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Reproductive Toxicity of Lead Acetate in Adult male Rats: Histopathological and Cytotoxic Studies

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Abstract

The present study was conducted to assess the histopathological and cytotoxic effect of lead exposure on male reproductive function.

Adult male rats were divided in 2 groups. The first as a control group was given distilled water as drinking, while the second was exposed to 2 g/l of lead acetate in distilled water during 35 days. The structural damage on tests parenchyma was investigated by histological study and supplemented by biochemical assay of testosterone level. DNA fragmentation in germinal cells was determined using terminal desoxynucleotidyl transferase mediated dUTP nick end-labeling (TUNEL) assay.

The histological study showed that lead can induce pronounced alterations on germ cells. The testosterone level was significantly decreased in treated rats compared to controls. TUNEL assay revealed a significant DNA fragmentation index in rats exposed to lead.

This study showed TUNEL assay can be used to determinate DNA fragmentation in germinal cells. Moreover, we conclude that lead acetate may be considered as strong cytotoxic and genotoxic agent on male reproductive function.

Keywords: DNA damage; Histopathology; Lead acetate; Testis; Testosterone; TUNEL assay

Introduction

In recent years, many researchers have focused on the potentially toxic effects of various chemical and physical agents in our environment on human fertility [1]. Many authors have reported adverse effects of environmental pollutants on sexual function, such as tobacco [2], pesticides [3] and heavy metals like lead [4], cadmium [5], mercury [6] or nickel [7]. Among these pollutants, lead seems to be one of the most abundant and largely distributed metal (water pipes, industrial pollution...) in our environment. Its bioconcentration in the seminal plasma [8] has encouraged researchers to investigate its potential effects on the reproductive function. Many studies have shown that exposure of humans and animals to lead induced testicular atrophy, disruption of spermatogenesis and cellular degeneration [9]. In addition, some previous studies have shown that lead can cross the blood-testis barrier, accumulate in the testicles and epididymis and/or affect the germ cells at any stage of their differentiation (spermatogonia, spermatocytes I, spermatids and spermatozoa) [10]. Research on lead toxicity has mainly focused on human semen quality, endocrine function, and birth rate. They have shown that blood concentration of lead higher than 40 µg/dl seems to be associated with some morphological alterations and decrease in sperm count, volume and motility [9]. Vaziri and Sica [11] reported that lead induces the generation of reactive oxygen species (ROS) and lipid peroxidation which may lead to testicular damage.

It also appears that exposure to lead for a long time could induce not only cytotoxic but also genotoxic effects in various tissues. In fact, lead acetate has been shown to induce a significant increase in chromosomal aberrations (CA) in human cells [12] and in the frequency of sister chromatid exchanges in cultures of Chinese hamster ovary cells [13]. In addition, DNA damage was observed after treatment with lead in mice blood cells [14]. Recently, Ahmed et al. [15] reported this genotoxic effect in male rabbit and showed that it may contribute to fertility reduction.

Our aim was to determine the structural damage in the testicular parenchyma by histological study and biochemical assay of testosterone concentration levels, and to correlate these findings to DNA fragmentation in germ cells using terminal desoxynucleotidyl transferase mediated dUTP nick end-labeling (TUNEL) assay.

Materials and Methods

Experimental animals

Adult male Wistar rats (170-230 g), aged four months were used. The animals were randomly divided in control (8 rat) and treatment (8 rat) groups. They were housed at 21°C and 55% humidity. The cages were provided with ventilation and a regulating 12 hr light/12 hr dark cycle system. They were fed with balanced plugs granules obtained

from SICO, Sfax, Tunisia. The experiments were conducted in accordance with the guidelines for animal-s 'care of the "Faculty of Medicine of Monastir", Tunisia.

Chemicals

Lead acetate ($(CH_3COO)_2{}^*3H_2O$ Pb) was dissolved in distilled water. The kit used for the determination of testosterone (Immunate c.) was obtained from Beckman Coulter. France. The kit used for the evaluation of DNA fragmentation (ApopTagR Apoptosis Detection Kit) was obtained from QBiogene, Paris, France.

Experimental protocol

Control rats were given distilled water while treated rats were received distilled water rich in lead acetate (2 g/l) during 35 days (6 days/7).

Follow-up of body weight and gonads

All animals were weighed weekly, before being fed and during the whole 35 days experimental period whereas the testes were weighed immediately after sacrifice.

Taking blood samples and immuno-radio detection of testosterone

The sacrifice of animals took place in the morning by decapitation to avoid the stress effect. A 5 ml blood sample was taken from each rat. Heparinised tubes of blood were then centrifuged at 4000 rpm during 15 min and plasma is store at -20°C until assay. The plasma testosterone level was measured by radio-immuno-assay (RIA), at the physiology department of the Faculty of Medicine of Sousse using the commercial kit (Immunate c. Beckman Coulter France).

Histopathological study

After decapitation, each rat's testes were removed, weighed and then immediately fixed in 10% formalin. Then they were processed in a series of graded ethanol solutions and embedded in paraffin. Five micrometer-thick paraffin sections were obtained by using rotary microtome and stained by Hematoxylin-Eosin (HE) or Masson Trichrome (MT). The sections were observed and photographed under light microscope Leica DM750, provided with a camera Leica ICC50.

DNA fragmentation assessment: TUNEL assay

The analysis of DNA fragmentation rate in germinal cells was performed by the terminal uridine nick endlabelling (TUNEL) technique using a commercial kit (ApopTagR Apoptosis Detection Kit, QBiogene, Paris, France). All procedures were performed according to the manufacturer's instructions. All sections were stained with methyl green. Germ cells with fragmented DNA were stained with brown while normal ones were greenish.

The number of TUNEL-positive germ cells (fragmented) was counted in percentage of in 10 randomly selected areas in each slide at a magnification of 100X in order to estimate a DNA fragmentation index (DFI).

Statistical analysis

Data were analyzed using the Statistical Package for social Sciences (version 17; SPSS). In each assay experimental data represent the mean of eight independent assays ± standard deviation (SD). Duncan's test was used to difference between analytical parameters means (one way analysis of variance, ANOVA). Statistical significance was considered at $p \leq 0.05$.

Results

Body weight gain (%)

In this study no significant change ($p \geq 0.05$) of the reduction of body weight was observed in treated group in comparison with the control (Table 1).

Parameters	Controls	Treated	p
body weight	35.23 ± 10.54	33.5400 ± 6.7800	NS
absolute testes weight	2.71 ± 0.018	2.6100 ± 0.0270*	<0.05
Relative testes weight	1.021 ± 0.0018	1.0140 ± 0.0015*	<0.05
Values are given as Mean ± SD in each group * Differs significantly at p<0.05 NS: not significant			

Table 1: Mean values of body, absolute/ relative weights of studied rat testes

Absolute and relative testes weights

In comparison with controls, a significant decrease ($p \leq 0.05$) in the absolute and relative testes weights was observed (Table 1).

Plasma testosterone concentration

Our results revealed a significant decrease in plasma testosterone concentration in treated groups compared with controls (Figure 1).

Histopathological alterations in testis

As shown in the histological sections, the parenchyma tests of control rats (Figure 2A and 2B) showed a normal architecture composed of tight seminiferous tubules with narrow interstitial spaces containing Leydig cells.

In their epithelium (Figure 2C), we observed the different stages of spermatogenesis, arranged in a centripetal disposition; from the periphery to the centre of the seminiferous tubules we can see small spermatogonia, larger spermatocytes I; sometimes with large nuclei in mitosis, smaller spermatids and finally spermatozoa with their flagella that fulfil the hole centre of the tubes. In treated rats, testis histopathological examination revealed the majority of seminiferous tubules were less cellular with a large centre and a reduced diameter. The interstitial tissue surrounding them becomes less abundant. In fact, spermatozoa were absent in almost all of seminiferous tubules which are severely empty (Figure 2D).

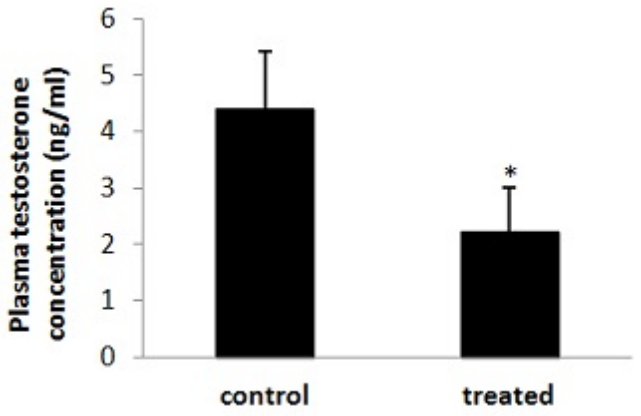

Figure 1: Measure of plasma testosterone concentration in rats treated in comparison with control group.*: Significantly different from control ($p \leq 0.05$).

In addition, it appears that lead induced a pronounced disruption of spermatogenesis. The germ epithelium became simple, apparently without Sertoli cells between the little germ cells in the destroyed walls (Figure 2E). Furthermore, there was a complete separation between the seminiferous tubules' wall and their basal lamina which became thick and irregular (Figure 2F and 2G). It was also found that the number of spermatogonia, spermatocytes I and especially spermatids decreased sharply and the leydig cells (Figure 2H). All these anomalies suggest that the cytotoxic effect of lead concerns particularly spermiogenesis more than the other stages of spermatogenesis. Added to all these alterations, hypertrophy and vacuolization of germ cells was noted (Figure 2I).

Results of TUNEL assay

However, as shown in Figure 3, exposure to lead resulted in a more significant fragmentation of germ cell DNA including spermatogonia, spermatocytes I and especially spermatids.

The results revealed that the testes of the treated group showed 35.12 ± 5.08% fragmented cells, whereas in the control group we found only 8.81 ± 2.57% fragmented cells. Our results showed a significantly increased of DNA fragmentation index (DFI) in the treated group compared with the control (Figure 4).

Discussion

Some heavy metals, such as lead, cadmium, arsenic and mercury can affect the male reproductive function, including sperm count [16], mobility, morphology [17] and spermatogenesis [18]. In the present study, the results clearly demonstrated that exposure of male rats to lead acetate may seriously affect the testicles. Indeed, we observed a decrease in the weight gain of treated rats but it was not statistically significant in our study. After using the same metal but with lower doses (100, 1000 ppm) for 28 days, Suradkar et al. [19] have found the same results. Minnema and Hammond [20] showed about 20% of weight loss in rats treated with a dose of 17.5 mg /l for only 10 days.

In addition, a significant decrease in absolute and relative testes weight was observed in lead treated rats. This decrease in testicular weight may be explained by a decrease in the number of Leydig cells, germ cells and inhibition of spermatogenesis. All this may be due to a deficiency in testosterone which is considered to be essential for the structural and functional integrity of reproductive organs [21]. The current results were concordant with studies conducted by Wang et al. [22] and Smith et al. [23]. Hamadouche et al. [24] also reported that a dose of 500 mg/l of lead acetate significantly reduced the weight of the testes and epididymis. They suggested that lead acetate affects hypothlamo-pituitary axis which may lead to a decrease in testosterone production and a spermatogenesis disruption.

We also observed a statistically significant decrease in plasma testosterone concentration of treated rats compared to controls. Similar results were found by Thoreux et al. [25]. They showed that the treatment of rats for 5 weeks with the dose of 8 mg/kg of body weight leads to an inhibition in the expression of enzymes involved in hormonal biosynthesis of steroids which include testosterone and luteinizing hormone (LH). This reduction in the testosterone concentration could be explained by different physiological mechanisms. First, the decrease in the number of LH binding sites in Leydig cells which were confirmed by Kempinas et al. [26]. Second, the reduction in plasma prolactin concentration as shown by Morris and Saxena [27] because prolactin plays a direct role in LH binding sites regulation in rodent's testes. All this could eventually have many consequences on the reproduction function as spermatogenesis and Leydig cells appear to be targets of lead.

The histopathological study of testes exposed to lead showed not only an atrophy of the seminiferous tubules but also an alteration of spermatogenesis. These results are consistent with those reported by various authors including Ghorbel et al. [4] who treated male rats with 3 and 6 mg/ml of lead acetate for 15, 30, 60 and 90 days. The same histological changes were also reported by Batra et al. [28]; El-Sayed and El-Neweshy [29] following chronic exposure of male rats to lead acetate. In addition, the fact that mainly spermatids were affected compared to other germ cells and the absences of sperm in the majority of seminiferous tubules were stated by several authors [19,30]. Indeed, the reduction of sperm observed in treated rats is mainly caused by an interruption in the different stages of spermatogenesis. In fact, normally the various stages of spermatogenesis form a smooth continuous chain and the decrease in spermatids number as they seem to be the most targeted cells leads to the disruption of spermatogenesis and the loss of spermatozoa in the seminiferous tubules. On the other hand, defects in the basal membrane of the seminiferous tubules could be the consequence of their atrophy by cellular degeneration or after the contraction of their myoid cells. Such changes can be attributed to a protein interaction mechanism. Similar observations were reported by Batra et al. [28]; Ghorbel et al. [4]; Makhlouf et al. [31] and El Shafai et al. [32]. It has also been demonstrated that lead acetate administration resulted in a decrease in the activity of some enzymes in the testes such as alkaline phosphatase, which may cause a structural damage to the membrane of the seminiferous tubules [33]. Besides, the thickening observed in this basal membrane could be the result of either an increase in collagen synthesis or a decrease in collagen phagocytosis by fibroblasts [34,35], since lead toxicity could also cause disruption of collagen binding to phagocytic fibroblasts [36].The presence of vacuoles in the germinal cells cytoplasm of the study rats was also observed. This vacuolization was reported by Ghorbel et al. [4]; El Shafai et al. [32]; Garu et al. [37] and Ahmed et al. [15]. They detected those vacuoles in Sertoli and spermatocytes I cells. According to Murthy et al. [38], this observation can be explained by an expansion in the smooth

endoplasmic reticulum (SER). In addition, we have noticed empty spaces between the germ line cells, which most likely correspond to the Sertoli cells loss. In accordance with our results, Adhikari et al. [39] observed that, 4-40 μM of lead caused a detachment of Sertoli and germ cells in vitro. They believe that this may be the cause of spermatogenesis disturbances observed in vivo. Actually, Sertoli cells have a dual role: firstly, they protect the germ cells from toxic blood circulating substances [40] due to their action as a blood-testis barrier. Secondly, in response to FSH and testosterone, they secrete various proteins which control sperm differentiation. Thus, the cytotoxicity of lead on Sertoli cells may therefore explain, at least in part, the observed spermatogenesis disruption.

In this study, the current results showed that the DNA fragmentation index (DFI) detected by the TUNEL technique in germ cells of treated rats was significantly higher compared to controls. Similar results were demonstrated by Shuying et al. [41] who treated the rats with three different doses of lead acetate. It is important to notice that DNA fragmentation in germinal cells is a physiological phenomenon that can occur during spermatogenesis. However, lead exposure has resulted in an increase in this nuclear DNA fragmentation in germ cells which could be explained by an excessive generation of reactive oxygen species (ROS). In fact, Vaziri and Sica [11] showed that lead exposure promotes the production of intracellular ROS that can induce morphological and genetic testicular damage. In general, ROS are reported to damage the polyunsaturated fatty acids of phospholipids in cells' membranes, causing of cellular functions impairment [42,43] and are also involved in gene mutations [44].

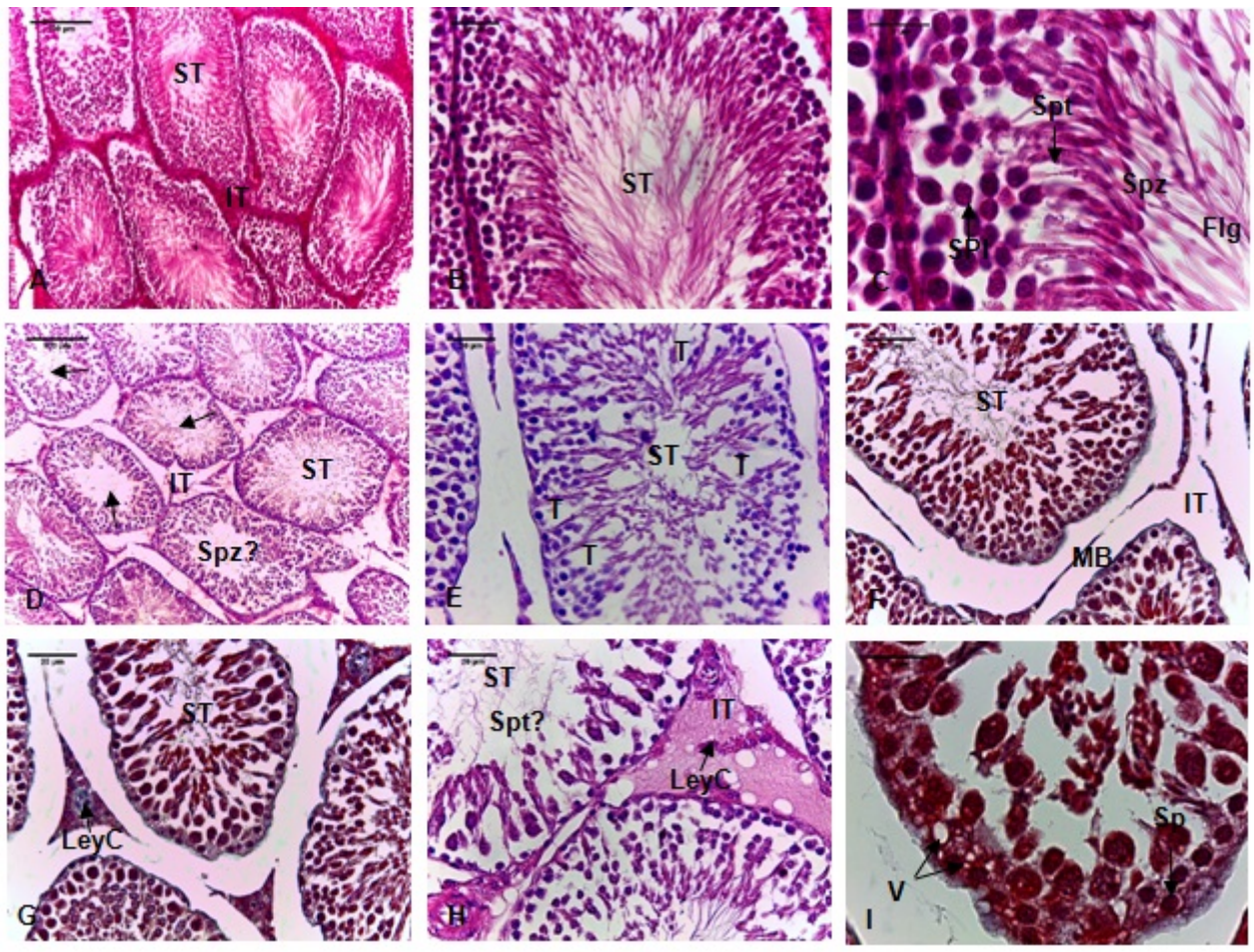

Figure 2: Photomicrographs of rats testicular control and treated. The control (A, 100x; B, 400x and C, 1000x; H&E) reveal interstitial tissues (IT) and seminiferous tubules (ST) with normal germ cells: spermatogonia (Sp), spermatocytes I (SPI), spermatids (Spt), spermatozoa (Spz) with flagellum (Flg). The rats treated showing change in the testicular parenchyma, abundant interstitial tissues (IT) and absence of sperm in lumen of tubules (Spz?) (D, H&E 100x), empty spaces after the disappearance of sertoli cells (T) (E, H&E 400x), basal lamina is separate from the tubules and is irregular (F and G, Trichrome 400x), germ cells in seminiferous tubules and leydig cells (LeyC) decreased sharply (H, H&E 400x), vacuolization of germ cells (I, Trichrome 1000x). ST: seminiferous tubules; Sp: spermatogonia; SPI: spermatocytes I; Spt: spermatids; Spz: spermatozoa; IT: interstitial tissue; LeyC: Leydig cell; Flg: flagellum; V: vacuole; T: disappearance of sertoli cells. (A, D: Scale bar 100 μm; B, E, F, G, H: Scale bar 20 μm; I: Scale bar 10 μm)

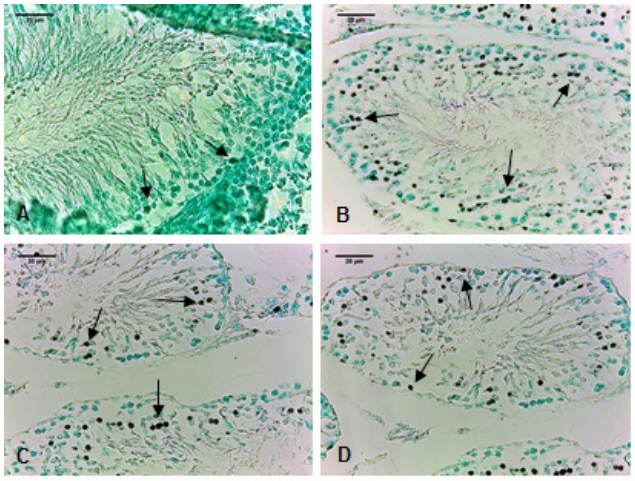

Figure 3: Photomicrographs of TUNEL-stained testicular sections from controls (A) and rats exposed to 2 g/l of lead acetate (B, C, and D). Magnification 400x. Arrows indicated TUNEL-positive cells (spermatogonia; permatocytes I; spermatids) (A, B, C, D: Scale bar 20 µm)

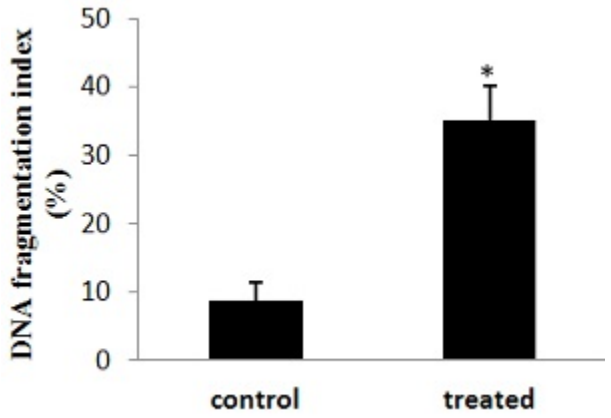

Figure 4: Variation in the DNA fragmentation index (DFI) in control and treated groups. *Significantly different from control ($p \leq 0.05$)

In addition, it was shown that the testes are more vulnerable to oxidative stress because of their high content of polyunsaturated fatty acids. Numerous studies have shown that the testes physiology, essentially characterized by the process of spermatogenesis, is disrupted at least in part because of ROS dependent mechanisms [45]. On the other hand, ROS are known to cause chromosomal aberrations and gene mutations, resulting in abnormal sperm and/or significantly reduced sperm count [46-50]. So, lead-induced ROS can cause some genic alterations in germ cells which lead to an abnormal sperm production [51]. It is known that sperm is extremely sensitive to oxidative damage because of its low antioxidant capacity [52]. However, oxidative stress is just one of the many mechanisms which are at the origin of DNA fragmentation, such as germ cell apoptosis during spermatogenesis [53-55], or defect in chromatin remodelling and compaction during the spermiogenesis process [56]. Adhikari et al. [57] and El Shafai et al. [32] reported that this damage to the DNA integrity of germ line after lead exposure could lead to a state of cellular apoptosis. In normal conditions, apoptosis is a physiological process that helps to maintain a well-defined number of cells in the testicular tissue and eliminating damaged cells. However, excessive apoptosis may cause an alteration of the male reproductive function. Furthermore, we have noticed that the germinal DNA fragmentation detected was more pronounced with spermatids, which confirmed the fact that lead affects more particularly spermiogenesis. This could be explained by a molecular mechanism as lead may interfere with the DNA of germ cells and prevent the normal progress of nuclear condensation which is a crucial step in the spermiogenesis process.

Conclusion

In conclusion, this study showed that TUNEL assay can be used to determinate DNA fragmentation index in germ cells of rat testes. Moreover, these cytogenetic findings were correlated with microscopic study of histological aspect, showing that lead acetate may be considered as a strong cytotoxic and genotoxic agent. However, further chronic studies are needed to explore mechanisms by which lead acetate led to these toxic effects at the molecular levels.

Acknowledgments

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