in fertility in Pb-exposed rats. According to Gorbel *et al.* (2002), chronic exposure to Pb causes a double sexual disorder in rats: the first disorder deals with hormonal functions, which are affected at early stages, and the second disorder deals with the genital tract, affecting the testis and ovary and resulting in reduced fertility in female rats in spite of the presence of a normal oestrus cycle. Interaction of Pb with human protamines (Quintanilla-Vega *et al.* 2000) may result in chromatin alterations, which in turn may lead to male fertility problems and eventually to DNA damage.

Other factors in the toxic manifestation of Pb might be involvement of Pb-binding proteins. It has been shown (N Babra, B Nehru and MP Bansal, unpublished results) that testicular high-affinity Pb-binding proteins are responsible for accumulation, intracellular distribution and mediating alterations at the biochemical and pathological level. Further, Fowler *et al.* (1985) stated that Pb-binding proteins act as tissue specific receptors for Pb; this could mediate the well-known alterations in renal gene expression produced by Pb exposure.

Alleviation of toxic manifestations found in the Zn-supplemented group suggests a crucial role for Zn in hormonal distribution as well as in fertilization. LH and FSH concentrations were close to normal with Zn supplementation. All the steroid hormone receptors require the divalent cation $Zn^{2+}$to maintain their secondary structure and normal function. Availability of more Zn might reactivate the normal functioning of receptors (Cox & Goding, 1991; Freedman, 1992). Further Zn stabilizes the cell membrane in seminal plasma and nuclear chromatin of spermatozoa. Further, it is likely that Zn might displace the loosely bound Pb from binding proteins. In quantitative studies (N Babra, B Nehru and MP Bansal, unpublished results), Zn ions were found to be efficient in displacing Pb that was already bound to varying extents. Hence, reproductive potential was close to normal with Zn supplementation, although the Zn dose was just 2 % of the Pb dose.

Conclusions

In conclusion, effects of high dose of Pb administration on spermatogenesis in the adult rat were dramatic, showing that the germ cells are a direct target for Pb. Abnormalities in sperm maturation and reduction in hormone concentrations indicate that Pb exposure impairs LH and FSH secretion. It is possible that there was a relationship between high concentration of Pb found in the testis and possible disturbance of different steps of spermatogenesis, leading to the observed reduction in fertility. Further, Zn supplementation could competitively and effectively reduce the availability of binding sites for Pb. These findings suggest that exposure to Pb might pose threat to male reproductive capability and potential. The therapeutic effect of Zn supplementation is also suggested.

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