

## Effects of long-term exposure of female rats to low levels of lead: ovary and uterus histological architecture changes

Eugenia DUMITRESCU<sup>1</sup>, Viorica CHIURCIU<sup>2</sup>, Florin MUSELIN<sup>3</sup>, Roxana POPESCU<sup>4</sup>, Diana BREZOVAN<sup>5</sup>, Romeo T. CRISTINA<sup>1</sup>

<sup>1</sup>Pharmacology and Pharmacy Departments, Faculty of Veterinary Medicine, Banat's University of Agriculture and Veterinary Medicine "King Michael I of Romania", Timisoara, Romania

<sup>2</sup>Drugs Production Department, Romvac Company, Voluntari, Romania

<sup>3</sup>Veterinary Toxicology Department, Faculty of Veterinary Medicine, Banat's University of Agriculture and Veterinary Medicine "King Michael I of Romania", Timisoara, Romania

<sup>4</sup>Cellular and Molecular Biology Department, University of Medicine and Pharmacy "Victor Babes", Timisoara, Romania

<sup>5</sup>Histology and Molecular Biology Department, Faculty of Veterinary Medicine, Banat's University of Agriculture and Veterinary Medicine "King Michael I of Romania", Timisoara, Romania

Received: 02.07.2014 • Accepted: 10.09.2014 • Published Online: 01.04.2015 • Printed: 30.04.2015

**Abstract:** The aim of the current study was to evaluate lead accumulation in the ovaries, fallopian tubes, and uterus and to take note of any consequent histo-architectural changes. The experiment involved a 12-month chronic exposure of 28 Wistar female rats at sexual maturity ( $221 \pm 0.88$  g/individual) to lead acetate in drinking water. The rats were divided into 4 groups based on the level of lead exposure: E1 at  $0.050$  mg L<sup>-1</sup>, E2 at  $0.100$  mg L<sup>-1</sup>, E3 at  $0.150$  mg L<sup>-1</sup>, and a control group that received tap water. Lead level evaluation was performed by atomic absorption spectrometry at 283.3 nm and the histo-architectonics in target organs were evaluated after hematoxylin and eosin staining and microscopy. The exposure to lead acetate produced significant histological alterations caused by lead accumulation in the sexual organs. These structural changes correlated with the level of exposure in the ovaries, uterus, and fallopian tubes. They were mainly edemas and necrosis, denudation, and/or different stages of follicle evolution. These alterations have been shown to indicate infertility in female rats.

**Key words:** Histo-architecture, lead, rat model, reproductive toxicology

### 1. Introduction

Lead is a heavy metal that is widely dispersed in the environment and remains in biotopes for long periods of time (Gidlow, 2004). Cases of high levels of lead exposure can be found in industrial areas and are more common in developing countries (ATSDR, 2007). Compared with other metals, lead does not play any physiological role in the body and is considered toxic even in small doses. Lead affects the cardiovascular, gastrointestinal, urinary, nervous, and reproductive systems. Exposure to lead usually occurs via dermal contact, oral ingestion, or inhalation (Gidlow, 2004). Lead is also involved in transplacental congenital intoxication (Taupeau et al., 2001). The available toxicology information about the effects of lead on the mammalian female reproductive system is sparser than what is found regarding the male system. The differences in the effects of lead on these systems are significant, particularly in terms of gametogenesis and the cyclic nature of the female reproductive function (Andrews, 1993).

For example, Borja-Aburto et al. (1999) argued that abortion and preterm delivery are the most reported effects of exposure to high lead levels in women. Decreased fertility has also been associated with continued exposure to lead and high levels of lead in the blood. Abortion, preterm delivery, and decreased fertility in women have been associated with blood lead levels above  $12$   $\mu$ g and  $30$   $\mu$ g dL<sup>-1</sup>. Similar values were reported concerning rats by Hilderbrand et al. (1973).

In order to protect the developing fetus, EU and US laws regarding lead industry workers include lower exposure criteria for women in 'reproductive capacity'. In Europe, the maximum permissible limit of lead in the blood is  $100$  mg L<sup>-1</sup>, and in the United States it is  $28$  mg L<sup>-1</sup>. Therefore, the assessment of fertility indicators and reproductive functions is of great importance in the toxicity evaluation of substances involved in reproduction (Gidlow, 2004).

There is a need to better understand the vulnerability of ovarian cells and sexual organs to lead and to clearly

\* Correspondence: [rtcristina@yahoo.com](mailto:rtcristina@yahoo.com)

demonstrate that oocytes can be damaged or destroyed by such an agent. The purpose of the present study was to evaluate lead accumulation in the ovaries, fallopian tubes, and uterus and to observe any consequent histo-architectural changes following 12 months of chronic exposure to lead. This is a follow-up study of our prior research on female rats and lead, including in utero exposure to lead.

## 2. Materials and methods

During the experiment, the following directives were respected: Council Directive 86/609/EEC, the European Convention principles for the protection of vertebrate animals used for experimental and other scientific purposes, adopted in 1986 in Strasbourg (European Commission, 1986); and Directive 2010/63/EU, 2010 of the European Parliament and the European Council adopted 22 September 2010, on the protection of animals used for scientific purposes (European Commission, 2010). The experiment was conducted in accordance with Romanian law on animal experimentation (Romanian Government, 2002a) and with the permission of the Scientific Ethics Committee of the Faculty of Veterinary Medicine in Timisoara.

### 2.1. Animals

All healthy animals were purchased from the authorized biobase of "Victor Babes" University of Medicine and Pharmacy in Timisoara. Our primary concern was to choose female specimens with approximately the same body weight. Before starting the experiment, the animals were subjected to a 7-day period of acclimatization, their health was clinically confirmed, and they were kept in the same room for the entire duration of the experiment. The specimens were housed in 4 polycarbonate cages with the following dimensions: 750 × 720 × 360 mm (L × W × H), 8 females in each cage/group. Wood shavings were used as bedding. The environmental temperature was maintained at 20 ± 2 °C at a relative humidity of 55 ± 10% and a 12/12-h light/dark cycle (NRC, 1996).

A nonsterile pelleted diet (Diet, Biovetimix, code 140-501, Romania) and tap water were offered ad libitum. The evaluation of the toxic effects of lead on the integrity of the reproductive system was carried out on 28 white Wistar female rats at sexual maturity (120 days old) divided into 4 groups: 3 experimental group (E1, E2, E3) and 1 control group (C). The registered average body weight for the groups was: E1 = 220.0 ± 0.72 g; E2 = 222.5 ± 1.16 g; E3 = 220.5 ± 0.82 g; and C = 221.0 ± 0.82 g.

The individuals from the E groups were exposed to lead as a soluble lead acetate (Merck, Darmstadt, Germany) in drinking water, administered ad libitum (from 2-L volume vessels, with the water solution being daily refreshed), for 12 months as follows: E1 at 0.050 mg

L<sup>-1</sup> (maximum admitted level in drinking water, according to the Romanian drinking water quality law) (Romanian Government, 2002b), E2 at 0.100 mg L<sup>-1</sup>, and E3 at 0.150 mg L<sup>-1</sup>.

The control group received only tap water ad libitum; all other conditions were kept the same. At 24 h following the last administration (day 361), all rats were euthanized in the same time period, from 0800 to 0900 hours, by overdosing anesthetic agents using 300 mg kg bw<sup>-1</sup> of ketamine (Ketamine 10%, CP Pharma, Burgdorf, Germany) and 30 mg kg bw<sup>-1</sup> of xylazine (Narcoxyll, Intervet International, Boxmeer, the Netherlands), in accordance with Directive 2010/63/EU (European Commission, 2010), and SVH AEC SOP.26, Euthanasia of Mice and Rats (Pierce, 2006).

### 2.2. Organ sampling and histological examination

Ovaries and uteri with fallopian tubes were freshly collected and fixed in alcohol (80% vol.) to be prepared histologically. Following sampling, a cytohistological examination was performed on the ovaries and separately on the uteri and the fallopian tubes. Fragments of tissue were fixed in 80% alcohol. Paraffin blocks containing tissue fragments were sectioned using a microtome, resulting in 5-µm-thick sections. The sections were stained by the hematoxylin and eosin method (Şincai, 2000). All histological images were captured using the Olympus CX 41 software program, at a magnification of 100×.

### 2.3. Sample digestion and atomic absorption spectrometry

Sample digestion was achieved using a CEM Mars X microwave digestion oven (CEM Microwave Technology Ltd., Buckingham, UK). Samples from the sexual organs (ovary and uterus with fallopian tubes) (1.0 g) were placed separately into digestion flasks with 10 mL of nitric acid (Merck, Germany) and 5 mL of perhydrols at 600 W for 20 min at 120 °C. The evaluation of lead levels in the sexual organs was performed after digestion by atomic absorption spectrometry (AAS), using an AA240 Zeeman with a graphite furnace (Varian Instruments Inc., Palo Alto, CA, USA) and a programmable sample dispenser (PSD 120) (with a detection limit of 0.06 µg L<sup>-1</sup>). The absorbance was determined by peak measuring and the concentration was achieved based on the new rational calibration algorithm in µg L<sup>-1</sup> units. The calibration curve was made to a 283.3 nm wavelength on 5 standard lead element levels in 0.1% HNO<sub>3</sub> with slit width of 0.5 nm. A recalibration was done every 10 measurements (RSD < 10%).

### 2.4. Statistical analysis

All data were analyzed using GraphPad Prism 5.0 (San Diego, CA, USA). The data in the different groups were compared by a one-way ANOVA with Bonferroni post hoc test. Statistical differences were considered significant when P < 0.05.

### 3. Results

The registered values of lead levels found in female rat ovaries, uteri, and fallopian tubes after 12 months of chronic exposure to lead acetate are presented in Figure 1.

#### 3.1. Ovaries

Compared with the control group, exposure to lead acetate showed a statistically significant increase of lead concentration ( $P < 0.001$ ) in almost all experimental groups as follows: E1 vs. C: +42.13%; E2 vs. C: 6.7-fold higher; E3 vs. C: 9.4 fold higher. The obtained results increased significantly ( $P < 0.001$ ) with the exposure level: E2 vs. E1: 4.4-fold greater; E3 vs. E2: 0.3-fold greater; E3 vs. E1: 6.3-fold greater.

#### 3.2. Uterus and fallopian tubes

The lead levels were also significantly higher ( $P < 0.01$ ) in the experimental groups versus the control (E1 vs. C: 3.6-fold greater; E<sub>2</sub> vs. C: 7.7-fold greater; E3 vs. C: 13.9-fold greater). They increased significantly ( $P < 0.001$ ) with the level of exposure (E2 vs. E1: 0.8-fold greater; E3 vs. E2: 0.7-fold greater; and E3 vs. E1: 2.2-fold greater).

The histological alterations of the studied organs are presented in Figure 2.

A large number of ovarian follicles in different stages of evolution were detected by a microscopic examination of the control group samples. Histological investigation of the ovaries from the control group revealed the presence of ovarian follicles in different stages of evolution, with primordial follicles and antral follicles. A microscopic examination of the uterus and the fallopian tubes of the controls revealed the presence of a normal structure of mucosa and uterine glands (Figures 2A and 2B).

Following exposure to 0.050 mg L<sup>-1</sup> lead, some areas with optical empty spaces were present in the ovarian tissue, as well as diffuse edemas and ovarian follicle denudation (Figure 2C).

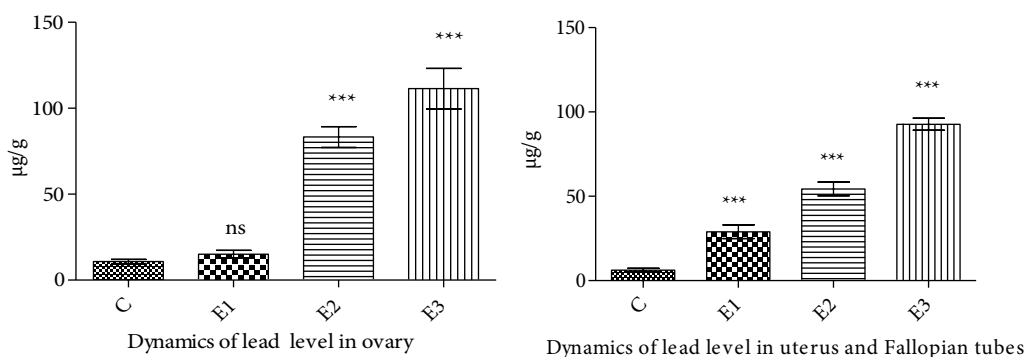
In Figure 2D, zones of necrosis can be seen in the uterus and fallopian tubes exposed to 0.050 mg L<sup>-1</sup> of lead.

Following exposure to 0.100 mg L<sup>-1</sup> of lead, the ovaries presented with large zones of necrosis and follicular edema (Figure 2E). The uterus and fallopian tubes, at the same level of exposure, revealed necrosis of the uterine glands (Figure 2F).

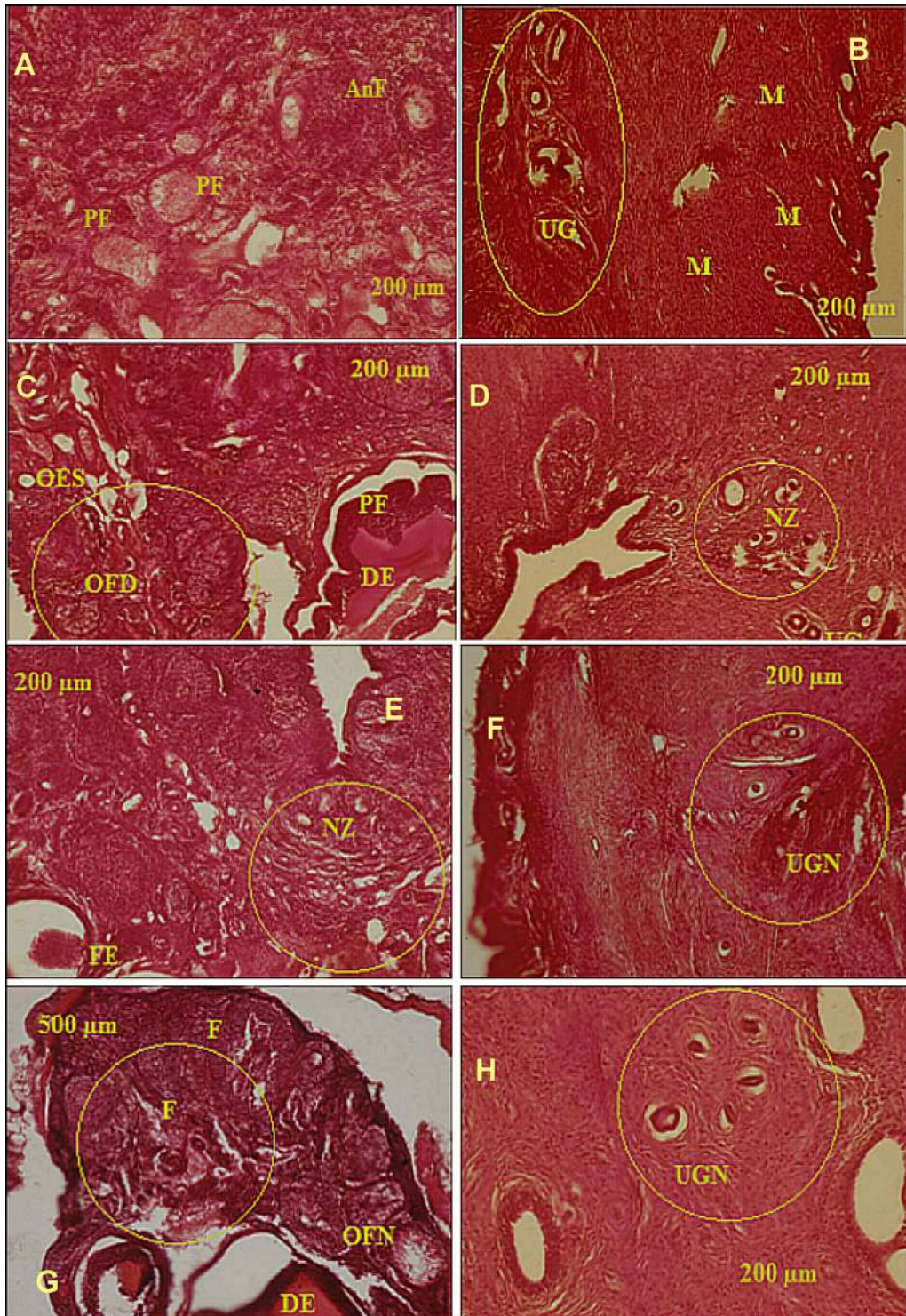
The histological sections of the ovary following exposure to 0.150 mg L<sup>-1</sup> of lead revealed the most noticeable alterations to this organ: edemas and necrosis of the ovarian follicles (Figure 2G). Finally, the microscopic examination of the uterus and fallopian tubes following exposure to 0.150 mg L<sup>-1</sup> lead also revealed necrosis of the uterine glands (Figure 2H).

### 4. Discussion

So far, most research on female specimens' exposure to lead has focused on clinically visible effects such as miscarriage, premature delivery, and infant mortality in humans and animals (Taupeau et al., 2001). Information about the presence of lead in the ovaries, fallopian tubes, and uterus is sparse, yet multiple authors have identified the presence of lead in the follicular fluid and showed that lead levels are higher in pregnant females as compared with nonpregnant cohorts. This strongly suggests that high levels of lead are linked to altered reproductive function (Piasek and Kostial, 1991; Taupeau et al., 2001; Silberstein et al., 2006). Dhir and Dhand (2010) reported that ovarian atresia was present in female rats following chronic exposure to lead, a finding also confirmed by other authors (Taupeau et al., 2001; Qureshi et al., 2010; Sharma et al., 2012). In a previous study we ascertained that when lead acetate was administered for a long period of time (12 months) to female rats, important changes in the rat serum panel took place. Those changes directly correlated with the different exposure levels ( $P < 0.01$ ). Compared with the controls, the serum levels in the experimental groups significantly decreased for follicle-stimulating hormone, significantly increased for luteinizing hormone (LH) and testosterone,



**Figure 1.** Registered lead level concentration values and lead's dynamics in the ovaries, uterus and fallopian tubes in the experimental groups as compared with the controls. ns: not significant; \*\*\*: highly significant ( $P < 0.05$ ).



**Figure 2.** The main histologic alterations observed in female rat ovaries, uterus, and fallopian tubes after different doses of lead acetate (H&E stain, 100 $\times$ ). **A)** Ovaries from the control group reveal the presence of ovarian follicles in different stages of evolution (PF- primordial follicles; AnF- antral follicles). **B)** Uterus from the control group reveals the presence of normal mucosa structure (M) and uterine glands (UG). **C)** Ovary image following 0.050 mg L<sup>-1</sup> lead exposure (OES- optical empty spaces; DE- diffuse edema; PF- primordial ovarian follicles; OFD- ovarian follicle denudation). **D)** Uterus following exposure to 0.050 mg L<sup>-1</sup> lead (NZ- necrotic zone; UG- uterine glands). **E)** Ovary following exposure to 0.100 mg L<sup>-1</sup> lead (NZ- necrosis zone; FE- follicular edema). **F)** Uterus following exposure to 0.100 mg L<sup>-1</sup> lead; (UGN- uterine glands necrosis). **G)** Ovary following exposure to 0.150 mg L<sup>-1</sup> lead (DE- diffuse edemas; OFN- necrosis of ovarian follicles; F- follicles in different stages of evolution). **H)** Uterus following 0.150 mg L<sup>-1</sup> lead exposure (UGN- uterine glands necrosis).

and significantly decreased for estradiol and progesterone (Dumitrescu et al., 2014).

Moreover, we observed that all these paraclinical results were also morphologically visible through alterations in interested organs and tissues, inducing changes in the reproductive system integrity biomarkers. The main structural changes we found in the ovaries were diffuse edema, necrosis in the ovarian follicles, optical empty spaces, denudation of the ovarian follicles, and different stages of follicle evolution. The main changes in the uterus and fallopian tubes were necrosis areas and necrosis of the uterine glands. Shah et al. (2008) found that after oral administration of high doses of lead, a reduced number of ovarian follicles and an increased number of atretic follicles were observed in all cases. It can be noted that the effects of lead on reproductive systems are complex and sex-specific, and they seem to involve multiple locations on the hypothalamic–pituitary–gonadal axis, confirming our findings on female rats.

We also agree with the research of Winder (1993), who ascertained that the body is most sensitive to lead exposure during its developing phase, when sexual maturity in all instances is delayed, and with other authors who suggested that intrauterine exposure to lead leads to a decrease in fetal weight (11% to 13%) and a reduced number of implantation sites in the uterus (Junaid et al., 1997; Nampoothiri and Gupta, 2006). Additionally, Saritha et al. (2012) showed that the exposure of female rats to lead in the perinatal period significantly increased the sexual cycle duration, which was also correlated with a decreased number of implantation sites, a fact also confirmed by our team in previous uncited studies. .

Franks et al. (1989) demonstrated that lead has adverse, measurable effects on the ovarian function in human reproduction. Tang and Zhu (2003) reported that women's occupational exposure to lead is undoubtedly

related to reproductive impairments. Baghurst et al. (1991) found great quantities of lead in the placental membranes. Tchernitchin et al. (2011, 2013) argued that prenatal exposure to lead in women, and also in female rats, can lead to decreased fertility, making a link between primate and murine fertility issues.

Evidence of the direct effects of lead exposure on the ovaries of murine females was also described in other extensive studies (Taupeau et al., 2001; Shah et al., 2008).

From a hormonal point of view, we agree that lead effects on steroids were accompanied by effects on LH hypophysary levels, suggesting a double action: upon the hypophysal-hypothalamic structural unit, or directly upon gonadal steroid synthesis. These effects were followed by histo-architecture changes (Andrews, 1993; Winder, 1993).

Lead toxicity is also known to significantly affect the red blood cells. Lead-associated changes in the nervous system, the kidneys, and the reproductive system reported in the literature show the importance and versatility of lead effects in mammals (Hilderbrand et al., 1973; Piasek and Kostial, 1991; Borja-Aburto et al., 1999).

The obtained lead values and the histomorphological structural changes found in the current study in the ovaries, the uterus, and the fallopian tubes of female rats have demonstrated the deleterious effects of lead. Based on these findings, we recommend the use of these exposure and integrity biomarkers of the reproductive system as early detection parameters of lead toxicity in lab animals.

### Acknowledgments

This work was cofinanced by the European Social Fund through the Sectorial Operational Program and Human Resources Development 2007-2013, POSDRU/89/1.5/S/62371 “Postdoctoral School of Agriculture and Veterinary Medicine”, USAMVB, Timisoara, Romania.

### References

- Andrews JS (1993). *Biologic Monitoring and Biomarkers*. ATSDR - Hazardous Waste Conference. Atlanta, GA, USA: Agency for Toxic Substances and Disease Registry.
- ATSDR (2007). *Toxicological Profile for Lead*. Atlanta, GA, USA: Agency for Toxic Substances and Disease Registry.
- Baghurst PA, Robertson EF, Oldfield RK, King BM, McMichael AJ, Vimpani GV, Wigg NR (1991). Lead in the placenta, membranes, and umbilical cord in relation to pregnancy outcome in a lead-smelter community. *Environ Health Pesp* 90: 315–320.
- Borja-Aburto VH, Hertz-Picciotto I, Rojas-Lopez MR, Farias P, Rios C, Blanco J (1999). Blood lead levels measured prospectively and risk of spontaneous abortion. *Am J Epidemiol* 18: 590–597.
- Dhir V, Dhand P (2010). Toxicological approach in chronic exposure to lead on reproductive functions in female rats (*Rattus norvegicus*). *Toxicol Int* 17: 1–7.
- Dumitrescu E, Cristina RT, Muselin F (2014). Reproductive biology study of dynamics of female sexual hormones: a 12-month exposure to lead acetate rat model. *Turk J Biol* 38: 581–585.
- European Commission (1986). Council Directive 86/609/EEC on the Approximation of Laws, Regulations, and Administrative Provisions of the Member States Regarding the Protection of Animals Used for Experimental and Other Scientific Purposes. Brussels, Belgium: European Commission.
- European Commission (2010). Directive 2010/63/EU of the European Parliament and the Council of 22 September 2010 on the protection of animals used for scientific purposes. Brussels, Belgium: European Commission.

- Franks AP, Laughlin NK, Dierschke DJ, Bowman RE, Meller PA (1989). Effects of lead on luteal function in *Rhesus* monkeys. *Biol Reprod* 41: 1055–1062.
- Gidlow DA (2004). Lead toxicity. *Occup Med-Oxford* 54: 76–81.
- Hilderbrand DC, Der R, Griffin WT, Fahim MS (1973). Effect of lead acetate on reproduction. *Am J Obstet Gynecol* 115: 1058–1065.
- Junaid M, Chowdhuri DK, Narayan R, Shanker R, Saxena DK (1997). Lead-induced changes in ovarian follicular development and maturation in mice. *J Toxicol Env Health* 1997; 50: 31–40.
- Nampoothiri LP, Gupta S (2006). Simultaneous effect of lead and cadmium on granulosa cells: a cellular model for ovarian toxicity. *Reprod Toxicol* 21: 179–185.
- NRC (1996). Guide for Care and Use of Laboratory Animals. 8th ed. Washington, DC, USA: The National Academies Press, pp 21–55.
- Piasek M, Kostial K (1991). Reversibility of the effects of lead on the reproductive performance of female rats. *Reprod Toxicol* 5: 45–51.
- Pierce S (2006). SVH AEC SOP.26. Euthanasia of Mice and Rats. Melbourne, Australia: Animal Ethics Committee of St. Vincent's Hospital.
- Qureshi N, Sharma R, Mogra S, Panwar K (2010). Amelioration of lead induced alterations in ovary of Swiss mice, by antioxidant vitamins. *J Herb Med Toxicol* 4: 89–95.
- Romanian Government (2002a). Law No. 471 of July 9th, 2002 Approving Government Ordinance No. 37/2002 for the Protection of Animals Used for Scientific or Other Experimental Purposes. Bucharest, Romania: Government of Romania.
- Romanian Government (2002b). Legea 458. Privind calitatea apei potabile (Romanian Law 458, About Drinking Water Quality). Bucharest, Romania: Government of Romania (in Romanian).
- Saritha S, Reddy PS, Reddy GR (2011). Partial recovery of suppressed reproduction by *Withania somnifera* Dunal in female rats following perinatal lead exposure. *Int J Green Pharm* 5: 121–125.
- Shah AS, Shariff MM, Khan AS, Tayyab M, Chaudary AN, Ahmed N (2008). Correlation of blood lead levels with atresia of ovarian follicles of albino mice. *Ann Pak Inst Med Sci* 4: 188–192.
- Sharma R, Qureshi N, Mogra S, Panwar K (2012). Lead induced infertility in Swiss mice and role of antioxidants. *Univ J Environ Res Technol* 2: 72–82.
- Silberstein T, Saphier O, Paz-Tal O, Trimarchi JR, Gonzales L, Keefe DL (2006). Lead concentrates in ovarian follicle compromises pregnancy. *J Trace Elem Med Biol* 220: 205–207.
- Șincai M (2000). Tehnici de citohistologie normală și patologică. Timisoara, Romania: Ed. Mirton (in Romanian).
- Tang N, Zhu ZQ (2003). Adverse reproductive effects in female workers of lead battery plants. *Int J Occup Med Env* 16: 359–361.
- Taupeau C, Poupon J, Nome F, Lefevre B (2001). Lead accumulation in the mouse ovary after treatment-induced follicular atresia. *Reprod Toxicol* 15: 385–391.
- Tchernitchin AN, Gaete L, Bustamante R, Sorokin YA (2003). Adulthood Prenatally Programmed Diseases: Health Relevance and Methods of Study. Hong Kong: iConcept Press.
- Tchernitchin AN, Gaete L, Bustamante R, Baez A (2011). Effects of prenatal exposure to lead on estrogen action in the prepubertal rat uterus. *Obstet Gynecol* 2011: 329692.
- Winder C (1993). Lead, reproduction and development. *Neurotoxicology* 14: 303–317.