Research Article

Journal of Applied Toxicology

Received: 21 April 2012, Revised: 6 June 2012, Accepted: 6 June 2012 Published online in Wiley Online Library: 13 July 2012

(wileyonlinelibrary.com) DOI 10.1002/jat.2795

Maternal lead exposure during lactation persistently impairs testicular development and steroidogenesis in male offspring

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ABSTRACT: Lead (Pb) is a testicular toxicant. In the present study, we investigated the effects of maternal Pb exposure during lactation on testicular development and steroidogenesis in male offspring. Maternal mice were exposed to different concentration of lead acetate (200 or 2000 ppm) through drinking water from postnatal day (PND) 0 to PND21. As expected, a high concentration of Pb was measured in the kidneys and liver of pups whose mothers were exposed to Pb during lactation. In addition, maternal Pb exposure during lactation elevated, to a less extent, Pb content in testes of weaning pups. Testis weight in weaning pups was significantly decreased when maternal mice were exposed to Pb during lactation. The level of serum and testicular T was reduced in Pb-exposed pups. The expression of P450scc, $P450_{17\alpha}$ and 17β-HSD, key enzymes for T synthesis, was down-regulated in testes of weaning pups whose mothers were exposed to Pb during lactation. Interestingly, the level of serum and testicular T remained decreased in adult offspring whose mothers were exposed to Pb during lactation. Importantly, the number of spermatozoa was significantly reduced in Pb-exposed male offspring. Taken together, these results suggest that Pb could be transported from dams to pups through milk. Maternal Pb exposure during lactation persistently disrupts testicular development and steroidogenesis in male offspring. Copyright © 2012 John Wiley & Sons, Ltd.

Keywords: lead; steroidogenesis; testis; endocrine disruptor; testosterone

Introduction

Lead (Pb) is a male reproductive toxicant in humans. Numerous reports have demonstrated that occupational exposure to Pb is associated with poor semen quality and increases in frequencies of spontaneous abortion and male infertility (Gennart *et al*., 1992; Kuo *et al*., 1997; Lancranjan *et al*., 1975; Lerda, 1992). Even a long-term blood Pb concentration below the currently accepted occupational health level (60 $\mu g\,dl^{-1}$) could adversely affect the sperm density and total sperm number (Alexander *et al*., 1996). According to a recent study, there was a significant reduction in sperm density and sperm number per ejaculum when the Pb level in seminal plasma was above 10 $\mu g\,l^{-1}$ (Xu *et al*., 2003). From Pearson's correlation analysis, positive correlations were demonstrated between Pb concentration in seminal plasma and sperm reactive oxygen species (ROS) level (Kiziler *et al*., 2007). In addition, a significant positive correlation was also observed between Pb in seminal plasma and the 8-hydroxy-2′-deoxyguanosine (8-OHdG) level (Xu *et al*., 2003). Pb is a testicular toxicant in experimental animals (Wang *et al*., 2008). The effects of Pb on spermatogenesis have been demonstrated in rat testes. An earlier study showed that nuclei and acrosomes in round spermatids were swelled and nuclei in Sertoli framentated in Pb-intoxicated rats, which may lead to disruption of spermatogenesis (Murthy *et al*., 1995). Another report demonstrated that Pb induced germ cell apoptosis in testes (Adhikari *et al*., 2001). According to a recent study, sperm nucleus took up Pb during the epididymal maturation and altered sperm chromatin condensation, which might finally interfere with the process of chromatin decondensation after fertilization (Hernandez-Ochoa *et al*., 2006).

In contrast, infants are susceptible to Pb-induced toxicity. According to several recent reports, maternal Pb exposure during pregnancy and lactation persistently impaired brain development and neurobehavioral alterations in adult offspring (Antonio *et al*., 2002; Devoto *et al*., 2001; Moreira *et al*., 2001; Reddy *et al*., 2007). Other previous studies showed that maternal Pb exposure during lactation delayed the timing of puberty in female offspring (Dearth *et al*., 2002, 2004). Indeed, Pb could be transported from dams to pups through milk (Hallen and Oskarsson, 1993). However, little is known about the effects of neonatal Pb exposure through milk on male reproduction.

In the present study, we investigated the effects of maternal Pb exposure during lactation on testicular development and spermatogenesis in male offspring. Our results found that maternal lead exposure during lactation persistently impaired testicular development and spermatogenesis in male offspring. Testosterone (T) is essential for normal testicular development and spermatogenesis. Steroidogenic acute regulatory (StAR) protein is a limiting factor in T biosynthesis, responsible for the transport of cholesterol into mitochondria (Miller, 2007). T synthetic enzymes including cytochrome P450 cholesterol side-chain cleavage enzyme

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(P450scc), cytochrome P450 17α-hydroxysteroid dehydrogenase (P45017α) and 17β-hydroxysteroid dehydrogenase (17β-HSD) play a critical role in the synthesis of T in Leydig cells (Payne and Youngblood, 1995). Thus, the present study investigated the effects of maternal Pb exposure during lactation on T production and the expression of StAR and T synthetic enzymes in the testes of male offspring. We found that maternal Pb exposure during lactation persistently disrupts steroidogenesis in male offspring through inhibiting the expression of T synthetic enzymes in testes.

Materials and Methods

Chemicals and Reagents

Lead acetate was purchased from Sigma Chemical Co. (St. Louis, MO, USA). StAR (Cat. No.: sc-25806), P45017α (Cat. No.: sc-66850), 17β-HSD (Cat. No.: sc-32872) and 3β-hydroxysteroid dehydrogenase (3β-HSD, Cat. No.: sc-30820) antibodies were purchased from Santa Cruz Biotechnologies (Santa Cruz, CA, USA). P450scc antibody was obtained from Chemicon International (Temecula, CA, USA; Cat. No.: AB1244). β-Actin antibody was from Boster Bio-Technology Co. Ltd (Wuhan, China). All other reagents were from Sigma or as indicated in the specified methods.

Animals and Treatments

The CD-1 mice (8- to 10-week-old; male mice: 28–30 g; female mice: 24–26 g) were purchased from Beijing Vital River whose foundation colonies were all introduced from Charles River Laboratories, Inc (Wilmington, MA, USA). The animals were allowed free access to food and water at all times and were maintained on a 12-h light/dark cycle in a controlled temperature (20–25 °C) and humidity (50 ± 5%) environment for a period of 1 week before use. For mating purposes, four females were housed overnight with two males starting at 09.00 hours. Females were checked by 07.00 hours the next morning, and the presence of a vaginal plug was designated as gestational day 0. Within 24 h after birth, excess pups were removed, so that 10 pups were kept per dam (five males and five females). Dams were divided into three groups. In Pb-treated groups, maternal mice drank different concentrations (200 ppm or 2000 ppm) of lead acetate dissolved in deionized water from postnatal day 0 (PND0) to weaning (PND21). The doses of lead acetate used in present study are according to Carmouche *et al*. (2005) and Deng and Poretz (2001). In the control group, maternal mice drank deionized water. At weaning (PND22), 12 male pups from six litters in each group were slaughtered. Sera were collected for measurement of T. The testes were excised, dissected, weighted and then divided in two parts: the left one was kept at −80 °C for subsequent measurement of T and immunoblotting. The other one was immersed in modified Davidson's fluid (mDF) for 12 h for histology and immunohistochemistry (Latendresse *et al*., 2002). At PND70, 12 male pups from 6 litters in each group were slaughtered. Serum was collected for the measurement of T. The epididymides were removed for analysis of sperm quality. The testes were excised, dissected, weighted and then divided in two parts: the left one was kept at −80 °C for subsequent measurement of T, RT-PCR and immunoblotting. The other part of the testes was immersed in mDF for 12 to 24 h for histology and immunohistochemistry. All the procedures performed on animals followed the guidelines for humane treatment set by the Association of Laboratory Animal Sciences and the Center for Laboratory Animal Sciences at Anhui Medical University.

Sperm Analysis

The epididymides were removed and immediately immersed into the F12 medium supplemented with 0.1% bovine serum albumin (BSA). Spermatozoa were released by mincing the epididymides in the collection medium. The sperm suspensions were incubated at 37 °C. After incubation, the sperm suspension was analyzed for the number of spermatozoa according to the WHO laboratory manual (WHO 2001).

Histology and Immunohistochemistry

Two cross sections from each testis were embedded in paraffin. Paraffin-embedded tissues were serially sectioned. At least two non-serial sections were stained with hematoxylin and eosin (H&E). Leydig cells were identified by staining for 3β-HSD. Sections of 5 μm were mounted onto coated slides, dewaxed and rehydrated. Antigen retrieval was performed by pressure cooking slides for 5 min in 0.01 M citrate buffer (pH 6.0). Slides were incubated for 30 min in 3% (vol/vol) hydrogen peroxide in methanol to block endogenous peroxidase activity and then washed in Tris-buffered saline [TBS; 0.05 M Tris, 0.85% (wt/vol) NaCl (pH 7.4)]. Non-specific binding sites were blocked with an appropriate normal serum diluted 1:5 in TBS containing 5% (wt/vol) BSA before the addition of goat polyclonal antibody against 3β-HSD (Santa Cruz Biotechnologies; Cat. No.: sc-30820) and overnight incubation at 4 °C. The 3β-HSD antibody was recommended for detection of 3β-HSD1 or 3β-HSD2 in mouse tissues. After washing in TBS, the slides were incubated for 30 min with the appropriate secondary antibody conjugated to biotin rabbit anti-goat (Santa Cruz Biotechnologies) and diluted 1:500 in the blocking mixture. This was followed by 30-min incubation with horseradish peroxidase-labeled avidin-biotin complex (Dako, Carpinteria, CA, USA). Immunostaining was developed by the application of diaminobenzidine (liquid DAB$^+$; Dako, Carpinteria, CA, USA), and the slides were counterstained with hematoxylin, dehydrated and mounted using Pertex mounting medium (Cell Path, Hemel Hempstead, UK). The number of 3β-HSD-positive cells was counted in 12 randomly selected fields from each slide at a magnification of × 400.

Radioimmunoassay

Serum was separated by centrifugation and stored at –80 °C until assay for T. Serum T was measured using ^{125}I-based radioimmunoassay (RIA) kits from the Beijing north institute of biological technology (Beijing, China). For measuring testicular T, testes were homogenized in 0.5 ml of PBS (PH7.4). T was extracted from homogenate using diethyl ether. After extraction, the organic phase was evaporated into dryness in a fume hood, the steroids were solubilized in an aliquot of PBS and measured using ^{125}I-based RIA kits (Beijing, China) according to the manufacturer's protocols for serum samples. The testicular T concentration was expressed as ng per testis.

Semiquantitative RT-PCR

Total cellular RNA was extracted using TRI-Reagent (Molecular Research Center, Cincinnati, OH, USA) according to the manufacturer's instructions. RNase-free DNase (Promega, Madison, WI) was used to remove genomic DNA. The integrity and concentration of RNA was determined by measuring absorbance at 260 nm followed by electrophoresis on agarose gels. For the synthesis of cDNA, 1.0 μg of total RNA from each sample was reverse transcribed in a 40-μl reaction volume containing 0.5 μg oligo(dT)$_{15}$ (Promega, Madison, WI, USA), 1 mM of each dNTP, 15 units AMV RT (Promega) and 1 U μl^{-1} recombinant RNasin RNase inhibitor (Promega) in 5 mM $MgCl_2$, 10 mM Tris–HCl, (pH 8.0), 50 mM KCl and 0.1% Triton X-100. The mixture was heated to 70 °C for 10 min, maintained at 42 °C for 30 min and then heated to 95 °C for 5 min to terminate the reaction. Reaction mixtures (25 μl) for PCR were assembled using 2 μl cDNA template, 0.625 units Hot Start Polymerase (Promega), 200 μM of each dNTP, 1.5 mM $MgCl_2$ and 1.0 μM of each primer in 1× Flexi buffer. The primers were synthesized using Sangon Biological Technology (Shanghai, China), according to sequence designs previously described (Akingbemi *et al*., 2003). The primer pairs, the number of cycles and annealing temperature are shown in Table 1. The amplified PCR products were subjected to electrophoresis at 75 V through 1.5 % agarose gels (Sigma) for 45 min. The pBR322 DNA digested with *Alu*I was used for molecular markers (MBI Fermentas, Vilnius, Lithuania). Agarose gels were stained with 0.5 mg ml^{-1} ethidium bromide (Sigma) TBE buffer.

Immunoblotting

Testis samples were homogenized in lysis buffer containing 50 mM Tris–HCl (pH 7.4), 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 1% sodium deoxycholate, 0.1% sodium dodecylsylphate (SDS) and 1 mM phenylmethylsulfonyl fluoride. Samples were then centrifuged at 15 000 *g* for 15 min. Supernatants from each sample were added to a gel loading buffer (100 mM Tris, pH 6.8, 20% glycerol, 200 mM DTT, 4% SDS, 0.03% bromophenol blue) and boiled for 5 min. Proteins (50 μg per sample) in loading buffer were subjected to electrophoresis in 10% SDS-polyacrylamide gel (PAGE) for 3 h. The gel was transferred electrophoretically onto a polyvinylidene fluoride membrane (Immobilon-P; Millipore Corp., Bedford, Massachusetts, USA) and blocked in 5% non-fat powdered milk in Dulbecco's PBS (DPBS) overnight at 4 °C. The membranes were then incubated for 2 h at room temperature with the following antibodies: StAR, 17β-HSD, P450$_{17\alpha}$, P450scc and β-actin. After washes in DPBS containing 0.05% Tween-20 four times for 10 min each, the membranes were incubated with goat anti-rabbit or goat anti-mouse IgG antibody for 2 h. The membranes were then washed four times in DPBS containing 0.05% Tween-20 for 10 min each, followed by signal development using an enhanced chemiluminescence (ECL) detection kit from Pierce (Pierce Biotechnology, Rockford, IL, USA).

Atomic Absorption Spectrometry

The determination of Pb in biological samples was performed by graphite furnace atomic absorption spectrometry (GFAAS) using the stabilized-temperature-platform-furnace coupled with a D_2-lamp background correction system. The external quality-control program did not show any time trend in the accuracy of Pb measurements. Detection limits were based on three times the relative standard deviation of 20 consecutive measurements of the blank solution (1% HNO_3) according to IUPAC, being 0.10 μg l^{-1} Cd. Precision of the method was measured by coefficients of variation (CV). The mean CV for measurement of Pb in biological samples was 3.5% for within-day determinations and 3.9% for day-to-day determinations. For blood samples, initially studied a 1 + 4, v/v dilution with 5% HNO_3. For other biological samples (testis, kidney and liver), samples were decomposed in duplicate according to the following procedure: 200 mg of samples was accurately weighed in a digestion tube, a 3 ml freshly prepared mixture of concentrated HNO_3-H_2O_2 was added to each tube and the solutions were kept at room temperature for 12 h, the clear transparent digests were obtained. After this period, the mixture was boiled nearly to dryness, and the residue quantitatively transferred to a 10-ml volumetric flask with 1.0% HNO_3. The resulting solution was analyzed by GFAAS by taking 10 μl of the digest under the aforementioned condition.

Statistical Analysis

For RT-PCR, StAR, P450$_{17\alpha}$, 17β-HSD and P450scc mRNA were normalized to the GAPDH mRNA level in the same samples. StAR, P450$_{17\alpha}$, 17β-HSD and the P450scc mRNA level of the control was assigned as 100%. For immunoblotting, StAR,

Table 1. Primers, the number of cycles and annealing temperature for RT-PCR

Name	Sequence	Denaturation (°C)	Annealing (°C)	Extension (°C)	Number of cycles (n)	Size (bp)
GAPDH	Forward: 5′-GAGGGGCCATCCACAGTCTTC-3′ Reverse: 5′-CATCACCATCTTCCAGGAGCG-3′	94	56	72	35	340
StAR	Forward: 5′-TGTCAAGGAGATCAAGGTCCTG-3′ Reverse: 5′-CGATAGGACCTGGTTGATGAT-3′	94	57	72	42	310
P450scc	Forward: 5′-AGGTGTAGCTCAGGACTTCA-3′ Reverse: 5′-AGGAGGCTATAAAGGACACC-3′	94	56	72	39	370
P450$_{17\alpha}$	Forward: 5′-CCAGGACCCAAGTGTGTTCT-3′ Reverse: 5′-CCTGATACGAAGCACTTCTCG-3′	94	56	72	39	250
17β-HSD 3	Forward: 5′- ATTTTACCAGAGAAGACATCT-3′ Reverse: 5′- GGGGTCAGCACCTGAATAATG-3′	94	52	72	43	367

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$P450_{17\alpha}$, 17β-HSD and P450scc were normalized to the β-actin level in the same samples. The densitometry unit of the control was assigned as 1. All quantified data were expressed as means ± SEM at each point. ANOVA and the Student–Newmann–Keuls post hoc test were used to determine differences among different groups.

Results

Pb concentration in Blood, Testis, Liver and Kidney at Weaning

To investigate whether Pb can be transported from dams to pups through milk, Pb concentration was measured in blood, testis, liver and kidneys of male pups. As shown in Fig. 1A, no significant difference on blood Pb concentration was observed between Pb-exposed pups and controls. However, the testicular Pb level was significantly increased in Pb-exposed pups (Fig. 1B). Moreover, the levels of hepatic and renal Pb were significantly increased in Pb-exposed pups in a concentration-dependent manner (Fig. 1C and D).

Testis weight and Testicular Histology at Weaning

As shown in Fig. 2A, maternal Pb exposure during lactation significantly decreased the testis weight of male pups at weaning. In addition, maternal Pb exposure during lactation significantly reduced the relative testis weight at weaning (Fig. 2B). The effects of maternal Pb exposure during lactation on histology for testes of weaning pups are presented in Fig. 2C–E. No abnormal morphology was observed in testes of weaning pups whose mothers were exposed to Pb. Interestingly, maternal Pb exposure during lactation markedly decreased the layers of spermatogenic cells (Fig. 2D and 2E).

The Numbers of Leydig Cells and T Production in Male Pups at Weaning

The effects of maternal Pb exposure during lactation on the numbers of Leydig cells were analyzed. As shown in Fig. 3A–D, the number of Leydig cells was significantly decreased in the testes of weaning pups whose mothers were exposed to a high concentration of Pb during lactation. The effects of maternal Pb exposure during lactation on testicular T production were analyzed. As shown in Fig. 3E, the serum T level was significantly decreased in weaning pups whose mothers were exposed to 2000 ppm Pb during lactation. Correspondingly, maternal Pb exposure during lactation significantly reduced the level of testicular T in weaning pups (Fig. 3F).

The Expression of Testicular StAR and T Biosynthetic Enzymes at Weaning

The effects of maternal Pb exposure during lactation on the expression of StAR and testicular T biosynthetic enzymes are presented in Fig 4. As shown in Fig. 4A, no significant difference on the expression of testicular StAR was observed between Pb-exposed mice and controls. Interestingly, the level of testicular P450scc, P45017α and 17β-HSD was significantly decreased in weaning pups whose mothers were exposed to Pb during lactation (Fig. 4B–D).

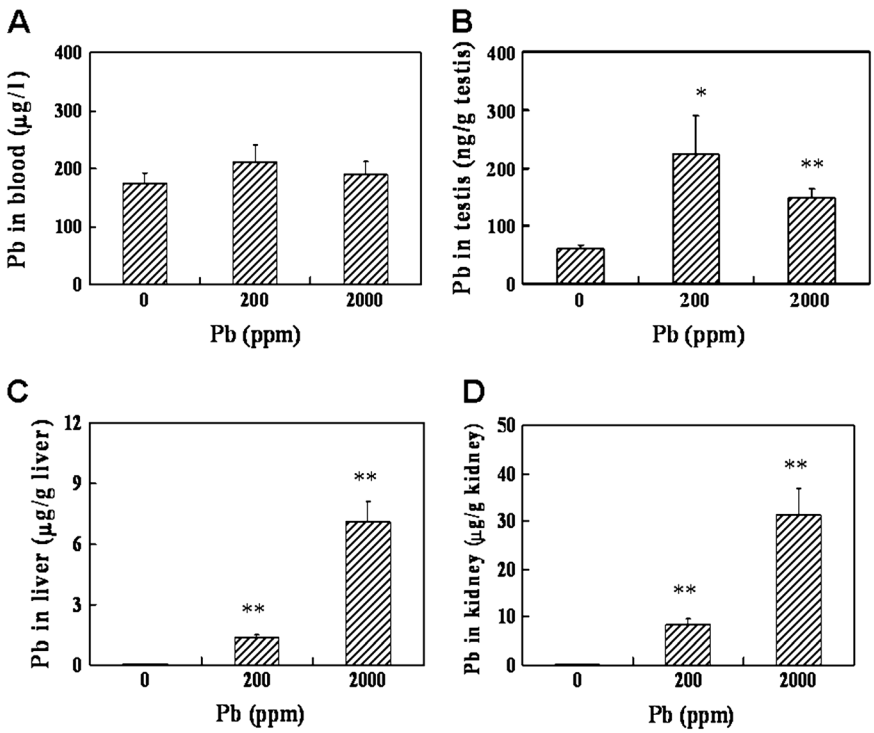

Figure 1. The effects of maternal lead (Pb) exposure during lactation on Pb concentration in blood, testis, liver and kidneys in male pups at weaning. Maternal mice drank different concentrations (200 or 2000 ppm) of lead acetate dissolved in deionized water from postnatal day 0 (PND0) to weaning (PND21). Blood, testis, liver and kidneys were collected from weaning pups at PND22. The Pb concentration was measured using graphite furnace atomic absorption spectrometry (GFAAS). Data were expressed as mean ± standard error of the mean (SEM) of 12 samples from 6 different litters. $^{*}P < 0.05$, $^{**}P < 0.01$ as compared with the control.

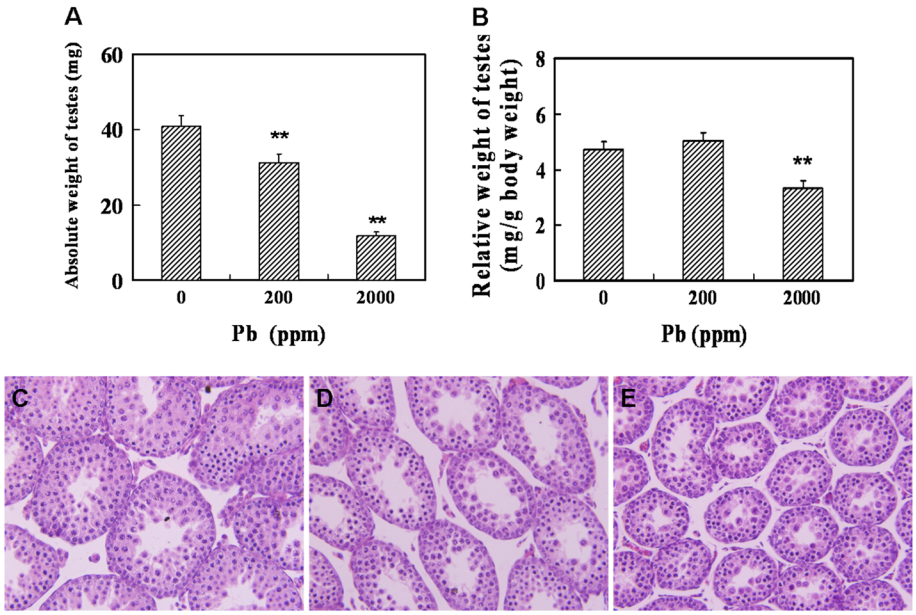

Figure 2. The effects of maternal lead (Pb) exposure during lactation on testis weight and testicular histology in male pups at weaning. Maternal mice drank different concentrations (200 or 2000 ppm) of lead acetate dissolved in deionized water from postnatal day 0 (PND0) to weaning (PND21). The testes were collected at PND22. (A) Absolute testis weight. (B) Relative testis weight. Data were expressed as mean ± standard error of the mean (SEM) of 12 samples from 6 different litters. $^{**}P < 0.01$ as compared with the control. (C–E) Testicular cross sections were stained with hematoxylin and eosin (H&E). (C) The testis from control mice. (D) The testis from Pb-treated (200 ppm) mice. (E) The testis from Pb-treated (2000 ppm) mice. Magnification: 400 ×.

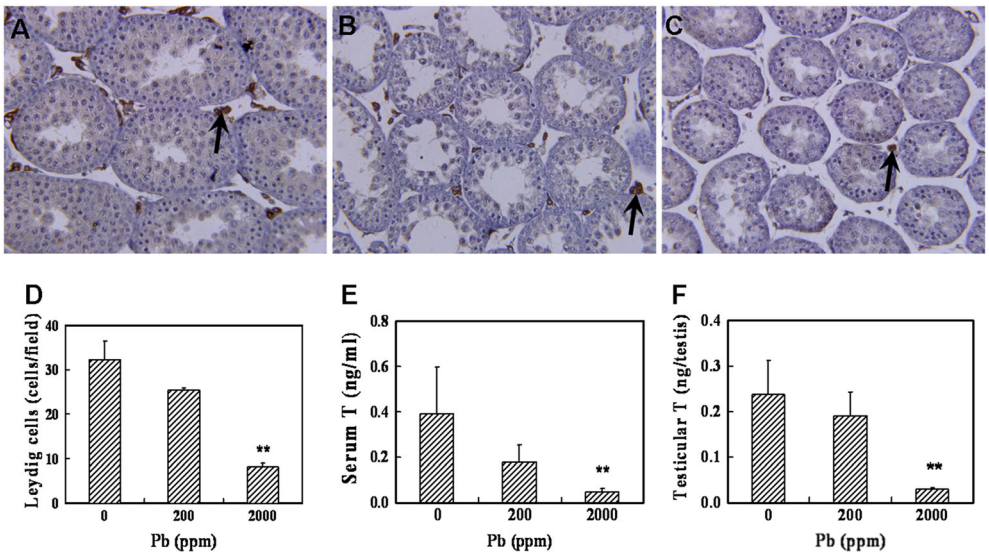

Figure 3. The effects of maternal lead (Pb) exposure during lactation on the numbers of Leydig cells and T level in male pups at weaning. Maternal mice drank different concentrations (200 or 2000 ppm) of lead acetate dissolved in deionized water from postnatal day 0 (PND0) to weaning (PND21). Sera and testes were collected at PND22. Leydig cells in testes were immunolocalized by staining with a polyclonal antibody specific for 3β-HSD. (A) Testis from control mice. (B) Testis from Pb-treated (200 ppm) mice. (C) The testis from Pb-treated (2000 ppm) mice. (D) The number of Leydig cells in testes was counted in 12 randomly selected fields from each slide at 400× magnification. Data were expressed as means ± standard error of the mean (SEM) of 12 samples from 6 litters. $^{**}P < 0.01$ as compared with controls. (E and F) T was measured by radioimmunoassay (RIA). (E) Serum T. (F) Testicular T. All data were expressed as means ± SEM of 12 samples from 6 litters. $^{**}P < 0.01$ as compared with the control.

Pb Concentration in the Blood, Liver, Kidneys and Testis at Adulthood

As shown in Figs. 5A–C, no significant difference in the level of Pb in the blood, liver and testis of adult male offspring was observed between Pb-exposed mice and controls. Interestingly, the renal Pb level was significantly increased in adult male offspring of Pb-exposed mice as compared with controls (Fig. 5D).

Weights of Male Reproductive Organs, Sperm Quality and Testicular Histology at Adulthood

As shown in Fig. 6A–C, no significant difference in the weight of testes, epididymides and prostate plus seminal vesicle in adult male offspring was observed between Pb-exposed mice and controls. The effects of maternal Pb exposure during lactation on sperm quality in adult male offspring were

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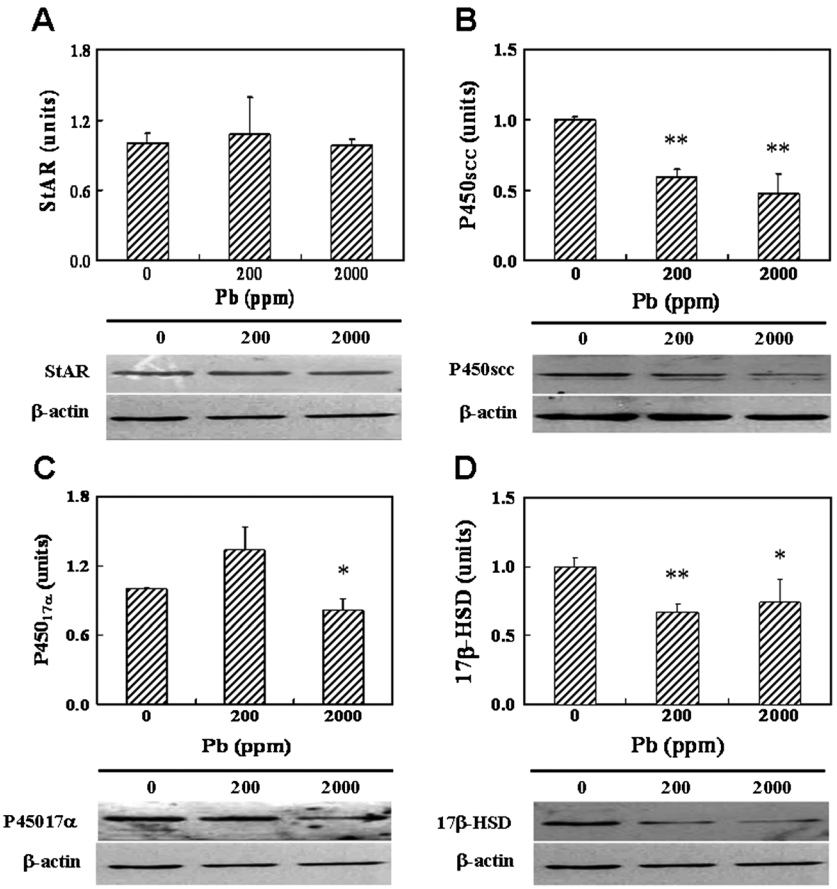

Figure 4. The effects of maternal lead (Pb) exposure during lactation on the expression of testicular StAR and T biosynthetic enzymes in male pups at weaning. Maternal mice drank different concentrations (200 or 2000 ppm) of lead acetate dissolved in deionized water from postnatal day 0 (PND0) to weaning (PND21). The testes were collected at PND22. The levels of StAR, P450$_{17\alpha}$, 17β-HSD and P450scc proteins were measured using immunoblotting. A representative gel was shown. All experiments were repeated for four times. Quantitative analyzes of scanning densitometry on four samples from four different litters were performed. StAR, P450$_{17\alpha}$, 17β-HSD and P450scc were normalized to β-actin level in the same samples. The densitometry unit of the control was assigned as 1. (A) StAR. (B) P450scc. (C) P450$_{17\alpha}$. (D) 17β-HSD. All data were expressed as means ± standard error of the mean (SEM) of four samples from four litters. *$P < 0.05$, **$P < 0.01$ as compared with the control.

analyzed. As shown in Fig. 6D, the number of spermatozoa in epididymides was significantly decreased in mice whose mothers were exposed to Pb during lactation. The effects of maternal Pb exposure during lactation on testicular histology in adult male offspring were analyzed. As shown n Fig. 6E–G, the layers of spermatogenic cells in seminiferous tubules were significantly decreased in the testes of Pb-exposed mice.

The Numbers of Leydig Cells and T Production at Adulthood

The effects of maternal Pb exposure during lactation on the numbers of Leydig cells in adult male offspring were analyzed. As shown in Fig. 7A–D, no significant difference in the number of Leydig cells in adult male offspring was observed between Pb-exposed mice and controls. The effects of maternal Pb exposure during lactation on testicular T production in adult male offspring were analyzed. As shown in Fig. 7E, the serum T level was significantly decreased in mice whose mothers were exposed to 2000 ppm Pb during lactation. In addition, a trend in the reduction of testicular T was observed in Pb-exposed mice (Fig. 7F).

The Expression of Testicular StAR and T Biosynthetic Enzymes at Adulthood

The effects of maternal Pb exposure during lactation on the expression of StAR and testicular T biosynthetic enzymes were analyzed. As shown in Fig. 8A, the level of testicular StAR mRNA in adult male offspring was significantly decreased in Pb-exposed mice, whereas no significant difference in the level of testicular StAR protein in adult male offspring was observed between Pb-exposed mice and controls (Fig. 9A). Interestingly, maternal Pb exposure during lactation significantly downregulated the levels of testicular P45017α mRNA and protein in adult male offspring (Figs 8C and 9C). However, maternal Pb exposure during lactation had no effect on the expression of testicular P450scc and 17β-HSD in adult male offspring (Figs. 8B, D and 9B, D).

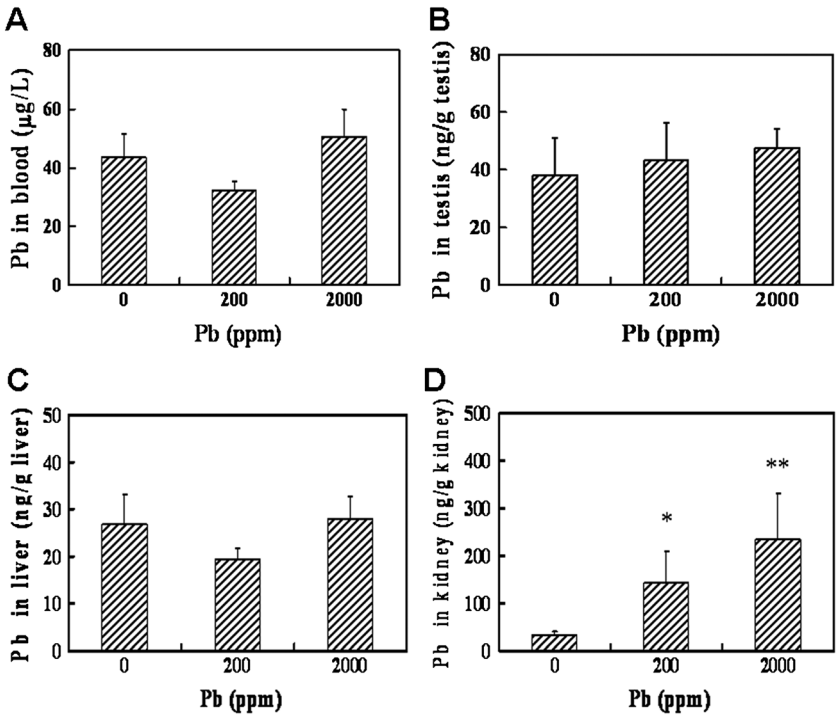

Figure 5. The effects of maternal lead (Pb) exposure during lactation on the Pb concentration of blood, testis, liver and kidneys in adult male offspring. Maternal mice drank different concentrations (200 or 2000 ppm) of lead acetate dissolved in deionized water from postnatal day 0 (PND0) to weaning (PND21). The blood, testis, liver and kidneys were collected from male offspring at PND70. Pb concentration was measured using graphite furnace atomic absorption spectrometry (GFAAS). Data were expressed as mean ± standard error of the mean (SEM) of 12 samples from 6 different litters. $^{*}P < 0.05$, $^{**}P < 0.01$ as compared with the control.

Discussion

Increasing evidence demonstrated that Pb could be transported from dams to pups through milk (Hallen and Oskarsson, 1993; Hallen *et al*., 1995;). In the present study, we measured the Pb level of blood, testis, liver and kidneys in male offspring whose mothers were exposed to Pb through drinking water during lactation. Although no significant difference in the blood Pb level was observed between Pb-exposed mice and controls, maternal Pb exposure during lactation significantly increased the level of hepatic and renal Pb in male pups. Importantly, we found for the first time that maternal Pb exposure during lactation significantly increased the testicular Pb level in weaning pups. These results suggest that Pb exposed through drinking water during lactation could be transported from dams to pups through milk.

Numerous studies showed that maternal Pb exposure during lactation impaired neurobehavioral development in offspring (Antonio *et al*., 2002; Gilbert and Lasley, 2007; Molina *et al*., 2011). Several previous studies found that maternal Pb exposure during lactation delayed the timing of puberty in female offspring (Dearth *et al*., 2002, 2004). Thus, whether maternal Pb exposure during lactation can impair testicular development in male offspring is especially interesting. The present results showed that the absolute and relative testis weights at weaning were significantly decreased in pups whose mothers were exposed to Pb during lactation. Moreover, maternal Pb exposure during lactation markedly reduced the layers of spermatogenic cells in the testes of male pups at weaning. Although testis weight had restored in adulthood, the number of spermatozoa in epididymides was significantly reduced in adult offspring whose mothers were exposed to Pb during lactation. These results suggest that an indirect exposure to Pb through milk can permanently impair testicular development and spermatogenesis in mice.

Androgens, primarily T, which act through the somatic cells to regulate germ cell differentiation, are a prerequisite for normal testicular development and spermatogenesis (Holdcraft and Braun, 2004; Wang *et al*., 2009). A Sertoli cell-selective knockout of the androgen receptor resulted in spermatogenic arrest in meiosis and infertility with defective spermatogenesis and hypotestosteronemia (Chang *et al*., 2004; De Gendt *et al*., 2004). Several previous studies showed that intratesticular T was significantly decreased in adult rodents chronically exposed to Pb (Rodamilans *et al*., 1988; Sokol, 1989, 1990; Thoreux-Manlay *et al*., 1995). The present study investigated whether maternal Pb exposure during lactation could disrupt testicular T production in male offspring. As expected, the level of serum and testicular T at weaning was significantly decreased in male pups whose mothers were exposed to PB during lactation. Importantly, the level of serum T remained decreased in adult male offspring whose mothers were exposed to PB during lactation. These results suggest that maternal Pb exposure during lactation can irreversibly disrupt steroidogenesis in testes.

StAR is an essential and limiting factor in testicular T synthesis, responsible for the transport of cholesterol into mitochondria (Miller, 2007). A previous *in vitro* study showed that lead acetate down-regulated the expression of StAR in MA-10 mouse Leydig tumor cells (Liu *et al*., 2001). The present

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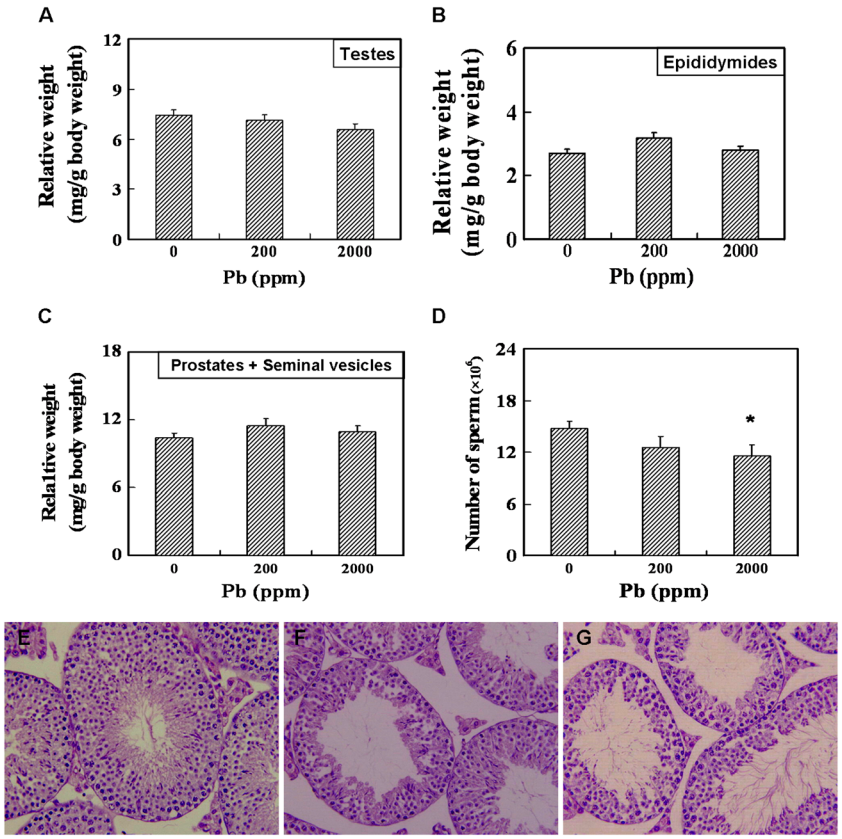

Figure 6. The effects of maternal lead (Pb) exposure during lactation on the weights of male reproductive organs, sperm quality and testicular histology in adult male offspring. Maternal mice drank different concentrations (200 or 2000 ppm) of lead acetate dissolved in deionized water from postnatal day 0 (PND0) to weaning (PND21). The testes, epididymides and prostate plus the seminal vesicle were collected from adult offspring at PND70. (A) Relative testis weight. (B) Relative epididymides weight. (C) Relative prostate plus seminal vesicle weight. (D) Sperm number in epididymides. Data were expressed as mean ± standard error of the mean (SEM) of 12 samples from 6 different litters. *$P < 0.05$ as compared with the control. (E–G) Testicular cross sections were stained with hematoxylin and eosin (H&E). (E) The testis from control mice. (F) The testis from Pb-treated (200 ppm) mice. (G) The testis from Pb-treated (2000 ppm) mice. Magnification: 400 ×.

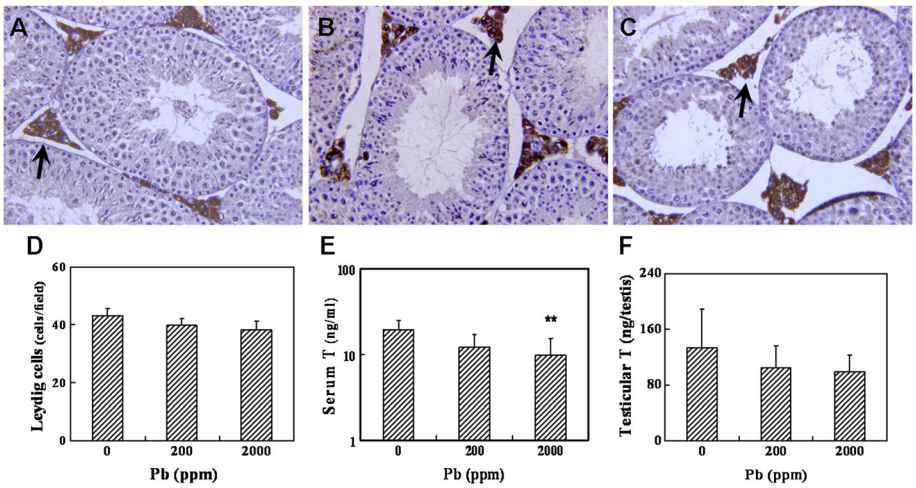

Figure 7. The effects of maternal lead (Pb) exposure during lactation on the numbers of Leydig cells and T level in adult male offspring. Maternal mice drank different concentrations (200 or 2000 ppm) of lead acetate dissolved in deionized water from postnatal day 0 (PND0) to weaning (PND21). Sera and testes were collected from male offspring at PND70. Leydig cells in testes were immunolocalized by staining with a polyclonal antibody specific for 3β-HSD. (A) The testis from control mice. (B) The testis from Pb-treated (200 ppm) mice. (C) The testis from Pb-treated (2000 ppm) mice. (D) The number of Leydig cells in the testes was counted in 12 randomly selected fields from each slide at 400× magnification. Data were expressed as means ± standard error of the mean (SEM) of 12 samples from 6 litters. (E and F) T was measured by radioimmunoassay (RIA). (E) Serum T. (F) Testicular T. Data were expressed as means ± SEM of 12 samples from 6 litters. **$P < 0.05$ as compared with the control.

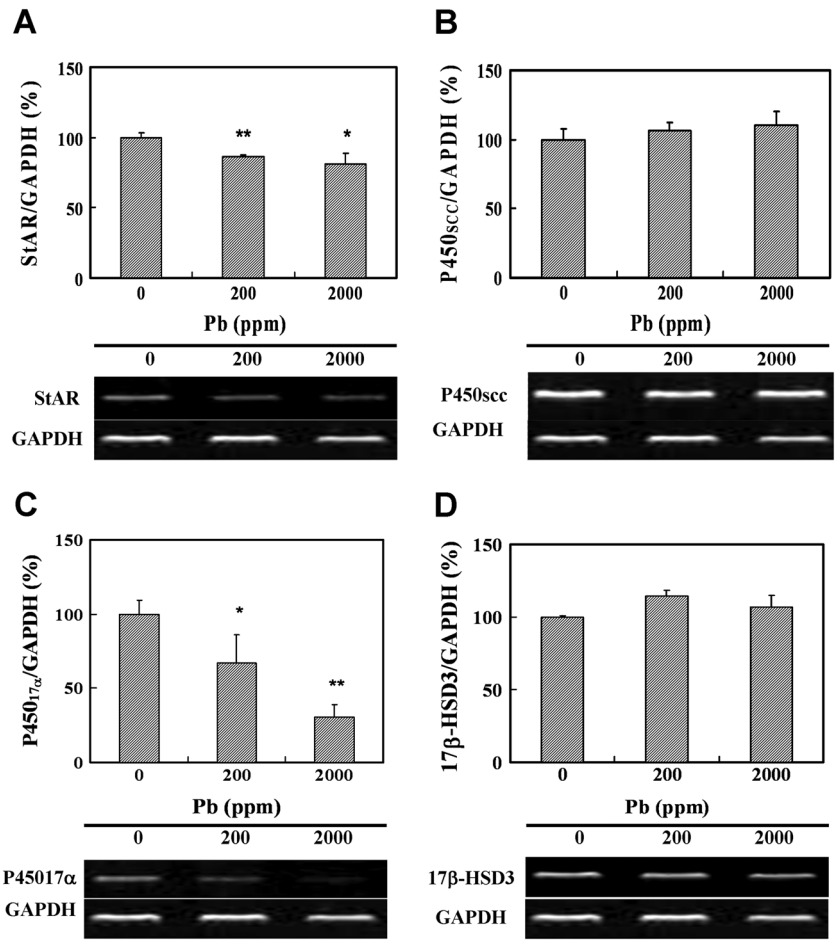

Figure 8. The effects of maternal lead (Pb) exposure during lactation on the mRNA level of testicular StAR and T biosynthetic enzymes in adult male offspring. Maternal mice drank different concentrations (200 or 2000 ppm) of lead acetate dissolved in deionized water from postnatal day 0 (PND0) to weaning (PND21). The testes were collected from male offspring at PND70. StAR, P450scc, P450$_{17\alpha}$ and 17β-HSD3 mRNA was measured using RT-PCR. A representative gel was shown. All experiments were repeated for four times. Quantitative analysis of StAR, P450scc, P450$_{17\alpha}$ and 17β-HSD3 mRNA from four individual RNA samples at each point was performed. StAR, P450$_{17\alpha}$, 17β-HSD3 and P450scc mRNA were normalized to GAPDH mRNA level in the same samples. The StAR, P450$_{17\alpha}$, 17β-HSD3 and P450scc mRNA level of the control was assigned as 100%. (A) StAR. (B) P450scc. (C) P450$_{17\alpha}$. (D) 17β-HSD3. All data were expressed as means ± standard error of the mean (SEM) of four samples from four litters. *$P < 0.05$, ** $P < 0.01$ as compared with the control.

study investigated the effects of maternal Pb exposure during lactation on the expression of testicular StAR in male pups. Results showed that maternal Pb exposure during lactation had little effect on protein expression of StAR in testes. T synthetic enzymes, primarily P450scc, P45017α and 17β-HSD, play a critical role in T synthesis in Leydig cells. P450scc initiates the first enzymatic step in T biosynthesis in the inner mitochondrial membrane of Leydig cells, where cholesterol is converted to pregnenolone. Pregnenolone is catalyzed by P45017α to produce 17-hydroxyprogesterone and androstenedione, with the latter then being converted to T by 17β-HSD3, expressed almost exclusively in the testes (Payne and Youngblood, 1995). In addition, T production is also catalyzed by 17β-HSD5, ubiquitously expressed in tissues including the testis, prostate, breast, ovary and endometrium (Day *et al.*, 2008). According to an earlier study, lead acetate inhibited the enzymatic activity of P450scc in MA-10 mouse Leydig tumor cells in a concentration-dependent manner (Huang *et al.*, 2002). The present study investigated the effects of maternal Pb exposure during lactation on the expression of testicular T synthetic enzymes. We found that the level of P450scc and P45017α was significantly decreased in testes of weaning pups whose mothers were exposed to Pb during lactation. Additionally, the expression of 17β-HSD, total isoforms including 17β-HSD3 and 17β-HSD5, was also obviously downregulated in Pb-treated testes. Surprisingly, the mRNA level of StAR and P45017α remained decreased in the testes of adult male offspring whose mothers were exposed to Pb during lactation. In addition, maternal Pb exposure during lactation irreversibly downregulated the protein level of testicular P45017α in male offspring. These results suggest that maternal Pb exposure during lactation persistently disrupts steroidogenesis through downregulating the expression of T synthetic enzymes in the testes.

A part from the expression of testicular StAR and T synthetic enzymes, we searched for other possible factors involved in the decreased T production in male pups whose mothers were exposed to Pb during lactation. Recently,

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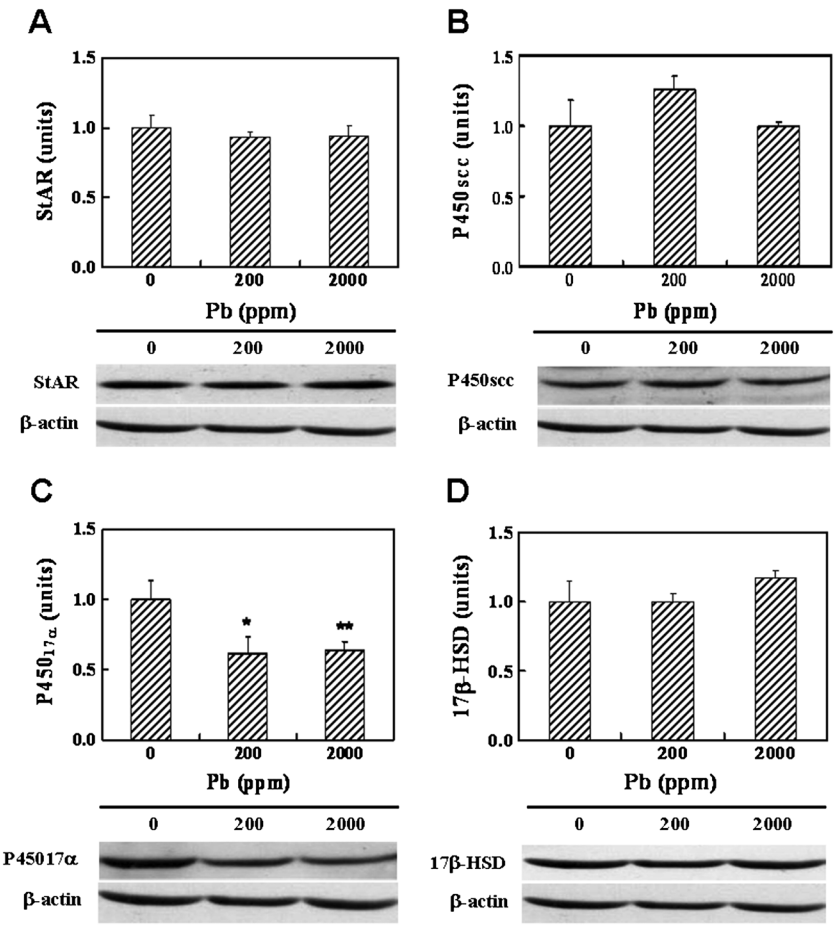

Figure 9. The effects of maternal lead (Pb) exposure during lactation on the protein level of testicular StAR and T biosynthetic enzymes in adult male offspring. Maternal mice drank different concentrations (200 or 2000 ppm) of lead acetate dissolved in deionized water from postnatal day 0 (PND0) to weaning (PND21). The testes were collected from male offspring at PND70. The levels of StAR, P450$_{17\alpha}$, 17β-HSD and P450scc proteins were measured using immunoblotting. A representative gel was shown. All experiments were repeated for four times. Quantitative analyzses of scanning densitometry on four samples from four different litters were performed. StAR, P45017α, 17β-HSD and P450scc were normalized to β-actin level in the same samples. The densitometry unit of the control was assigned as 1. (A) StAR. (B) P450scc. (C) P450$_{17\alpha}$. (D) 17β-HSD. All data were expressed as means ± standard error of the mean (SEM) of four samples from four litters. *$P < 0.05$, **$P < 0.01$ as compared with the control.

several studies showed that abnormal Leydig cell aggregation in the fetal testes of rats exposed to phthalates was associated with the decreased T synthesis in testes (Lin *et al*., 2008; Mahood *et al*., 2005, 2007). The present study investigated the effects of maternal Pb exposure during lactation on the number of Leydig cells in male pups. Our results showed that the number of Leydig cells in the testes at weaning was markedly decreased in mice whose mothers were exposed to Pb during lactation. Thus, the present study does not exclude that maternal Pb exposure during lactation disrupts testicular T synthesis via injuring Leydig cells. Additional work is required to determine whether Pb can induce Leydig cell apoptosis and abnormal Leydig cell aggregation in the testes.

In summary, the present results allow us to reach the following conclusions. First, Pb could be transported from dams to pups through milk; second, neonatal Pb exposure through milk persistently disrupts testicular T synthesis via downregulating the expression of StAR and T synthetic enzymes; third, the resultant decrease in serum and testicular T leads to adverse effects on the testicular development and spermatogenesis in male offspring.

Acknowledgments

This work was supported by National Natural Science Foundation of China (30671786, 31000664) and the Key Project of Chinese Minstry of Education (208060).

REFERENCES

Adhikari N, Sinha N, Narayan R, Saxena DK. 2001. Lead-induced cell death in testes of young rats. *J. Appl. Toxicol.* **21**: 275–277.

Akingbemi BT, Ge R, Rosenfeld CS, Newton LG, Hardy DO, Catterall JF, Lubahn DB, Korach KS, Hardy MP. 2003. Estrogen receptor-alpha gene deficiency enhances androgen biosynthesis in the mouse Leydig cell. *Endocrinology* **144**: 84–93.

Alexander BH, Checkoway H, van Netten C, Muller CH, Ewers TG, Kaufman JD, Mueller BA, Vaughan TL, Faustman EM. 1996. Semen quality of men employed at a lead smelter. *Occup. Environ. Med.* **53**: 411–416.

Antonio MT, Lopez N, Leret ML. 2002. Pb and Cd poisoning during development alters cerebellar and striatal function in rats. *Toxicology* **176**: 59–66.

Carmouche JJ, Puzas JE, Zhang X, Tiyapatanaputi P, Cory-Slechta DA, Gelein R, Zuscik M, Rosier RN, Boyce BF, O'Keefe RJ, Schwarz EM. 2005. Lead exposure inhibits fracture healing and is associated with increased chondrogenesis, delay in cartilage mineralization, and a

decrease in osteoprogenitor frequency. *Environ. Health Perspect.* **113**: 749–755.

Chang C, Chen YT, Yeh SD, Xu Q, Wang RS, Guillou F, Lardy H, Yeh S. 2004. Infertility with defective spermatogenesis and hypotestosteronemia in male mice lacking the androgen receptor in Sertoli cells. *Proc. Natl. Acad. Sci. U. S. A.* **101**: 6876–6881.

Day JM, Tutill HJ, Purohit A, Reed MJ. 2008. Design and validation of specific inhibitors of 17beta-hydroxysteroid dehydrogenases for therapeutic application in breast and prostate cancer, and in endometriosis. *Endocr. Relat. Cancer* **15**: 665–692.

De Gendt K, Swinnen JV, Saunders PT, Schoonjans L, Dewerchin M, Devos A, Tan K, Atanassova N, Claessens F, Lecureuil C, Heyns W, Carmeliet P, Guillou F, Sharpe RM, Verhoeven G. 2004. A Sertoli cell-selective knockout of the androgen receptor causes spermatogenic arrest in meiosis. *Proc. Natl. Acad. Sci. U. S. A.* **101**: 1327–1332.

Dearth RK, Hiney JK, Srivastava V, Burdick SB, Bratton GR, Dees WL. 2002. Effects of lead (Pb) exposure during gestation and lactation on female pubertal development in the rat. *Reprod. Toxicol.* **16**: 343–352.

Dearth RK, Hiney JK, Srivastava V, Les Dees W, Bratton GR. 2004. Low level lead (Pb) exposure during gestation and lactation: assessment of effects on pubertal development in Fisher 344 and Sprague–Dawley female rats. *Life Sci.* **74**: 1139–1148.

Deng W, Poretz RD. 2001. Lead exposure affects levels of galactolipid metabolic enzymes in the developing rat brain. *Toxicol. Appl. Pharmacol.* **172**: 98–107.

Devoto P, Flore G, Ibba A, Fratta W, Pani L. 2001. Lead intoxication during intrauterine life and lactation but not during adulthood reduces nucleus accumbens dopamine release as studied by brain microdialysis. *Toxicol. Lett.* **121**: 199–206.

Gennart JP, Buchet JP, Roels H, Ghyselen P, Ceulemans E, Lauwerys R. 1992. Fertility of male workers exposed to cadmium, lead, or manganese. *Am. J. Epidemiol.* **135**: 1208–1219.

Gilbert ME, Lasley SM. 2007. Developmental lead (Pb) exposure reduces the ability of the NMDA antagonist MK-801 to suppress long-term potentiation (LTP) in the rat dentate gyrus, in vivo. *Neurotoxicol. Teratol.* **29**: 385–393.

Hallen IP, Jorhem L, Oskarsson A. 1995. Placental and lactational transfer of lead in rats: a study on the lactational process and effects on offspring. *Arch. Toxicol.* **69**: 596–602.

Hallen IP, Oskarsson A. 1993. Dose dependent transfer of 203lead to milk and tissue uptake in suckling offspring studied in rats and mice. *Pharmacol. Toxicol.* **73**: 174–179.

Hernandez-Ochoa I, Sanchez-Gutierrez M, Solis-Heredia MJ, Quintanilla-Vega B. 2006. Spermatozoa nucleus takes up lead during the epididymal maturation altering chromatin condensation. *Reprod. Toxicol.* **21**: 171–178.

Holdcraft RW, Braun RE. 2004. Hormonal regulation of spermatogenesis. *Int. J. Androl.* **27**: 335–342.

Huang BM, Lai HY, Liu MY. 2002. Concentration dependency in lead-inhibited steroidogenesis in MA-10 mouse Leydig tumor cells. *J. Toxicol. Environ. Health A* **65**: 557–567.

Kiziler AR, Aydemir B, Onaran I, Alici B, Ozkara H, Gulyasar T, Akyolcu MC. 2007. High levels of cadmium and lead in seminal fluid and blood of smoking men are associated with high oxidative stress and damage in infertile subjects. *Biol. Trace Elem. Res.* **120**: 82–91.

Kuo HW, Wang CS, Lai JS. 1997. Semen quality in workers with long-term lead exposure: a preliminary study in Taiwan. *Sci. Total Environ.* **204**: 289–292.

Lancranjan I, Popescu HI, GAvănescu O, Klepsch I, Serbănescu M. 1975. Reproductive ability of workmen occupationally exposed to lead. *Arch. Environ. Health* **30**: 396–401.

Latendresse JR, Warbrittion AR, Jonassen H, Creasy DM. 2002. Fixation of testes and eyes using a modified Davidson's fluid: comparison with Bouin's fluid and conventional Davidson's fluid. *Toxicol. Pathol.* **30**: 524–533.

Lerda D. 1992. Study of sperm characteristics in persons occupationally exposed to lead. *Am. J. Ind. Med.* **22**: 567–571.

Lin H, Ge RS, Chen GR, Hu GX, Dong L, Lian QQ, Hardy DO, Sottas CM, Li XK, Hardy MP. 2008. Involvement of testicular growth factors in fetal Leydig cell aggregation after exposure to phthalate in utero. *Proc. Natl. Acad. Sci. U. S. A.* **105**: 7218–7222.

Liu MY, Lai HY, Yang BC, Tsai ML, Yang HY, Huang BM. 2001. The inhibitory effects of lead on steroidogenesis in MA-10 mouse Leydig tumor cells. *Life Sci.* **68**: 849–859.

Mahood IK, Hallmark N, McKinnell C, Walker M, Fisher JS, Sharpe RM. 2005. Abnormal Leydig Cell aggregation in the fetal testis of rats exposed to di (n-butyl) phthalate and its possible role in testicular dysgenesis. *Endocrinology* **146**: 613–623.

Mahood IK, Scott HM, Brown R, Hallmark N, Walker M, Sharpe RM. 2007. In utero exposure to di(n-butyl) phthalate and testicular dysgenesis: comparison of fetal and adult end points and their dose sensitivity. *Environ. Health Perspect.* **115**(Suppl. 1): 55–61.

Miller WL. 2007. StAR search--what we know about how the steroidogenic acute regulatory protein mediates mitochondrial cholesterol import. *Mol. Endocrinol.* **21**: 589–601.

Molina RM, Phattanarudee S, Kim J, Thompson K, Wessling-Resnick M, Maher TJ, Brain JD. 2011. Ingestion of Mn and Pb by rats during and after pregnancy alters iron metabolism and behavior in offspring. *Neurotoxicology* **32**: 413–422.

Moreira EG, Vassilieff I, Vassilieff VS. 2001. Developmental lead exposure: behavioral alterations in the short and long term. *Neurotoxicol. Teratol.* **23**: 489–495.

Murthy RC, Gupta SK, Saxena DK. 1995. Nuclear alterations during acrosomal cap formation in spermatids of lead-treated rats. *Reprod. Toxicol.* **9**: 483–489.

Payne AH, Youngblood GL. 1995. Regulation of expression of steroidogenic enzymes in Leydig cells. *Biol. Reprod.* **52**: 217–225.

Reddy GR, Devi BC, Chetty CS. 2007. Developmental lead neurotoxicity: alterations in brain cholinergic system. *Neurotoxicology* **28**: 402–407.

Rodamilans M, Martinez-Osaba MJ, To-Figueras J, Rivera-Fillat F, Torra M, Perez P, Corbella J. 1988. Inhibition of intratesticular testosterone synthesis by inorganic lead. *Toxicol. Lett.* **42**: 285–290.

Sokol RZ. 1989. Reversibility of the toxic effect of lead on the male reproductive axis. *Reprod. Toxicol.* **3**: 175–180.

Sokol RZ. 1990. The effect of duration of exposure on the expression of lead toxicity on the male reproductive axis. *J. Androl.* **11**: 521–526.

Thoreux-Manlay A, Velez de la Calle JF, Olivier MF, Soufir JC, Masse R, Pinon-Lataillade G. 1995. Impairment of testicular endocrine function after lead intoxication in the adult rat. *Toxicology* **100**: 101–109.

Wang L, Xun P, Zhao Y, Wang X, Qian L, Chen F. 2008. Effects of lead exposure on sperm concentrations and testes weight in male rats: a meta-regression analysis. *J. Toxicol. Environ. Health A* **71**: 454–463.

Wang RS, Yeh S, Tzeng CR, Chang C. 2009. Androgen receptor roles in spermatogenesis and fertility: lessons from testicular cell-specific androgen receptor knockout mice. *Endocr. Rev.* **30**: 119–132.

WHO. 2001. Laboratory manual of the WHO for the examination of human semen and sperm-cervical mucus interaction. *Ann. Ist. Super. Sanita* **37**: I–XII, 1–123.

Xu DX, Shen HM, Zhu QX, Chua L, Wang QN, Chia SE, Ong CN. 2003. The associations among semen quality, oxidative DNA damage in human spermatozoa and concentrations of cadmium, lead and selenium in seminal plasma. *Mutat. Res.* **534**: 155–163.