Lactational Lead Exposure Perturbates Androgenesis in Juvenile and Pubertal Wistar Rats

Odukoya SOA, Akinola OB

Department of Anatomy and Cell Biology, Obafemi Awolowo University, Ile-Ife, Nigeria, Department of Anatomy, University of Ilorin, Ilorin, Nigeria

Abstract:

Background: High to low lead (Pb) concentrations in breast milk has been found to perturb some biological events in the postnatal life. While postnatal Pb exposure has been reported to impair some andrological parameters in mammals, the age-dependent andrological signature of lactational Pb poisoning is not clear. Aims and Objectives: This study investigated the effects of Pb exposure during lactational period on the testicular andrological profiles of rats at certain post-lactational ages using varying doses of Pb. Material and Methods: Lactating mothers and their pups were randomly divided into 4 groups comprising 24 pups each. The treatment groups received 10mg/dL, 30mg/dL, and 70mg/dL of lead acetate in their drinking water from postnatal day one (P1) to P21 of the lactational period. The control rats received distilled water. At P22, P60, P90 and P120, the pups from each group were euthanized, testes were collected, homogenized and the supernatant was used to assay for testosterone and oestrogen using standard methods. Results: Lactational lead poisoning was associated with depressed testicular testosterone productions (P<0.05 compared with controls) and abnormally high levels of testicular oestrogen. These statistically significant differences (P<0.05) in androgens levels were corrected to near normal with increasing postnatal ages at low doses. Conclusion: These results show that lactational Pb intoxication causes reversible androgenic perturbations at low doses but irreversible damage at high doses during postnatal life. Conclusively, high lactational Pb is associated with post-lactational irreversible impairment of androgenic profiles.

Keywords: Lactational Lead, Androgenesis, Pubertal Wister Rats, Juvenile

Introduction:

Lead (Pb) is an environmentally persistent element and a major global environmental hazard. The Centers for Disease Control (CDC) currently consider Pb poisoning the leading environmental health threat to children in the US [1]. No safe threshold for Pb exposure has been discovered; that is, there is no known sufficiently small amount of Pb that will not cause harm to the body. Existing literature indicates that there is no safe level of blood Pb [2].

The fact that testosterone exerts its effects on somatic cells rather than germ cells was highlighted by the germ cell transplantation studies [3-5], in which spermatogonia from AR-deficient animals developed into spermatozoa in wild-type recipients. FSH has a key role in the development of the immature testis, particularly by controlling sertoli cell proliferation [4, 6]. Following many conflicting data in animal and human models, there is now general agreement that some degree of complete spermatogenesis can be initiated and maintained in the apparent absence of FSH. However, quantitatively normal spermatogenesis in adulthood is dependent on FSH. FSH secretion is regulated by negative feedback from
the testicular hormone, inhibin B, and through testosterone, either alone or by its aromatization to oestradiol [4, 7].

Estrogens are synthesized from androgens by the aromatase complex, which contains the cytochrome P450 enzyme encoded by the Cyp19 gene [8]. Aromatase expression is detected in Sertoli-Leydig cells, spermatogonia, spermatocytes, elongate spermatids and spermatozoa in adult mice and rats [8], and in Sertoli-Leydig cells, spermatocytes, spermatids and spermatozoa in man [9]. Moreover, aromatase has been immunolocalized in epithelial cells of human efferent ducts and in rat epididymis [8-10] suggesting additional sources of estrogen in the male reproductive system and the possibility of paracrine/autocrine effects [11].

Estrogens play key roles in the development and maintenance of reproductive function and fertility [9, 12, 13]. Estrogens also have an important role in pathological processes observed in tissues of the reproductive system [14, 15].

The crucial role of estrogen for the reproductive function has been demonstrated by studies with mice with targeted disruption of ESRs (Esr1-/-, Esr2-/-, Esr1-/-/Esr2-/-), aromatase enzyme (Cyp19-/-), and animals treated with the antiestrogen (ICI 182,780) [16, 17]. The studies with knockout animals have shown that the spermatogenesis, steroidogenesis and fertility of Esr1-/-, Esr1-/-/Esr2-/- and Cyp19-/- animals are affected [16, 17]. A recent study showed that a new Esr2-/- mouse mutant, in which exon 3 of Esr2 was deleted by Cre/LoxP-mediated excision, was completely devoid of any downstream transcripts, and the males were also sterile [18-20].

Materials and Methods:
Experimental Animals
Animals bred in the Animal holdings of the department of Anatomy, Bowen University, Iwo were used. Pure strain was obtained after 18 months of continuous breeding. Sexually matured animals (120-150 days old) were paired 1 male to 2 females (10 males and 20 females) and kept under standard laboratory conditions at a constant light/dark cycle. The pregnant rats were isolated into separate cages and fed with standard rat chow.

![Fig. 1: Pattern of Increased Estradiol Level Behavior in the Male Animals][1]

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[1]: https://example.com/fig1.png
from Ladokun feeds, Ibadan and watered *ad libitum*, the day the dam littered was marked as day 0.5. After parturition, the dams and 96 pups were randomly divided into 4 equal groups of 24 pups per group: Group A (control) were given distilled water only while 3 treatment groups (B, C and D) received 10 mg/dL, 30 mg/dL, and 70 mg/dL of lead acetate respectively in their drinking water *ad libitum* from day 0.5 to day 21 of the lactational period. The animal handling and care was in line with the rules and guideline of the Health Research and Ethics Committee of the Institute of Public Health, Obafemi Awolowo University Ile-Ife, Nigeria.

**Oral Lead Acetate Dosing of Lactating Rats:**
About 100mg, 300mg and 700mg of lead acetate powder were weighed on a Metler Toledo sensitive weighing balance and prepared in 1000ml of water respectively to obtain 10mg/dL, 30mg/dL and 70mg/dL of lead acetate. These solutions were administered orally to lactating rats *ad libitum*, while the pups sucked from the Pb contaminated breast milk from the mothers.

**Euthanasia of Pups following Lactational Pb Exposure:**
Throughout the nursing periods (3 weeks postnatal), 3 groups of nursing albino rats, each group comprising 24 pups were exposed to varying doses of lead acetate in their drinking water [low dose: 10 mg/dL [21]; moderate dose: 30 mg/dL [22]; and high dose: 70 mg/dL [23], while the pups were allowed to suck freely from their mothers' Pb contaminated breast milk, as blood Pb levels has been reported to be just 3% higher than breast milk Pb levels in Pb intoxicated individuals [24]. At postnatal day 22 (PND22) i.e. one day post-weaning, 6 pups from each group were euthanized using intra-muscular injection of 40 mg/kg b/w of pentobarbital sodium [25]. The testes were harvested following whole body perfusion using phosphate buffered saline and abdominopelvic incisions. The testes were stored in plain specimen bottles in the freezer at -4°C for testicular hormones assay. The remaining 18 animals per group were kept under standard laboratory conditions of constant light and dark cycle and room temperature, fed with standard rat chow and distilled water *ad libitum* and sacrificed 6 per group at 60, 90 and 120 days post partum respectively, following the same procedures as at PND 22.

**Blood Lead Levels Quantification (Atomic Absorption Spectrometry-AAS):**
The standard procedure was carried out to establish the concentration of Pb in the blood. For each of the experimental animals, a sample solution of whole blood and 2M concentrated 

$\text{H}_2\text{SO}_4$ was prepared by diluting into a specimen bottle, 1 volume of blood with 3 volumes of 2M concentrated 

$\text{H}_2\text{SO}_4$ (ratio 1:3), and the mixture was thoroughly shaken. The solutions were centrifuged for 15 minutes at 4000 rpm. Each solvent was decanted into a new heparinized specimen bottle and the centrifugation was repeated again to ensure that the supernatant was clear. The clear supernatants were decanted into plain specimen bottles and each sample was analyzed by the Atomic Absorption Spectrometer (AAS) method. The AAS was set at the standard wavelength for Pb analysis of 283.3nm. Five standard Pb analysis solutions (0, 2.5, 5, 10 and 20 ppm) were run through the AAS followed by each sample. The Pb concentration in the serum was recorded by the AAS.
Tissue Homogenization:
The tissues previously stored in the freezer were brought out to thaw. Fresh phosphate buffer (pH 7.4) was prepared. Each tissue was weighed and put in a mortar that had already been rinsed with distilled water followed by the phosphate buffer. As per standard procedure, 10 volume of the buffer by mass of the tissue was measured and added to the tissue in the mortar; the tissue was then gently but firmly ground with the pestle until a uniform consistency of the tissue was obtained. The homogenate was then transferred into test tube each and centrifuged. The clear supernatant obtained was decanted into a new sample bottle for further analyses.

Statistical Analysis of Data
SPSS (Version 20) statistical analysis software was used for testing differential hypothesis on parameters stated in the quantitative analysis section above. Two way ANOVA (Analysis of Variance) with Tukey's and Duncan's Post-hoc tests were performed. The P-value for statistical significance was set at P<0.05 and P<0.01, unless where otherwise stated. Data are presented as mean ± SEM (standard error of means). Using the Graph Pad Prism (version 6.0, 2014, Graph Pad Inc.) and Microsoft Excel 2013 softwares, values obtained from the statistical analyses were presented in descriptive forms using Histograms.

Results:
Testicular Testosterone Levels:
From figure 2A, no statistically significant difference was observed at PND 21 between the control and the treated groups, whereas by PND 60, the control (4.750±0.765) showed a statistically significant higher testosterone level compared with the treated groups at P<0.05, 10mg/dl (1.783±0.209), 30mg/dl (1.367±0.176) and 70mg/dl (1.367±0.204). Up to this level, a dose dependent low testosterone outputs were observed across the groups. At P90, no statistical significant difference was observed between group 10mg/dl and the control (6.700±0.638) which is significantly higher than groups 30mg/dl (3.008±0.364) and 70mg/dl (1.833±0.204). At P120, groups 10mg/dl (10.025±0.752) and 30mg/dl (12.533±0.781) were significantly higher compared with the control (6.867±0.719). Group 70mg/dl (1.100±0.107) remained significantly low compared with the control. Values are expressed as mean±SEM at P<0.05.

Figure 2B depicts changes in testosterone levels within each of the groups. While the control group maintained a time based consistent rise in testosterone level across the different postnatal days within the normal testosterone range per time, groups 10mg/dl and 30mg/dl showed consistently low levels of testosterone till P90 where group 10mg/dl rose to the normal range level, and 30mg/dl exhibited some increase, still below the normal range. At P120, the two groups exhibited an abnormal, statistically significant rise in testosterone levels above the normal range. Group 70mg/dl maintained consistently low testosterone levels below normal ranges throughout the experiment.
Mean Testicular Testosterone Levels

Fig. 2A: Mean Testicular Testosterone Levels Across the Groups
Values are Mean ± SEM, n=6* indicates statistical significant difference from the control at P<0.05.

Fig. 2B: Mean Testicular Testosterone Levels Within the Groups
Values are Mean ± SEM, n=12** indicates statistical significant difference from the control at P<0.05.

Testicular 17ß Oestradiol Levels

From figure 3A, at PND 21 and PND 60, 30mg/dl (389.10±29.938; 440.46±60.518 respectively) and 70mg/dl (381.82±30.833; 358.92±33.413 respectively) groups showed statistically significant higher testicular oestradiol levels compared with the control (280.68±21.279; 252.31±15.999 respectively). No statistically significant difference was observed between the treated groups and the control at PND 90. At PND 120, groups 30mg/dl (370.56±15.093) and
70mg/dl (417.58±58.639) showed statistically significant higher levels of oestradiol compared with the control (266.51±38.679). Group B (10mg/dl) was consistently higher than the control group except at PND 120, but differences are not statistically significant at any level, P<0.05. Values are expressed as mean ± SEM.

Figure 3B shows the mean testicular oestradiol levels within each group. The control (A) and 10mg/dl (B) groups showed fairly constant and consistently normal oestradiol levels while groups 30mg/dl (C) and 70mg/dl (D) exhibited consistently abnormal high levels of oestradiol except at PND 90.

![Fig. 3A: Mean Testicular 17β Oestradiol levels Across the Groups](image1)

Values are Mean ± SEM, n=6** indicates statistical significant difference from the control at P<0.05.

![Fig. 3B: Mean Testicular 17β Oestradiol levels Across the Groups](image2)

Values are Mean ± SEM, n=12** indicates statistical significant difference from the control at P<0.05.
Discussion:
Androgens, primarily testosterone, which act through the somatic cells to regulate germ cell differentiation, is a prerequisite for normal testicular development and spermatogenesis [26, 27]. The concentration of testosterone in the rat testis is normally 50-fold higher than that in serum [4]. In this experiment, at PND 21, no significant difference in testicular testosterone levels between the control and the treated groups was observed, but subsequent testosterone production from PND 60 was significantly reduced in a dose related manner among the Pb treated groups. At PND 120, recoveries were observed in groups 10mg/dl and 30mg/dl at an above normal production (hypertestosteronemia), whereas the 70mg/dl group showed consistent hypotestosteronemia throughout the experiment, which observed reduction in testosterone (serum) with increasing semen Pb concentration [28]. The observed hypertestosteronemia in adult Wistar rats could be an evidence of testicular function being the target of Pb action in the testis without really explaining the molecular mechanisms behind it [29].
A sertoli cell-selective knockout of the androgen receptor resulted in spermatogenic arrest in meiosis, leading to infertility through defective spermatogenesis and hypotestosteronemia [30, 31]. Several previous studies showed that intratesticular testosterone was significantly decreased in adult rodents chronically exposed to Pb [32-35]. Maternal Pb exposure during lactation can irreversibly disrupt steroidogenesis in the testes as the level of serum and testicular testosterone at weaning was significantly decreased in male pups whose mothers were exposed to Pb during lactation. Importantly, the level of serum testosterone remained decreased in the adult male offspring whose mothers were exposed to Pb during lactation [36].
Steroidogenic Acute Regulatory (StAR) protein is an essential and limiting factor in testicular testosterone synthesis, responsible for the transport of cholesterol into mitochondria [37]. A previous in vitro study showed that lead acetate down-regulated the expression of StAR in MA-10 mouse Leydig tumor cells [38] and the effects of maternal Pb exposure during lactation on the expression of testicular StAR in male pups [36]. Their results showed that maternal Pb exposure during lactation had little effect on protein expression of StAR in the testes. Testosterone synthetic enzymes, primarily P450scc, P45017a and 17b-HSD, play a critical role in testosterone biosynthesis in the inner mitochondrial membrane of Leydig cells, where cholesterol is converted to pregnenolone. Pregnenolone is catalyzed by P45017a to produce 17-hydroxyprogesterone and androstenedione, with the latter then being converted to testosterone by 17b-HSD3, expressed almost exclusively in the testes [39]. In addition, testosterone production is also catalyzed by 17b-HSD5, ubiquitously expressed in tissues including the testis, prostate, breast, ovary and endometrium [40]. 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 [41]. Their study investigated the effects of maternal Pb exposure during lactation on the expression of testicular testosterone synthetic enzyme and found that the levels of P450scc and P45017a were significantly decreased in the testes of weaning pups whose mothers were exposed to
Pb during lactation. Additionally, the expression of 17b-HSD, total isoforms including 17b-HSD3 and 17b-HSD5, were also obviously downregulated in Pb-treated testes. Surprisingly, the mRNA level of StAR and P45017a remained decreased in the testes of adult male offspring whose mothers were exposed to Pb during lactation [41]. In addition, maternal Pb exposure during lactation irreversibly downregulated the protein level of testicular P45017a in male offspring. This might be the occurrence in the 70mg/dl group where consistent and irreversible hypotestosteronemia was observed all through the experiment.

They concluded that maternal Pb exposure during lactation persistently disrupts steroidogenesis through downregulating the expression of testosterone synthetic enzymes in the testes [41]. These findings suggest the reason for the low testicular testosterone recorded in the lactational Pb exposed pups in this present research, but in contrast to their findings, with low and medium doses of 10mg/dl and 30mg/dl respectively, full recovery to abnormally high levels of testicular testosterone between postnatal days 90 and 120 was observed, while its level in the 70mg/dl group remained very low even at post pubertal stages. This suggests that their observations might be a function of Pb dose concentration during exposure.

On the other hand, testicular oestradiol levels were consistently higher in the treated groups compared to the control all through this experiment, though not dose dependent but statistically significant, that is, well above the normal approximate 250 pg/mL levels. Oestrogen had been reported to be present in very high concentrations in the rete testis and seminal fluids of several species. In concert with the findings in this study, it had been reported earlier high levels of serum oestradiol and low testosterone levels, including LH, FSH and prolactin in Pb treated rats [42]. Current evidence indicates that germ cells, in addition to Leydig cells, synthesize oestrogen, and that sperm in the upper epididymis serve as the major source of oestrogen in the male reproductive tract [43].

The presence of P450 aromatase in male germ cells has been demonstrated in several species, including the mouse and rat. Thus, the conversion of androgens to oestrogen by sperm remains the primary source of oestrogen in the lumen of the tract [44-45]. In the reproductive tract, oestrogen can reach relatively high concentrations and in rete testis fluid of the rat, oestradiol is approximately 250 pg/mL, which is higher than the average serum concentrations in the female [44, 45]. Hess, 2001 submitted that, it appears that oestrogen is required for normal fertility in the male, however, the mechanisms of oestrogen action in the epididymis and vas deferens, remain to be determined.

An earlier report had claimed significantly higher serum oestradiol levels in lead intoxicated men than the reference group, with low serum FSH, LH, prolactin, and testosterone levels [42]. Oestradiol suppressed male sexual behavior like intromission and ejaculation when introduced during critical period in sexual development [46]. Also, oestradiol in vivo decreases testosterone production by a direct inhibitory effect on testicular steroidogenesis, hence the probable suppression of testosterone production by the high testicular oestradiol levels recorded in this experiment [46]. The relationship between high oestradiol levels and low testosterone associated with environmental pollution by heavy metals.
There may be an increase in circulating oestradiol levels, leading to an altered testosterone:oestradiol ratio[48, 49].

Contrary to the findings in this study and several other studies, any negative correlations between oral Pb poisoning and steroidogenic profiles and semen characteristics in their studies were not finding, despite the testicular histoarchitectural disruptions and seminiferous epithelium anomalies with numeric and morphologic germ cells abnormalities they reported[50, 51].

**Conclusion:**
Lactational Pb intoxication causes reversible androgenic perturbations at low doses but irreversible damage at high doses during postnatal life. Conclusively, high lactational Pb is associated with post-lactational irreversible impairment of androgenic profiles.

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**References**


