Ovarian Histometric study of offspring in rats exposed to lead acetate using stereology technique

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Abstract
With the expansion of industrial processes, heavy metal pollution for example lead has become a serious problem. They have the ability to pass through organs and causes them to malfunction. One of the target organs of lead is ovarian tissue. Lead is also able to cross the blood-placental barrier and transferred to the fetus. It is also possible to pass lead through milk to the newborn. This study was performed to investigate the effect of low dose mother's lead consumption on the development of the ovaries using stereology technique in rats as animal models. In the present study, Wistar rats were randomly divided into five groups. The groups included the Control group and 4 experimental groups of Pre-pregnancy, Pregnancy, Lactation and Pre-pregnancy-pregnancy-lactation. The experimental groups received 0.2% lead acetate, with 0.5cc glacial acetic acid through drinking water. Acetate consumption was in such a way that in the pre-pregnancy group 30 days before mating, in the pregnancy group during 21 days of pregnancy, in the lactation group during 21 days of lactation, and in the pre-pregnancy-lactation group from 30 days before mating to the end of lactation they received lead acetate through drinking water. On the 65th day after the birth, all infants were killed in the laboratory and left ovarian specimens were collected for stereological studies. Examination of the results showed that the total volume of ovary, cortex, medulla, interstitial tissue and total volume of adult reproductive follicles were not significantly different between the experimental groups of pre-pregnancy, pregnancy, lactation and pre-pregnancy-pregnancy-lactation and the control group. Examination of the corpus luteum volume results showed that the pre-pregnancy group had a significant decrease compared to the pregnancy group. The present study showed that exposure of mothers to low-dose lead acetate reduces the volume of corpus luteum, especially during pre-pregnancy.

Key words: Lead acetate, Ovary, Stereology, Rat

Introduction
Contamination of living environment with heavy metals such as lead and their use for production and industrial processes is a major challenge and increasing. Human beings are indirectly faced with lead by biological compounds such as food, and weather (Pirooty and Ghasemzadeh 2013). World health organization (2013) conducted studies on contamination with lead and reported death for 853000 people (Ericson et al. 2016). Ovaries are spherical or oval formed which are composed from cortex and medulla. The Medulla portion is composed from intensive fibrous connective tissue and blood vessels. Cortex section contains growing follicles types (initial, secondary and mature), corpus luteum, interstitial tissue and blood vessels. Initial follicles are placed into tunica albuginea and under germinal epithelial...
tissue. Single oocyte core has a chromatin and a nucleus. These follicles have an ovum, but multiple ovulations are not common in rats. Zona Pellucida is found beside oocyte. Granulosa layer surrounds zona Pellucida and itself is surrounded by internal and external theca (Treuting et al. 2012). Ovarian tissue is a subject tissue for heavy metals that the metals can be accumulated in the tissue and cause physiological disorders, tissue alterations, changes in follicles growth and ovulation fluctuations (Grau 2011, Rafati Rahimzadeh et al. 2015). Studies have shown that presence of lead in ovarian tissue induces apoptosis in granulosa cells of antral follicles (Restanty et al. 2018). Facing with lead acetate reduced initial and mature follicles in BALB/c mice (Waseem et al. 2014). Lead accumulations in ovarian tissues, fallopian tube, and uterus cause edema, changes in follicle development and in fertilization (Dumitrescu et al. 2015). In addition, lead can also be transferred by blood-placenta barrier to embryo and delays development and has teratogenic effect. Maternal contamination with lead is transferred to child by milk (Patriarca et al. 2000, Masso-Gonzalez and Antonio-Garcia 2008).

Study on lead toxicity effects on development of ovary after birth showed that most infants died and also abortion and preterm birth rates were increased. Lead decreases ovarian weight, follicle size and prevents the embryogenesis period development (Sharma et al. 2012). Studies on the adverse effects of lead acetate on ovarian tissue in Albino rats showed that consumption of lead acetate with low dose caused histopathologic changes, such as degeneration of oocyte, degenerative changes of crown cells and increased atretic follicles, but it did not cause significant changes in follicles (Sodani 2017). With regards to increasing contamination with lead in world, much efforts have been conducted for decreasing contamination in mothers, pregnant women, and children, but it is impossible to avoid lead exposure. The purpose of this study is to examined the effects of mother’s low dose lead toxicity on development of infant’s ovaries using stereology technique in rat model, but so far, no study has not been conducted on this topic. The obtained results of this research can be used for assessing the effects of lead on ovarian tissue changes and deciding for selecting appropriate methods for protecting mothers against toxicity with lead and its adverse effects on ovary.

Materials and Methods

In the present study, adult male and female Wistar rats were purchased from Pasteur Institute of Iran and kept in embryology and histology laboratory under standard conditions (12 hours of light, 12 hours of darkness and a temperature of 20-26 degrees Celsius and enough water and food). To adapt the animal to the laboratory environment, rats were kept for one week under these conditions. This project has been approved under registration number 30029/6/17 in the Faculty of Veterinary Medicine, University of Tehran.

For mating, in each cage, two female animals were considered for each male animal, and 12 hours after mating, vaginal plaque was examined and if there was vaginal plaque, that day was considered as zero day of pregnancy (p = 0). Fertile rats were isolated and randomly divided into five groups. The control group (group 1) had access to normal drinking water with 0.5 ml/liter of glacial acetic acid during pre-pregnancy, pregnancy and lactation. The pre-pregnancy group (group 2) received 0.2% lead acetate and 0.5 ml / liter of glacial acetic acid in1 liter drinking water 30 days before mating, and had access to normal drinking water after mating until the end of the study period. The pregnancy group (group 3) received 0.2% (or 2gr) lead acetate and 0.5 ml / liter of glacial acetic acid in 1 liter drinking water during the 21-day gestation period. The lactation group (group 4) received 0.2% lead acetate and 0.5 ml / liter
Ovarian Histometric study of offspring in rats exposed to glacial acetic acid in 1 liter drinking water during the 21-day lactation period. Pre-pregnancy-pregnancy-lactation groups (group 5) received 0.2% lead acetate and 0.5 ml/liter glacial acetic acid in 1 liter drinking water 30 days before gestation until the end of lactation. In fact, all mothers in the experimental groups, except the control group, received lead acetate with drinking water at different periods (Jaako-Movits et al. 2005, Barkur and Bairy 2014).

At the end of the lactation period, the female offspring were separated from the mothers and kept in separate cages until the end of the 65th day to reach sexual maturity. At the end of day 65, female rats were euthanized using 300 mg/kg 10% ketamine and 30 mg/kg xylazine (Dumitrescu et al. 2015). In order to perform the stereology technique, the left ovaries were removed and placed in 4% paraformaldehyde, and after 24 hours, the formalin was replaced so that the samples were well fixed. Ovarian weight was recorded with a digital scale. Ovarian samples were molded with paraffin after performing the usual tissue preparation steps by Orientator method, and using systematic uniform random sampling method, 7 μm thick sections were prepared and stained with hematoxylin-eosin. Tissue sections were imaged with a light microscope (Jenamed 2) and a camera (uEye). Finally, tissue images were analyzed using Image J software and specific stereology plugins. Calculation of total ovarian volume was obtained using point grids and the following formula (Howard and Reed 2005).

\[ V = T \times (a/p) \times \Sigma P \]

T: the incisions distances or (inter-plane spacing)

a/p: area per point

p: point count

The following equation was used to calculate ovarian volume parameters (cortex volume, medulla volume, follicles, corpus luteum) using the test point system (Gundersen 1988).

\[ V_v (structure) = \Sigma P \text{ structure} / \Sigma P \text{ ovary} \]

\[ V (structure) = V_v (structure) \times V (ovary) \]

\[ V_v: \text{ Volumetric density of the desired structure} \]

\[ \Sigma P \text{ structure}: \text{The sum of the points that collide with the structure of the ovary} \]

\[ \Sigma P \text{ ovary}: \text{The sum of the points of impact on the ovary} \]

Figure 1. A point grid for calculating the volume of the cortex, the medulla in adult ovaries exposed to lead acetate, the yellow arrow indicates that the right and top angles of a cross are considered for counting (scale bar: 500 μm)
Figure 2. Point grid for calculating corpus luteum volume (A) and follicle volume (B) in adult births exposed to lead acetate, the yellow arrow indicates that the right and top angles of a cross are for counting (scale bar: 100 μm).

Data were analyzed using SPSS software version 16 and averaged with standard deviation tolerance. Normal distribution of data was performed using Kolmogorov-Smirnov test. In case of normal distribution of data from one-way ANOVA statistical test and Tukey supplementary test, and in case of abnormal distribution of data, Kurskal-Wallis and Mann-whithey tests were used. A value of P < 0.05 was considered as a significant criterion.

Results

Total ovarian volume

The total volume of the ovary is (in group 1 or control 0.05 ± 0.01 cm³), in group 2 or pre-pregnancy (0.05 ± 0.00 cm³), in group 3 or pregnancy (0.06 ± 0.01 cm³), in group 4 or lactation (0.06 ± 0.00 cm³), and in group 5 or pre-pregnancy-pregnancy-lactation (0.08 ± 0.02 cm³); and no significant difference was observed between the groups (Figure 2, A).

Ovarian medulla volume

In the study of medulla volume, the results were in groups 1 or control (9.6 ± 1.7%), in group 2 or pre-pregnancy (16.6 ± 4.6%), in group 3 or pregnancy (12.3% ± 5%), in group 4 or lactation (6.8 ± 4.9%), and in group 5 or pre-pregnancy-pregnancy-lactation (11.1 ± 4.7%). No significant difference was observed between the groups (Figure 2, B).

Ovarian cortex volume

Cortex volume was reported in group 1 or control (90.3 ± 1.7%), in group 2 or pre-pregnancy (83.3 ± 4.7%), in group 3 or pregnancy (87.6 ± 5%), in group 4 or lactation (93.1% ± 4.9%), and in group 5 or pre-pregnancy-pregnancy-breastfeeding (88.8 ± 4.7%). No significant difference was observed between the groups (Figure 2, C).

Interstitial tissue volume of the ovary

Interstitial ovarian tissue was in group 1 or control (35.6 ± 1.2%), in group 2 or pre-pregnancy (34.5% ± 0.7%), in group 3 or pregnancy (33 ± 4%), in group 4 or lactation (35.5 ± 1.5%), and in group 5 or pre-pregnancy-pregnancy-lactation (34.9 ± 5.3%); and there was no significant difference between the groups (Figure 2, D).
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The total volume of follicles
The volume of follicles was in group 1 or control (16.1 ± 4.7%), in group 2 (pre-pregnancy) (14.6 ± 2.7%), in group 3 (pregnancy) (10.7 ± 2.8%), in group 4 (lactation) (17.6 ± 4.2%), and in group 5 (pre-pregnancy-pregnancy-lactation) was (11.8 ± 4%); and no significant difference was observed between experimental and control groups (Figure 3, A).

The volume of corpus luteum
The corpus luteum volume in group 1 or control was (35.4 ± 4.1%), in group 2 or pre-pregnancy (29.5% ± 5.5%), in group 3 or pregnancy (38.1% ± 6), in group 4 or lactation (36.6% ± 2.3%), and in group 5 or pre-pregnancy-pregnancy-lactation was (35.3 ± 7.1%).

The results showed that the pre-pregnancy group had a significant decrease compared to the pregnancy group. Also, the pregnancy group showed a significant increase compared to the control group, but the difference was not significant (p = 0.06) (Figure 2, B).

Figure 2 Comparison of total ovarian volume of adult (A), volumetric density of ovarian medulla in adult (B), volumetric density of the ovarian cortex in adults (C), and volumetric density of interstitial tissue (D), Between group 1 (control), group 2 (pre-pregnancy), group 3 (pregnancy-), group 4 (lactation), group 5 (pre-pregnancy-pregnancy-lactation). Mismatched letters indicate a significant difference (p <0.05) between all groups.
Figure 3 Volumetric density of follicles in adults (A) and volumetric density of corpus luteum in adults (B) between group 1 (control), group 2 (pre-pregnancy), group 3 (pregnancy), group 4 (lactation) and group 5 (pre-pregnancy-pregnancy-breastfeeding). Dissimilar letters indicate a significant difference (p<0.05) between all groups.

Discussion

In the present study, the effect of low-dose lead exposure on ovarian tissue was investigated by Stereology Technique. The results showed that the cortex volume in the pre-pregnancy group decreased compared to the control group and other experimental groups and the medulla volume increased, but this difference was not significant. On the other hand, in the lactation group, the cortex volume increased compared to the other groups and the medulla volume decreased, but this difference was not significant. The cause of decreased or increased cortex volume can be inferred due to decreased or increased folliculogenesis because lead in low doses also inhibits the process of folliculogenesis and impairs its function (Taupeau 2001). Interstitial tissue volume decreased in the pregnancy group compared to the control group and other experimental groups but was not significant. In addition, total ovarian volume in the pre-pregnancy-pregnancy-lactation group increased compared to the control group and other experimental groups but was not significant. According to the results of this study and the ability of lead to cross the blood-placental barrier and also the transmission of lead poisoning through milk to infants, the existence of changes in pregnancy and lactation is not far from the mind, but it seems that the amount of destruction and poisoning with lead is directly related to the dose. In other words, the higher the dose of lead, the greater the rate of destruction and its negative effects on ovarian tissue. There are several studies in this field that prove the results of this study and its reasons, including the study of Gertrude et al (Gertrude et al. 1960).

They reported an increase in connective tissue, inhibition of follicle growth and damage to the primordial oocytes in the ovaries of Rhesus monkeys while consuming 10 mg of lead per month. However, they stated that follicular growth had improved immediately after discontinuation of Ovarian lead injection. A study by Sharma et al showed that female mice exposure to 160 mg of lead caused changes and damage to the cortex and ovarian medulla (Sharma et al. 2012). Omar Balubaid in his research showed that lead acetate reduced the interstitial tissue of the ovary (Omar Balubaid 2012). Dahmardeh et al examined the morphometry of the ovary in two periods of 30 and 60 days following the use of oxaliplatin in the pre-pregnancy, pregnancy and lactation periods by stereology technique. They stated in their
results that the total volume of ovaries and cortex in the pregnancy group in the 60-day period was significantly reduced compared to the control group (Dahmardeh et al. 2020).

The results of the present study showed that the total volume of follicles in the pregnancy group decreased compared to the control group and increased in the lactation group compared to other groups but was not significant. According to the results obtained in the lactation group, the increase in cortex volume can be attributed to the increase in follicle volume. Lead has the ability to cross membranes and can accumulate in soft tissues such as the ovaries and directly affect the production of follicles and their growth (Garu et al. 2011) or through the hypothalamic-pituitary-ovarian axis reduces gonadotropin hormone (LH and FSH) and changes in the tissue structure of the ovary, including the number of follicles (Albishtue et al. 2018). It should be noted that Mitotic divisions and the beginning of meiotic division begin in the embryonic period and due to the property of lead in the production of free radicals and apoptosis, it causes ovarian atrophy and reduction of follicles during pregnancy. Numerous studies have been conducted in this field. Junaid et al examined the toxicity of lead in the ovaries and reported that lead reduced the number of follicles (Junaid et al. 1997). Azarnia et al in their study entitled “The protective role of L-cysteine against atresia follicles exposed to lead at a dose of 10 mg in the short term”, showed that the number of initial follicles did not differ in the experimental and control groups (Azarnia et al. 2004). Dorostghoal et al stated that maternal exposure to lead acetate, especially at high doses during lactation, can affect the growth of primary follicles and reduce their number (Dorostghoal et al. 2011). Sharma et al also reported a decrease in the number of follicles following the consumption of lead during pregnancy and lactation (Sharma & et al, 2012). Waseem et al also showed a reduction in follicles following exposure to lead acetate at a dose of 30 mg (Waseem et al. 2014). Sodani also reported a decrease in ovarian follicles following the consumption of low-dose lead acetate (Sodani 2017). Dahmardeh et al reported a decrease in total follicle volume during pregnancy following oxaliplatin administration compared with the control group (Dahmardeh et al. 2020).

The results of the present study showed that the corpus luteum volume decreased subsequently with lead acetate consumption in the pre-pregnancy group in comparison with other groups (control, pregnancy, lactation and pre-pregnancy-lactation). But only in comparison with the pregnancy group, this difference was significant. The corpus luteum volume increased in the pregnant group compared with the control group (p = 0.06) and other experimental groups but was not significant (decreased corpus luteum may be due to the effect of lead on gonadotropin receptors, and affect the production of steroids in the ovaries). In other words, with a decrease in progesterone levels, the corpus luteum also decreases (Nampoothiri and Gupta 2006, Li et al. 2020). The results of the present study were somewhat consistent with the research of (Dorostghoal et al. 2011). They stated that prolonged exposure to lead at a dose of 300 mg during lactation reduces the corpus luteum in the fetus (Dorostghoal et al. 2011). Also, the results of McGivern et al showed that exposure of rats to low-dose lead (2.5 g) during pregnancy reduces the corpus luteum during estrus, which was consistent with the results of the present study (McGivern et al. 1991).

Omar Balubaid, in his study reported a decrease in corpus luteum following exposure to lead which is consistent with the results of the present study (Omar Balubaid 2012). Shubina and Dudenkova in their study entitled Ovarian morphology
and astral cycle of female rats subsequently reported a decrease in corpus luteum with the use of lead acetate, they also showed irregular shapes of yellow bodies in their results (Shubina and Dudenkova 2014). The results of the study by Albishtue et al showed that the corpus luteum was reduced by exposure to lead acetate at a dose of 10 mg (Albishtue et al. 2018). Dahmardeh et al stated that the corpus luteum volume following oxaliplatin consumption in 60-day-old offspring of the pre-pregnancy, pregnancy and lactation groups was not significantly different from the control group (Dahmardeh et al. 2020). Although the present study did not contradict previous studies, but it seems that the reason for the difference in results is due to the low dose of lead consumed, different phases of the estrous cycle at the time of animal sampling.

Extensive studies have been conducted on the effect of lead acetate on different organs of the body, especially female reproductive system during Pregnancy and Lactation. However, no comprehensive research has been conducted on offspring that their mothers received lead during Pregnancy or Pre-pregnancy-pregnancy-lactation periods and their results were not compared with pregnancy and lactation periods (Especially with the stereology technique).

As stated in previous studies, lead in high doses and over a long period of time can damage ovarian tissue. In fact, exposure to lead reduces glutathione levels and increases Malondialdehyde (MDA) levels in the ovary. Lead inhibits glutathione synthetase and reductase enzymes (which are involved in glutathione metabolism). On the other hand, due to exposure to lead, changes in pituitary gonadotropin or steroid secretion are made and through the hypothalamus axis or disturbance in the balance of antioxidants, it exerts its negative effects and causes changes in ovarian tissue.

According to previous studies and the results of this study, it can be stated that the toxic effects of lead are dose-dependent and its destructive effects are directly related to the dosage, which can cause adverse effects on total ovarian volume, medulla and cortex volume, interstitial tissue volume and corpus luteum volume. The results of this study showed that exposure to lead with low dose of 0.2% reduced corpus luteum volume but did not affect total ovarian volume, cortex volume, medulla and total volume of follicles and over time and stay away from lead, its negative effects will be improved.

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Conflict of Interest
There’s no conflict of interest between authors.

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References


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مطالعه هیستوتومتری تخمدان موالید در موش‌های صحرایی مواجهه شده با استات سرب با استفاده از تکنیک استریولوژی

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چکیده

با گسترش فرآیندها صنعتی، آلودگی با فلزات سنگین به عنوان مثال سرب، به یک مشکل جدی تبدیل شده است. این‌ها توانایی عبور از ارگان‌ها را داشته و سبب اختلال در عملکرد آن‌ها می‌شود. یکی از ارگان‌های مورد هدف سرب بافت تخمدان است. همچنین سرب قادر است از سد خویش جنگی عبور و به جنس متنقل شود. همچنین سرب از طریق شیر به نوزاد منتقل می‌شود. همچنین سرب از طریق شیر به نوزاد امکان پذیر است. همچنین سرب از طریق شیر به نوزاد امکان پذیر است. این مطالعه جهت بررسی اثر آلودگی سرب با دوز پایین در مادر بر روی تکیه‌گاه موالید با استفاده از تکنیک استریولوژی در مدل حیاتی رت انجام شد. در پژوهش، موش‌های صحرایی نژاد ویستار به صورت تصادفی به پنج گروه تقسیم‌بندی شدند. گروه‌ها شامل کنترل و 1، 2، 3، 4 و 5 گروه تجربی پیش‌آبستنی، پیش‌آبستنی، پیش‌آبستنی، پیش‌آبستنی و پیش‌آبستنی-آبستنی بودند. بررسی حجم جسم زرد نشان داد که گروه پیش‌آبستنی در مقایسه با گروه آبستنی کاهش معنی‌داری کاهش حجم جسم زرد نشان داد. مطالعه حاضر نشان داد که مواجهه مادران با استات سرب با دوز کم سبب کاهش حجم جسم زرد موالید به خصوص در دوران پیش‌آبستنی می‌شود.

کلمات کلیدی: استات سرب، تخمدان، استریولوژی، موش صحرایی

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