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Lead Induced Oxidative Stress and Affected the Expression of Steroidogenesis -related Genes in Testis of Male Mice

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Abstract

Background: Lead (Pb) is classified as a major risk factor affecting the male reproductive system; however, its precise mechanisms of action are poorly understood and inconsistent. This work aimed to investigate the effect of Pb toxicity on male reproductive function.

Methods: Accordingly, in this study, adult male mice treated with Lead acetate (PbAc) by gavage (200 mg/kg/day) for 28 days. We analyzed sperm count and morphology, oxidative stress, and the expression of antioxidant and steroidogenesis -related genes in the testes of male mice.

Results: Pb significantly ($P<0.05$) showed decreased body weight and sperm count, as well as significantly ($P<0.05$) increased the number of abnormal sperms and plasma testosterone level. The activities of superoxide dismutase (SOD), glutathione peroxidase (GSH-Px) and catalase (Cat) were significantly decreased, whereas the level of malondialdehyde (MDA) was significantly increased in the testis of mice treated by Pb. The mRNA levels of antioxidant-related genes (SOD1, GPX1, and CAT) were significantly decreased following Pb treatment. Furthermore, the expression of genes involved in the steroidogenic pathway, including steroidogenic acute regulatory protein (Star), cytochromeP-450scc (Cyp11a1), 17β-Hydroxysteroid dehydrogenase (17β-HSD), 3β-Hydroxysteroid dehydrogenase (3β-HSD) and cytochrome P450, family 17 (Cyp17), were significantly ($P<0.05$) decreased after exposure to Pb.

Conclusion: In conclusion, Pb disrupts male reproductive function by inducing oxidative stress, negatively regulating the mRNA expressions of steroidogenesis and antioxidant -related genes, and ultimately reducing sperm quality and quantity.

Keywords: Lead; testis; spermatogenesis, oxidative stress; steroidogenesis.

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Introduction

The environmental and occupational exposure to lead (Pb) has been well-documented and is considered as major public health issues.[1] The male reproductive system has been recognized as a primary target of Pb-induced toxicity.[2,3] previous studies have suggested that Pb can accumulate in the testis and alter its function by causing oxidative stress through excessive generation of reactive oxygen species (ROS) and disruption of the activities of antioxidant enzymes such as Catalase (Cat), Superoxide dismutase (SOD), and Glutathione peroxidase (GSH-Px).[4] Oxidative stress results in apoptosis of spermatozoa, DNA damage, and the disruption of spermatogenesis events that lead to changes in sperm quality and function.[5-8] However, the precise mechanisms underlying Pb toxicity to male reproductive function and spermatogenesis are unclear.

Pb is reported to affect the hypothalamic-pituitary testis axis,[9,10] and alter the levels of luteinizing hormone (LH), follicle stimulating hormone (FSH) and testosterone (T). The testis is responsible for the biosynthesis of steroid hormones in males and it requires continuous androgenic stimulation for normal growth and functions.[11] Testosterone is gonadal steroids produced by the Leydig cells and acts to maintain spermatogenic processes.[11,12] The biosynthesis of testosterone is initiated by the transport of cholesterol from the outer to inner mitochondrial membrane; this step is mediated by steroidogenic acute regulatory (Star) protein.[13] Cyp11a1 mediates the cleavage of the side chain of cholesterol to form pregnenolone. In the endoplasmic reticulum, pregnenolone is converted to progesterone by 3β-HSD. Finally, progesterone is metabolized into testosterone by Cyp17 and 17β-HSD (14). Pb can modify hormonal metabolism by altering the synthesis and breakdown of testosterone, FSH and LH.[9] In albino rats, Pb affects testis steroidogenic activity, as well as plasma testosterone and gonadotropin levels.[15] Nevertheless, data concerning the effect of Pb on testosterone production and steroidogenesis are scarce and inconstant.

Therefore, this study aimed to investigate the effect of Pb toxicity on male reproductive function. We analyzed sperm count and morphology, oxidative stress, and the expression of antioxidant, steroidogenesis and apoptosis-related genes in the testes of male mice.

Materials and methods

Animals and treatment

In this experiment, twenty adult male Kunming mice (8weeks old) weighing 25 to 30g were purchased from Nanjing Qinglongshan Experimental Animal Factory (Nanjing, China), housed in the animal room, and provided with a standard diet and water *ad libitum*. Animals were maintained with a 12 to 12 light-dark cycle, 55± 5% humidity, good ventilation, and a temperature of 25 ± 2˚C. Mice were acclimatized for seven days before treatment. After acclimatization, mice were randomly divided into two groups and housed five per cage (10 mice per group). The control group was received 0.3 mL of water only, whereas the treatment group received the same volume of distilled water containing lead acetate (PbAc) obtained from Ding Si, Nanjing, China by gavage at a dose of 200 mg/kg/bw for 28 days. All mice were killed by euthanasia with ether, followed by cervical dislocation. Blood samples were collected and centrifuged at 3000 rpm at 4 °C for 15 min to separate the plasma from the blood cells and stored at -20°C for later use. Testes were surgically obtained and frozen immediately in liquid nitrogen and stored at -80˚C for further analysis.

Sperm morphology and count

Sperm analysis was performed at the Quality Supervision & Test Center of Cattle Frozen Sperm (Nanjing Agricultural University, Nanjing, China). Sperm morphology was assessed. After staining with Giemsa, a total of 600 spermatozoa were counted in random fields, and the percentage of abnormal sperm was calculated. Classification of sperm morphology was based on the criteria of abnormality described previously Wyrobek and Bruce.[16] For sperm count, the number of sperm in five squares in the hemocytometer was counted as described previously.[17]

Determination of plasma testosterone

Plasma testosterone levels were measured using a mouse testosterone ELISA kit (Jiancheng, Nanjing, China), following the manufacturer's instructions.

Testicular antioxidant enzyme activity and MDA level

Testis samples (50 mg) from each mouse were homogenized in 10 volumes of precooled physiological saline; the homogenate was centrifuged at 3000 rpm for 15 minutes and the supernatant was used for

biochemical assays. The activities of superoxide dismutase (SOD), glutathione peroxidase (GSH-Px), catalase (Cat) and the content of malondialdehyde (MDA) in testis were detected using commercial Assay Kits (Jiancheng, Nanjing, China).

RNA extraction and real-time quantitative reverse transcription polymerase chain (qRT-PCR) analysis.

Total RNA from each sample was extracted from 50 mg testis tissue using TRIzol® reagent (Invitrogen, USA) according to the manufacturer's instruction. Purity and concentration of total RNA were determined by obtaining the absorbance values at the wavelength of 260/280 nm using NanoDrop™ (Thermo Fisher Scientific, Waltham, MA, USA) and the ratio between the absorbance values at 260 nm and at 280. Total RNA was reverse transcribed into cDNA using Prime Script™ RT Master Mix (TaKaRa, Tokyo, Japan). The reaction mix for qRT-PCR consisted of 2 µL of diluted cDNA, 0.3 µL of forward and reverses primers, 5 µL of SYBR green PCR master mix (Roche, Switzerland), and 2.4 µL of PCR grade water. qRT-PCR performed on Applied Biosystems 7500 HT Sequence (Thermo Fisher Scientific, USA). Mice-specific primers used were synthesized by Genscript® (Nanjing, China) and are listed in Table 1. The relative levels of gene expression were determined by the $2^{-\Delta\Delta Ct}$ method.[18] GAPDH was used as housekeeping gene.

Statistical analysis

All data were expressed as means ± SEM. The differences between groups were analyzed using independent samples t-test with SPSS Statistical Package for Social Sciences software for Windows, version 20.0 (SPSS; Chicago, Illinois, USA). P ≤ 0.05 was considered statistically significance.

Results and Discussion

Effect of Pb on sperm count and morphology

Pb-treated mice showed a significant reduction ($P < 0.05$) in sperm count as well as an increase in the percentage of sperm with abnormal morphology compared to the control group (Figure 1A, B). Several studies revealed defective spermatogenesis and altered production and maturation of sperm in the testis after Pb accumulation.[2,3] Similarly, significant reductions in both the quantitative and qualitative characteristics of spermatozoa in the testes of albino rats exposed to PbAc have also been reported.[19] Furthermore, an in vitro study suggested that Pb might inhibit testis spermatogenesis by disturbing the metabolic activities of the Sertoli cells.[20]

Effect of Pb on the activity of antioxidant enzymes, MDA level, and the expression of antioxidant-related genes of testis tissues

The activities of SOD, GSH-Px and Cat in the testis of mice were significantly ($P < 0.05$) lower in the treatment group compared to those in the control. Furthermore, MDA levels were significantly ($P < 0.05$) higher in the treatment group than in the control (Table 2). These results were further confirmed by the decreased mRNA expression levels of SOD1, GPX1 and CAT after Pb exposure (Figure 2).

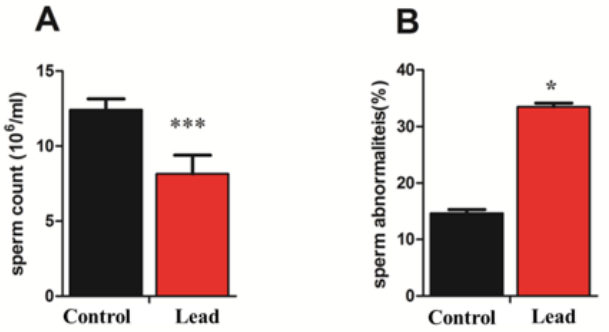

Figure 1 Sperm parameters. A) Sperm count. B) Sperm morphology. Data are represented as mean ± SEM, * P < 0.05, ** P < 0.01, *** P < 0.001 indicate significant difference from the control group.

Table 1: Primers sequences used for qRT-PCR analysis

Genes	Accession number	Primer sequence (5 to 3)	PCR products (bp)
Star	NM_011485.5	F:TCGCTACGTTCAAGCTGTGT R:ACGTCGAACTTGACCCATCC	152
Cyp11a1	NM_019779.4	F:CTAAAGGACTTTCCCTGCGCT R:CCCTCCAGAAGTGGTACAGG	186

Contd... Table 1: Primers sequences used for qRT-PCR analysis			
17β-HSD	NM_008291.3	F: AGACCGCCGATGAGTTTGTT R: TCAGGAGGAATCGTTGAGCG	153
3β-HSD	NM_001304800.1	F: GGCCTGTGTTCAAGCAAGTG R: TCTGTTCCTCGTGGCCATTC	107
Cyp17	NM_007809.3	F:TGGAGGCCACTATCCGAGAA R: CACATGTGTGTCCTTCGGGA	119
SOD1	>NM_011434.2	F: GGAACCATCCACTTCGAGCA R: CGTCCTTTCCAGCAGTCACA	232
GPX1	>NM_008160.6	F: CACAGTCCACCGTGTATGCC R: CTTGCCATTCTCCTGGTGTCC	230
CAT	>NM_009804.2	F: CACTGACGAGATGGCACACT R: TGTGGAGAATCGAACGGCAA	175
GAPDH	XM_001476707.5	F:AGAAACCTGCCAAGTATGATGAC R:CCTGTTGCTGTAGCCGTATTC	221

Table 2: Effects of Pb on superoxide dismutase (SOD), glutathione peroxidase (GSH-Px), Catalase (CAT) and malondialdehyde (MDA) in testis of mice

Groups	SOD (U/mg prot)	GSH-Px (U/mg prot)	Cat (U/mg prot)	MDA (nmol/mg prot)
control	58.22 ± 2.85	0.22± 0.01	0.042±.001	0.74± 0.10
lead	38.56 ± 2.00***	0.14± 0.01**	0.018± 0.00*	1.12± 0.08*

Data are represented as mean ± SEM. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ indicate significance from the control group

Oxidative damage due to Pb administration was previously reported by Rao and coworkers.[21] Pb could inhibit the activities of antioxidant enzyme by binding to the sulfhydryl groups of catalase and SOD with higher affinity.[22] It is well-established that Pb induces cellular damage through generation of ROS and reduction of antioxidant enzymes, which might be responsible for disruption of testicular functions and spermatogenesis.[23]

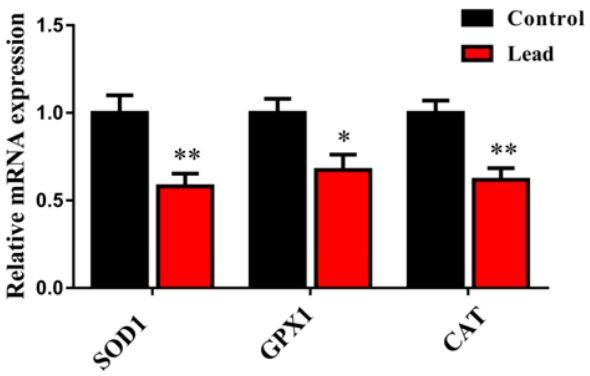

Figure 2: Effect of Pb treatment on mRNA expression of SOD1, GPX1, and CAT in the testis tissues of male mice. Data are represented as mean ± SEM, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ indicate significant difference from the control group.

Effects of Pb on plasma testosterone levels and expression of steroidogenesis-related genes in testis tissues

In this study, a significant increase ($P < 0.05$) in the plasma testosterone levels of mice in the treatment group compared to those in the control group was observed (Figure 3A). qRT-PCR analysis revealed that the expression levels of Star, Cyp11a1, 17β-HSD, and Cyp17 in the treatment group were significantly ($P < 0.05$) lower compared to those in the control group. However, no significant difference in the mRNA levels of 3β-HSD between groups was observed (Figure 3B–D, F). Despite the reduction in the expression of genes responsible for testosterone biosynthesis, the plasma levels of testosterone were

increased by treatment with Pb. Moreover, in the treatment group, we detected lower mRNA level of Star, Cyp11a1, Cyp17 and 17-β HSD, which are genes that play a significant role in steroid hormone synthesis in the testis. Testosterone is synthesized by Leydig cells under the stimulation of LH, which binds to the androgen receptors (AR) found in Sertoli cells to initiate the functional responses required to support spermatogenesis.[11,24] It appears that Pb may accumulate in the testis and disrupt its function either through the abnormal metabolism of testosterone or through an abnormal interaction between androgens and their receptors in Sertoli cells.[25]

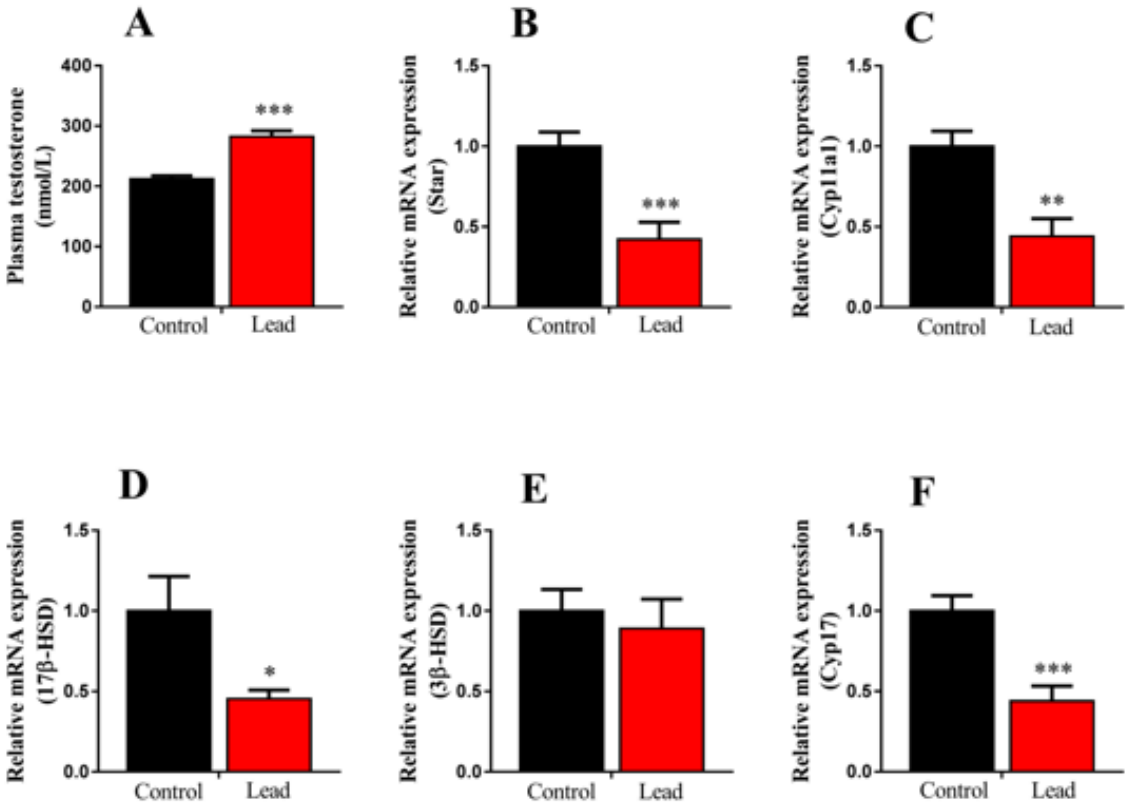

Figure 3: Effect of Pb treatment on plasma testosterone level and mRNA expression of Star, Cyp11a1, 17β-HSD, Cyp17, and 3β-HSD in the testis tissues of male mice. Data are represented as mean ± SEM, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ indicate significant difference from the control group

The stimulatory events include the enzymatic conversion of substrates to testosterone [(26)]. Inhibition of testis steroidogenesis has previously been reported [(3)], wherein the researchers described the reduced enzymatic activity of 3β-HSD and 17β-HSD in the testis of Pb-exposed rats. Furthermore, several studies reported the accumulation of plasma testosterone in rats that consumed Pb.[27] Conversely, other studies reported decreased plasma testosterone level after lead exposure in rats.[2,23] However, the increase in plasma testosterone levels despite the reduced expression of steroidogenic enzymes requires further investigation. We hypothesized that Pb induced the aberrant metabolism of testosterone and caused its accumulation in the plasma, followed by a feedback control of testis steroidogenesis. The oxidative stress induces after Pb administration might affect the expression of key enzymes involved in testicular steroidogenesis. Our result is line with Anjum who reported reduced the enzyme activity of 3β-HSD and 17β-HSD in the testis of Pb-exposed rats.[3,23]

Conclusion

Taken together, our findings indicate that Pb administration reduces sperm quality by increasing oxidative stress and inducing apoptosis-related events in the testis, which is associated with reduction in the expression of testicular steroidogenesis genes.

Ethics statement

The experiments were approved by The Ethics Committee of Nanjing Agricultural University (Nanjing, China) according to the guidelines of the Care and Use of Laboratory Animals prepared by the Institutional Animal Care.

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Conflict of Interest: The authors declare that they have no Conflict of Interest.

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