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Testicular histomorphometry and semen quality of adult Wistar rats following juvenile oral lead intoxication

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SUMMARY

Empirical evidence has shown that childhood lead (Pb) intoxication impairs cognitive function and heightens the risk of dementia in young adult humans. However, the effects of juvenile Pb toxicity on testicular morphometry and semen quality in young adulthood require further studies. Using a rodent model, juvenile (4 weeks old) Wistar rats were randomized to receive either normal saline or lead acetate in their drinking water at 0.5% (5000 ppm), 1.5% (15000 ppm) and 2.5% (25000 ppm) for 7 weeks. At 11 weeks postnatal, young adult rats were anaesthetized and their cauda epididymides excised for sperm analysis. The left testicles were homogenized in phosphate buffer and the supernatants assayed for testosterone by the enzyme immunoassay technique. The right testicles were processed for photomicroscopy by the haematoxylin and eosin technique. Using ImageJ software, testicular sections were subjected to morphometric analysis. Our findings showed that juvenile Pb intoxication alters testicular histomorphometry in young adulthood as indicated by significant ($P<0.05$) reductions in seminiferous tubule diameters and lumen, and marked attenuation of the germinal epithelium in the Pb-exposed rats compared with the non-exposed age-matched controls. Furthermore, findings from sperm analysis showed significantly low sperm density and motility following oral Pb exposure, while the percentage of deformed and dead sperm cells had increased significantly ($P<0.05$). Meanwhile, testicular interstitial compartment and the associated Leydig cells were unperturbed, and testicular testosterone levels were not significantly different between the control and Pb-treated groups ($P>0.05$). These findings show that Pb intoxication initiated prior to pubescence alters testicular histomorphometry and lowers semen quality in young adult Wistar rats, and these changes were unconnected with testicular testosterone levels.

Key words: Lead – Testis – Morphometry – Seminiferous tubule – Testosterone – Juvenile

INTRODUCTION

Male reproductive health hazards are numerous, and the testes are especially sensitive to toxicants that include physical hazards such as ionizing radiation and heat; chemical hazards such as carbon disulphide and dibromochloropropane; and heavy metals such as cadmium, mercury and lead (Bonde, 2002, 2010).

Lead (Pb) is a known occupational hazard and a potent male reproductive toxicant. *In vitro* study by Adhikari et al. (2000) showed that the addition of variable doses of lead (Pb) to a mixed culture of Sertoli and germ cells at 0.4, 4 and 40 nM for 24 and 48 hours resulted in progressive detachment of male germ cells from Sertoli cells in a concen-

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tration- and duration-dependent manner. *In vivo* studies in adult rodents have also demonstrated the effects of Pb exposure on spermatogenesis, testicular steroidogenesis and male reproductive functions. While several of these studies showed deleterious effects of Pb on male reproductive anatomy and biochemistry (Batra et al., 2001, 2004; Graca et al., 2004; Priya and Reddy, 2012; Wang et al., 2013), a few studies reported the contrary (Boscolo et al., 1988; Kempinas et al., 1988; Coffigny et al., 1994). In humans, recent findings indicated reduction of sperm density in Belgian lead smelter workers whose sperm count diminished to 35 million/ml compared with 51 million/ml in the non-exposed group (Mahmoud et al., 2005). Similarly, studies among paint factory workers in Bangalore showed reduced semen volume and low sperm motility from exposure to Pb (Naha and Manna, 2007). Moreover, clinical evaluation of 23 men exposed to Pb poisoning showed increased frequency of asthenospermia, hypospermia and teratospermia, perhaps due to a direct effect of the metal on the male gonad (Lancranjan et al., 1975).

A number of mechanisms have been advanced to explain the testicular toxicity of Pb. Recent studies by Kumar et al. (2013) in Wistar rats showed excessive generation of free radicals and impairment of testicular antioxidant defence system following treatment with Pb and cadmium (Cd). Moreover, *in vitro* studies (Liu et al., 2001; Liu et al., 2003; Pandya et al., 2012) showed that Pb impairs steroidogenesis in Leydig cells by decreasing the expression of steroidogenic acute regulatory (StAR) protein and the activity of 3beta- and 17beta hydroxysteroid dehydrogenase, suggesting that Pb impairs spermatogenesis by perturbing androgen biosynthesis in Leydig cells. Besides, those rats that received 0.2% Pb in their drinking water showed significantly increased expression of caspase-3 and transforming growth factor beta 1 (TGFβ1); as well as increased DNA damage and apoptotic index in testicular cells – an indication that Pb increases apoptotic germ cell loss (Wang et al., 2006).

Nonetheless, humans are continually exposed to the untoward reproductive effects of Pb, and such exposure is one of the several factors contributing to the declining semen quality in men in reproductive age. Even children are not exempted from the adverse effects of Pb toxicity. For example, in the Nigerian Northern state of Zamfara, high morbidity and mortality were recorded in children exposed to environmental Pb intoxication arising from illegal mining activities and ore processing in villages. In such children, blood Pb levels were as high as 45-708 µg/dl, much higher than the internationally acceptable level of 10 µg/dl (Medecins Sans Frontieres, 2012). In human longitudinal studies, findings have shown that early childhood environmental Pb exposure is a risk factor for cognitive impairment in young adulthood (Mazumdar et al., 2011; Mazumdar et al., 2012). It is known that Pb can gain entry into cells via the calcium ion (Ca^{2+}) channels (Atchison, 2003) and can perturb cellular processes that involve Ca^{2+} signalling, such as intercellular communication that occurs between neurons at synapses. Moreover, Pb can impair cell motility by disturbing Ca^{2+} homeostasis, as it is capable of displacing Ca^{2+} from binding to Ca-binding proteins, especially calmodulin (Shirra and Barran, 2009). Pb may also exacerbate tissue oxidative stress-induced lesions due to its interference with enzymes that maintain the reducing state of cells (Flora et al., 2008). These biological properties of Pb could impact negatively on sperm production and quality, as well as alter testicular microanatomy. In the present study, we report the effects of Pb intoxication initiated in the juvenile stage of postnatal development on semen quality and testicular morphometry in young adult Wistar rats.

MATERIALS AND METHODS

Chemicals

Lead acetate (Kiran Light Laboratories, India) was procured from Yomi-Esthony Enterprise, Nigeria. Other chemicals and reagents were of analytical grade.

Animals

Juvenile male Wistar rats (4 weeks old; 35 g body weight) were sourced from John Alfred Animal Holdings, Ilorin, and were transferred to the Small Animal Facility of the College of Health Sciences, University of Ilorin, Nigeria. Rats were maintained on standard rodent chow and water was given freely. Care and handling of animals conformed to international standards, according to the National Academy of Sciences' Guide for the Care and Use of Laboratory Animals.

Oral administration of lead acetate and collection of biological samples

Juvenile Wistar rats (at 4 weeks of postnatal development) were randomized to receive either normal saline (control) or high daily doses of lead acetate in their drinking water at 0.5% (5000 ppm), 1.5% (15000 ppm) and 2.5% (25000 ppm) (Nathan et al., 1992; Pinon-Lataillade et al., 1995) for 7 weeks (n=6 rats in each group). Thereafter, animals were anaesthetized with diethyl ether. The cauda epididymides were excised for sperm analysis. For each rat, the left testicle was homogenized in phosphate buffer (0.1 M, pH 7.4) and centrifuged at 10,000 rpm for 10 minutes to obtain supernatant for testicular testosterone assay. Furthermore, all animals were subjected to whole body perfusion, first with normal saline, and then with the fixative (4% paraformaldehyde in phosphate buffered saline), and the testicles were

transferred into 4% paraformaldehyde solution for histological preparation.

Sperm analysis

Caudal epididymal sperm was analysed for motility, count, viability and morphology as previously described by Oyewopo and Togun (2005). Briefly, Sperm count was estimated with the aid of the Neubauer improved cell-counting chamber. The ratio of the motile to non-motile sperm cells (sperm motility) was estimated for the treatment and control groups. The total number of sperm cells with normal morphology was estimated and expressed as a percentage of the total number of sperm cells. The life-death ratio of the sperm cells was also estimated as the number of live sperm cells divided by the total number of sperm cells multiplied by 100.

Bioassay for testicular testosterone

Supernatants from testicular homogenates were assayed for testosterone concentrations by the enzyme immunoassay (EIA) technique as described in Gower (2010) using the testosterone EIA kit from Cayman Chemical (MI, USA).

Testicular histology and histomorphometry

Paraformaldehyde-fixed testicles were sectioned at 7 µm with the aid of the rotary microtome and then processed for light microscopy by the haematoxylin and eosin (H&E) method as described in Bancroft and Stevens (1982). Using ImageJ (NIH) software, haematoxylin and eosin sections of the testes were subjected to morphometric analysis. In the control and Pb-treated rats, the diameter (external diameter) of the seminiferous tubules, thickness of the seminiferous epithelium and diameter of seminiferous tubule lumen were estimated to the nearest µm.

Statistical analysis

Data were analysed using one-way analysis of variance (ANOVA), with the aid of the SPSS software version 20 (IBM, USA). Results are presented as mean ± standard error of the mean (mean±SEM). The means of the variables measured among the Pb-exposed and non-exposed age-matched control groups were compared using Tukey *post hoc* test. P value less than 0.05 ($p<0.05$) was taken as statistically significant. All graphs were drawn using the GraphPad Prism (GraphPad Software Inc., USA).

RESULTS

Oral Pb intoxication initiated before pubescence markedly reduces sperm count and motility in young adult Wistar rats

In young adult Wistar rats (11 weeks old), oral Pb intoxication initiated in the prepubescent stage of postnatal life results in marked impairment of sperm production as indicated by the significantly low sperm count in all the Pb-treated rats. In rats that received the maximum Pb dose of 2.5% in their drinking water, sperm count had reduced significantly to 4.2 (x10^6)/ml compared to a control value of 49.4 (x10^6)/ml. Moreover, rats that received 0.5% of Pb had significantly higher sperm count compared to those that received higher doses of Pb in their drinking water (Fig. 1A). In addition, juvenile Pb intoxication was associated with reduced sperm motility in adult rats (Fig. 1B). Those rats that received higher doses of Pb acetate in their drinking water (1.5 and 2.5%) had zero number of motile sperm.

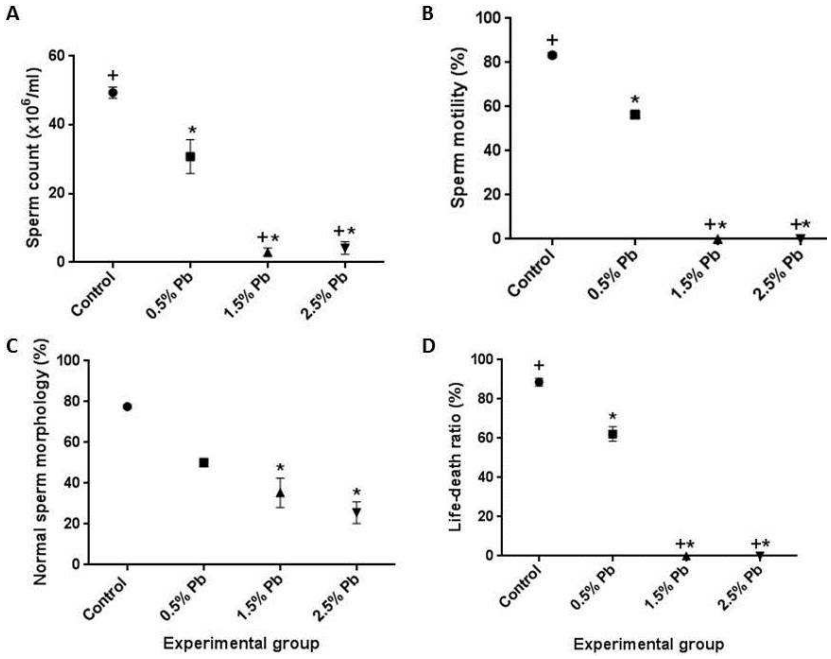

Fig. 1. Caudal epididymal sperm analysis showing significant reductions in sperm density **(A)** and motility **(B)**, as well as decreased percentages of sperm cells with normal morphology **(C)** and viability **(D)** following chronic exposure to Pb acetate. Data are mean±SEM (n=6 for each group). *P<0.05 compared with control; $^+$P<0.05 compared with the group on 0.5% Pb acetate solution.

Oral juvenile Pb exposure increases the percentage of dead and malformed sperm cells in young adult Wistar rats

In young adult Wistar rats, oral Pb treatment initiated in the juvenile stage of postnatal life increased the number of sperm cells with subnor-

mal or abnormal anatomy (multiple tails, absent tail, coiled tail, duplicated head, and pin head) (Fig. 1C), and reduced the viability of sperm cells (increased percentages of dead sperm cells) (Fig. 1D).

Oral Pb intoxication started prior to pubescence alters seminiferous tubule morphometry in young adult Wistar rats

Lead (Pb) intoxication initiated in the prepubescent stage of postnatal development was associated with markedly attenuated seminiferous epithelium in adult Wistar rats. The thickness of the seminiferous epithelium was significantly reduced ($P<0.05$) from 142.6±2.8 µm in the control rats to 98.1±4.4 µm in rats that received 2.5% aqueous Pb solution (Fig. 2A, Fig. 3).

Histologically, young adult rats that received Pb solutions starting in the prepubescent stage of postnatal life had their germinal epithelium largely eroded, with histological evidence of germ cell loss. Thinning of the germinal epithelium was observable, with massive loss of spermatocytes, spermatids and spermatozoa, but the spermatogonial stem cells were largely preserved in their peripheral location in the seminiferous epithelium (Fig. 3). Furthermore, the interstitial tissue between adjoining seminiferous tubules was intact and there was no evidence of Leydig cell loss from oral Pb treatment (Fig. 3). In addition, Pb treatment resulted in significant reduction in the external (cross-sectional) diameters of the seminiferous tubules (Fig. 2B), with concomitant reductions in the lumens (internal diameter) of these tubules in rats that received higher doses of Pb (Fig. 2C).

Oral Pb treatment initiated in prepubescence does not perturb testicular testosterone in young adult Wistar rats

Testicular testosterone levels did not differ significantly between rats in the control and Pb-treated groups, irrespective of the doses of Pb administered (Fig. 2D). This finding is supported by the intact morphologic appearance of the extratubular interstitial compartment observed in the haematoxylin and eosin sections of the testis in our model. There was no histologic evidence suggesting loss of the androgenic interstitial cells of Leydig in the Pb-treated rats at the oral doses tested (Fig. 3A-3D).

DISCUSSION

We report some testicular histomorphometric characteristics and semen quality of young adult rats on oral Pb intoxication initiated in the prepubescent stage of postnatal development (starting four weeks after birth). Juvenile exposure to oral Pb resulted in the diminution of the diameter and lumen of the seminiferous tubules, and produced thinning of the germinal epithelium in young adult Wistar rats. Besides, Pb treatment induced significant reduction in sperm count and sperm motility, and elevated sperm cell death and deformity in these rats.

Some of the factors that could account for the attenuation of the seminiferous epithelium in young adult Wistar rats following juvenile Pb exposure are germ cell death by apoptosis and/or necrosis. Wang et al. (2006) studied DNA damage in germ cells by single cell gel electrophoresis (SCGE) and apoptosis by TUNEL in mice that received 0.2% Pb via oral gavage, and reported accelerated DNA damage, elevated apoptotic cell death and increased expression of TGFβ1 and caspase-3 in the germ cells of Pb-treated male mice.

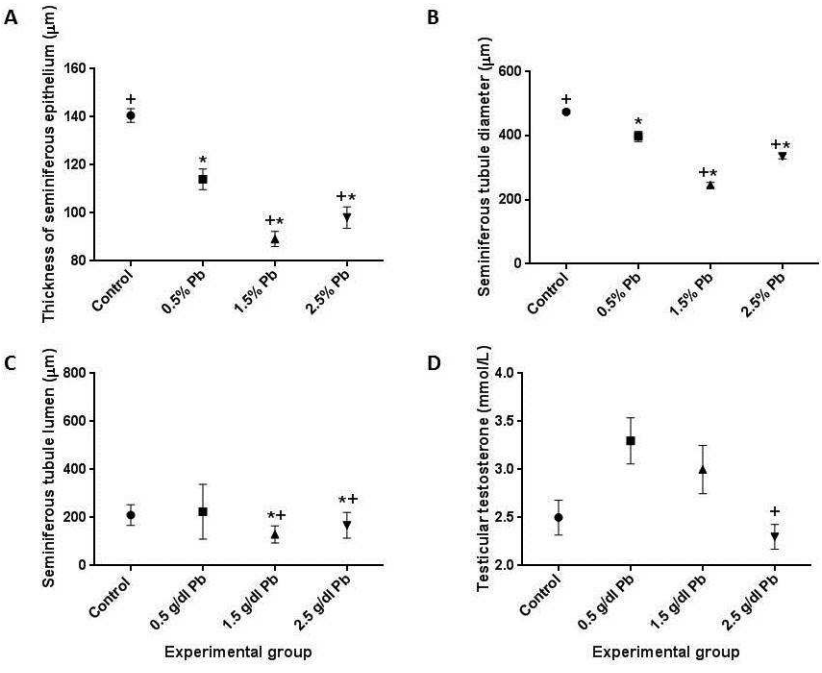

Fig. 2. Testicular histomorphometric findings in the Pb-treated and non-treated control rats. Significant reductions in the thickness of the seminiferous epithelium **(A)** and diameter of seminiferous tubule **(B)** were seen in the Pb-treated rats. Higher doses of Pb (1.5% and 2.5%) caused significant reductions in the lumen of seminiferous tubule ©, but no significant differences were found in the testicular testosterone levels among the treatment groups **(D)**. Data are mean±SEM (n=6 for each group). *$P<0.05$ compared with control; $^{+}P<0.05$ compared with the group on 0.5% Pb acetate solution.

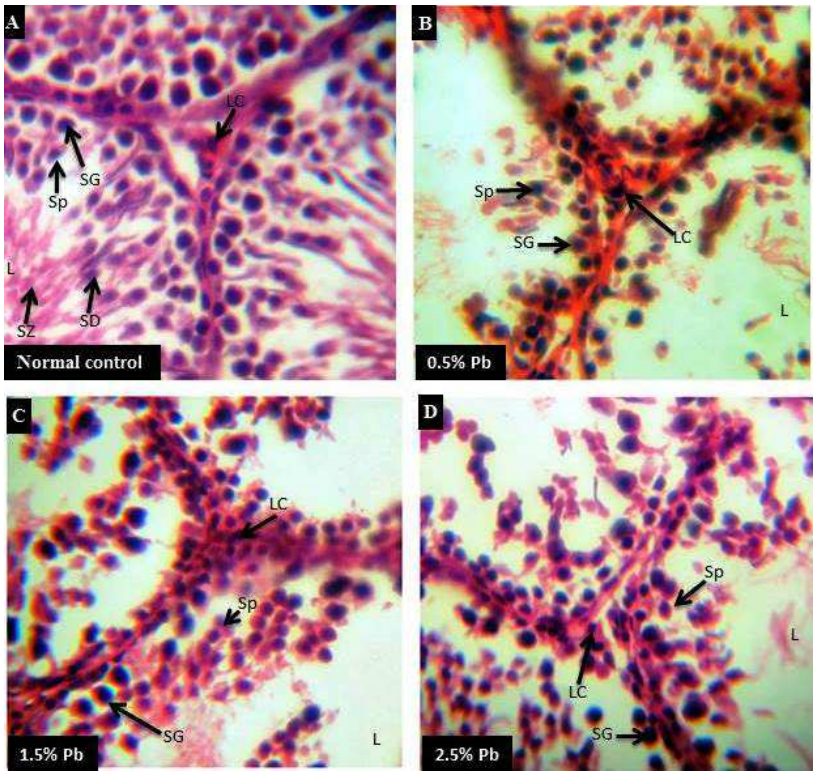

Fig. 3. Representative photomicrographs of the testes in the control rats **(A)**, and rats on 0.5% **(B)**, 1.5% **(C)**, and 2.5% **(D)** aqueous solutions Pb acetate. Massive erosion of germinal epithelium characterized by diminished population of spermatocytes (Sp), spermatids (SD), and spermatozoa (SZ) can be seen; but the spermatogonial stem cells (SG) and Leydig cells (LC) are largely preserved. The lumen (L) of the seminiferous tubule appears empty when compared with the control. Haematoxylin and eosin stain; x400.

Moreover, using spectra analysis, isothermal titration calorimetric studies and molecular docking investigations, Zhang et al. (2014) showed that Pb could bind directly to DNA with four binding sites to form Pb-DNA complex by minor groove binding effects and electrostatic forces, thereby causing damage to the DNA double helix structure. Thus, the germ cell loss that accounts for the decreased seminiferous tubule diameter and attenuated germinal epithelium in adult rats in our model could result from the genotoxic effect of Pb on germ cell DNA, leading to germ cell death.

Moreover, in addition to its ability to damage germ cell DNA by direct interaction, Pb could also elevate testicular free radical generation as well as weaken antioxidant defence system of the testis in adult Wistar rats treated with Pb alone or in combination with cadmium (Kumar et al., 2013). This and related studies (Marchlewicz et al., 2007; Ayinde et al., 2012) support a role for oxidative damage of the germ cells from Pb exposure, and may as well explain the thinning of the germinal epithelium and reduction in seminiferous tubule diameter in our study model. It is known that germ cells are especially susceptible to damage by oxidative stress, as has been reported in studies where certain antioxidants such as zinc chloride, alpha tocopherol and phytochemicals from the leaf extract of *Anacardium occidentale* (Linn.) have either offered protection against, or ameliorated the toxic effects of reactive oxygen species on testicular germ cells (Saalu et al., 2013; Ukwenya et al., 2013b).

Meanwhile, despite the marked erosion of the germinal epithelium produced by oral treatment with Pb acetate in our rodent model, the spermatogonial stem cells are mostly unaffected, and are to a large extent, preserved (Fig. 3). But most spermatocytes, spermatids and spermatozoa are lost. Such histological alterations are not only associated with Pb-induced testicular damage but have also been reported in diabetic Wistar rats induced with streptozotocin, an islet beta cell toxin; and in rats treated with gossypol, a testicular toxin in cottonseed oil (Ukwenya et al., 2013a; Akinola et al., 2006). The sparing of spermatogonial stem cells from the toxic effects of Pb suggests that these cells are available in relatively large numbers to facilitate the reconstruction of the germinal epithelium following withdrawal of the heavy metal insult. This is supported by the findings of Graca et al. (2004) in mice treated with 74 mg/kg $PbCl_2$, where partial recovery from testicular damage was reported after 32-day post-withdrawal of Pb treatment.

Moreover, data from our studies and those of others (Batra et al., 2001, 2004; Eduardo and Ricardo, 2008) further suggest that Pb is able to cross the blood-testis barrier to exert its deleterious effects on spermatocytes, spermatids and spermatozoa; and that the reversibility of such lesion is feasible after withdrawal of Pb treatment, owing to the relative abundance of testicular spermatogonial stem cells in Pb-treated rodents (Fig. 3).

Meanwhile, despite several bodies of evidence supporting the testicular toxicity and teratogenicity of Pb (Corpas and Antonio, 1998; Wadi and Ahmad, 1999; Marchlewicz et al., 2007; Wang et al., 2008; Ayinde et al., 2012; Wang et al., 2013a), a few findings do not support such evidence. For example, the work of Coffigny et al. (1994) in 90-day old offspring of pregnant Sprague-Dawley (SD) rats that inhaled lead oxide for 13 days during gestation showed that foetal exposure to lead oxide by maternal inhalation has no adverse effects on testicular morphology, testicular steroidogenesis or semen quality in post-

natal life.

Nonetheless, sperm analysis findings in the present study support the hypothesis that Pb intoxication initiated prior to pubescence could arrest spermatogenesis in young adulthood, as suggested by the significantly low caudal epididymal sperm density and motility in the Pb- exposed rats, as well as the increased percentages of deformed and dead sperm cells (Fig. 1). The elevated loss of sperm cells (indicated by a reduced life-death ratio and low sperm density) in our rodent model might be due to the genotoxic property of Pb on germ cell DNA (Wang et al., 2006), as well as to potential oxidative damage from Pb exposure (Marchlewicz et al., 2007; Kumar et al., 2013). The same factor could account for the higher percentages of malformed sperm cell population in Pb-treated rats. The significant reduction in sperm motility in the Pb - exposed rats could arise from the perturbation of cellular energy metabolism essential for cell motility (Naha and Manna, 2007); or to impaired Ca^{2+} homeostasis induced by exposure to lead acetate (Shirra and Barran, 2009).

Furthermore, it appears that the deleterious effects of juvenile Pb exposure on adult semen quality and testicular histomorphometric profile are disconnected from testicular testosterone levels. As shown in Fig. 2, testicular testosterone levels were not significantly different between the control and Pb-treated rats. Similarly, Pb treatment did not impair the architectonics of the testicular interstitial compartment. The intertubular interstitium and the associated Leydig cells are unperturbed by Pb treatment (Fig. 3). Our data thus suggests that juvenile Pb exposure does not result in the loss interstitial cells of Leydig; neither does it alter the levels of testicular testosterone in young adult Wistar rats. These findings support the hypothesis that testicular lesions associated with Pb toxicity may occur independent of testicular testosterone levels; and agree with data from human studies, which shows that Pb exposure does not perturb plasma testosterone concentrations despite diminished semen quality in exposed men (Ng et al., 1991; Naha and Manna, 2007).

However, a number of *in vitro* and animal studies shows that exposure to Pb does impair the biosynthetic function of the Leydig cells by means of reduced activity of the enzymes of testicular steroidogenesis, viz, 3β hydroxysteroid dehydrogenase and 17β hydroxysteroid dehydrogenase (3β HSD and 17β HSD), with the associated reductions in plasma and testicular testosterone levels (Liu et al., 2001; Liu et al., 2003; Priya and Reddy, 2012; Wang et al., 2013). Nonetheless, our findings in the present work do not support the latter.

To conclude, data from the present studies in Wistar rats shows impairment of testicular histomorphometry and poor semen quality that is unconnected with testicular testosterone levels in young adult Wistar rats on oral Pb intoxication initiated in the prepubescent stage of postnatal development. Thus, juvenile Pb intoxication portends male reproductive risk in early adulthood. Meanwhile, additional mechanistic studies are recommended. These may include elucidation of the expression profile of testicular divalent metal transporters and calcium-binding proteins under similar experimental paradigm. In addition, the structural and functional integrity of the sustentacular Sertoli cells may be studied, as impaired activity of these cells may contribute to some of our findings.

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