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Study on the effect of maternal administration of oxaliplatin on offspring testes using unbiased design-based stereology

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Abstract

Oxaliplatin (Ox) is widely used for the treatment of various tumors. Since Ox prevents DNA replication and transcription, it may affect organs with rapid cell divisions such as the testes. Although its use during pregnancy has been reported, no information regarding its effects on the testes of the offspring is not available yet. Thirty-two mice were randomly divided into four groups. The control group (1) was administered intraperitoneally 0.2 ml of saline three times a week for the 21 days of pre-pregnancy, pregnancy and lactation. Experimental groups 2, 3 and 4 received 3 mg/kg of oxaliplatin three times a week for 21 days during pre-pregnancy, pregnancy and lactation, respectively. The left testis was removed from male offspring 30 and 60 days after birth. The volume of the testes, seminiferous tubules and interstitial tissue, the surface area and height of the seminiferous epithelium, as well as the length and diameter of seminiferous tubules, were analyzed by means of stereology. Results showed a decrease in the evaluated parameters in experimental groups, in comparison with the control group. Due to the ameliorating effect of Ox on offspring testes, cautiousness is needed during maternal administration in order to preserve the fertility of male offspring.

Keywords: oxaliplatin, perinatal administration, stereology, testis

1. Introduction

Oxaliplatin (Ox) is a third-generation platinum anti-neoplastic drug which is mainly used alongside other chemotherapeutic agents, such as 5-fluorouracil and folinic acid, to treat various cancers in both pediatric and adult patients (Bano et al., 2016; Geohagen et al., 2020). It is used for the treatment of the tumors of the colon (Demontoux et al., 2019), stomach (Bang et al., 2012), ovary (Bogliolo et al., 2015), liver (Shi et al., 2019), pancreas (Springfeld et al., 2019) and lung (Scagliotti et al., 2005).

Although the positive effects of Ox have been proven, it is known to have toxic side effects such as peripheral neuropathy, gastrointestinal and liver toxicity (Hoff et al., 2012), hematological toxicity (Moskovitz et al., 2015), pulmonary toxicity (Moskovitz et al., 2015), gonadal toxicity (Levi et al., 2015) and, rarely, ototoxicity (Güvenç et al., 2016; Perde-Schrepler et al., 2020), thus limiting the use of this drug.

Ox carries out its anti-cancer effect by causing cytotoxicity through the prevention of DNA replication (Alian et al., 2012) and the interruption of the base stacking of DNA in the platination process (Zou et al., 2016). This mechanism may affect organs with rapid cell divisions such as the testes. Consistently, Chater et al. (2007) showed the apoptotic effect of platinum compounds including Ox on testicular germ cells. Levi et al. (2015), demonstrated that Ox affects the weight of the testes and epididymites, as well as sperm concentration, and causes pathological lesions in the seminiferous tubules.

Until now, researches have reported the effects of oxaliplatin on adult testes; however, there is no detailed information in the literature regarding its effects on offspring testes in case of prenatal administration. Although the FDA rated Ox as pregnancy category D due to its risks of fetal injury, there are still several reports describing its use during pregnancy thanks to its benefits (Spanos et al., 2008; Gensheimer et al., 2009; Kanate et al., 2009; Makoshi et al., 2015). Given the growing interest in research in the field of fertility preservation strategies in cancer patients (Le Bihan-Benjamin et al., 2018; Selter et al., 2019), this study aimed at evaluating, by means of unbiased design-based stereology, the testicular morphometrical features in the offspring of mice

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which were administered Ox during the prenatal period.

Stereology is an unbiased technique that can transform 2D- into 3D data and provide quantitative information on a tissue as a whole (Théroux-Rancourt et al., 2019). This method is applicable to reproductive medicine for an accurate determination of the length, surface and size of testicular structures (Liew et al., 2013).

In the present study, the volume of the testes, seminiferous tubules and interstitial tissue, surface area and height of seminiferous epithelium, as well as the length of seminiferous tubules, were analyzed by means of stereology on the testes of 30- and 60-days old offspring, after maternal administration of oxaliplatin during pre-pregnancy, pregnancy and lactation.

2. Materials and methods

2.1. Experimental Animals

Male and female NMRI mice were purchased from the Pasteur Institute of Iran. The animals were housed in cages and given ad libitum access to food and water. Environmental temperature was maintained between 23–25°C while a 12 h light / 12 h dark daily cycle was initiated. The experiment was in accordance with the standard guide for the care and use of laboratory animals (Faculty of veterinary medicine, University of Tehran, Tehran, Iran. N. 6067543). After the mice had adapted to their environment, they were prepared for mating by placing two females with one male in each cage. The vaginal plaques were examined 12 h after mating and the pregnant mice were separated and were considered to be at day 0 of gestation.

2.2. Experimental design

Thirty-two animals were randomly divided into 4 groups of animals including group 1 (control), group 2 (pre-pregnancy), group 3 (pregnancy) and group 4 (lactation). The control group were administered intraperitoneally (IP) 0.2 ml of saline three times a week for 21 days during the 21 days of the pre-pregnancy, pregnancy and lactation periods. The mice from experimental groups 2, 3 and 4 were administered 3 mg/kg of oxaliplatin three times a week for 21 days, during pre-pregnancy, pregnancy and lactation, respectively. The drug dosing schedule was based on Wafai et al. (2013) and illustrated in Figure 1. 21 days after birth, neonates were separated from their mothers and kept in separate cages.

2.3. Tissue sampling and stereological methods

The male offspring were euthanized by cervical dislocation 30 and 60 days after birth. The left testis was removed, weighed and then immersed in buffer formalin solution for fixation. The orientator method was employed for obtaining isotropic uniform slabs from each testis which is necessary for the estimation of length and surface area. Briefly, each testis was embedded in agar and placed on a first circle divided into 10 equal parts. A random number between 0 and 10 was selected and the testis was cut into two halves in the direction of the selected number. The cut surface of each half was placed on a second circle unequally divided into 10 parts on its perimeter. Again, a random number was selected and each half was cut into several parallel slabs in the direction of the selected number in order to obtain 8-10 slabs from each testis (Noorafshan, 2014).

The slabs were routinely processed for light microscopy, embedded in paraffin and cut into 5 µm-thick sections. Afterwards, the sections were stained using Hematoxylin-eosin. A microscope (CX40, Olympus) connected to a digital camera (MB-2250, Germany) was employed in order to capture systematic uniform random fields of view by moving the microscope stage in equal step lengths in the x and y axis directions for each section. Geometrical probes were created using ImageJ software (https://imagej.nih.gov/ij/).

The total volume of each testis was calculated by transforming its weight into a volume using the specific testis tissue density:

$$V(testis) = W(testis) / \rho,$$

where ρ is the weight to volume ratio of testicular tissue (1.04 g/cm^3).

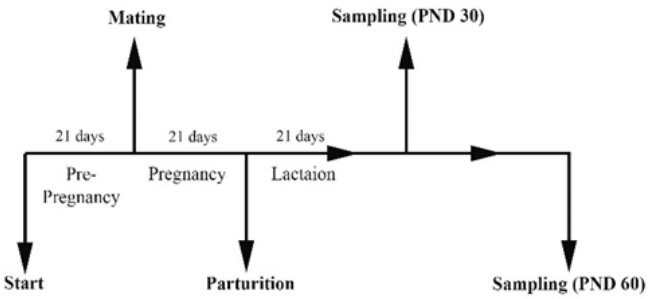

Fig.1. Schematic representation of the experimental procedure

2.4. Volume fraction testis structures

The volume fraction of testicular structures (seminiferous tubules, interstitial tissue and germinal layer) was estimated by applying the test point system (Figure 2) and the following formula (Gundersen et al., 1988).

$$Vv\ (structure) = \sum P\ structure / \sum P\ testis$$

where ΣP structure is the total number of points hitting seminiferous tubules, interstitial tissue or germinal layer in the testis and ΣP testis is the total number of points hitting the whole testis. Subsequently, the total volume of each structure was calculated by multiplying the volume fraction by the volume of the testis.

2.5. Length of seminiferous tubules

The length density of the seminiferous tubules was estimated by superimposing an unbiased counting frame on each microscopic field of view. Then, the tubule profiles which were inside the counting frame or touched the inclusion line and did not touch the exclusion lines were counted (Figure 3) and the length density was calculated as:

$$Lv\ (seminiferous\ tubules) = 2\sum Q / (\sum P.\ a/p)$$

where ∑Q is the total number of the counted profiles, ∑P is the total number of counted frames and a/p is the area per frame (Gundersen et al., 1988). The total length was subsequently obtained by multiplying the length density by the total volume of the testis.

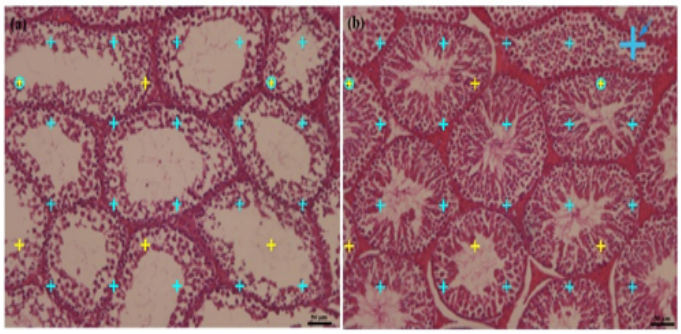

Fig. 2. Estimation of the volume fractions using the point counting system in 30-day (a) and 60-day old (b) mice. The encircled points represent the collision sites with the testis, the full points (blue) those with the tubules, and the dense points (yellow) with the interstitial tissue. The right upper corner of each point (arrow) hitting the structure was considered.

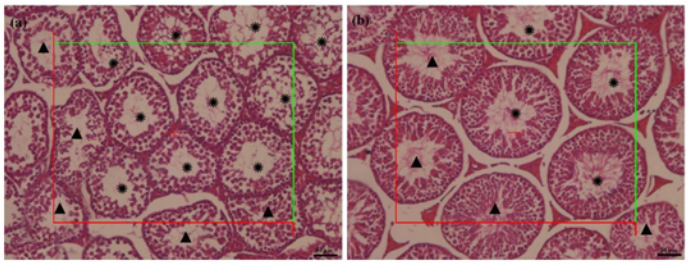

Fig. 3. Estimation of the length density of seminiferous tubules using the unbiased-counting frame in 30- (a) and 60-day old (b) mice. The tubule profiles included in the frame or touching the accepted lines were counted (asterisk), while those outside of the frame or touching the forbidden lines were not (arrowheads).

2.6. Diameter of seminiferous tubules

The diameter of the seminiferous tubules was estimated for the tubules sampled by employing the counting frame principle. The largest diameter perpendicular to the longest axis of the tubule passing over the center of tubule was considered as its diameter (Noorafshan, 2014).

2.7. Surface area of germinal layer

Test lines were superimposed on selected microscopic fields of view (Fig. 4) in order to obtain the surface density of the germinal layer using the following formula (Gundersen et al., 1988): Sv (germinal layer) = 2∑l / (∑P. l/p) where ∑l is the number of intersections of the luminal surface of the germinal layer and the test lines, ∑P is the number of points hitting the germinal layer and l/p represents the length of each test line associated to each point of the test grid.

The surface area of seminiferous tubules was calculated by multiplying the surface density by the volume of the germinal layer.

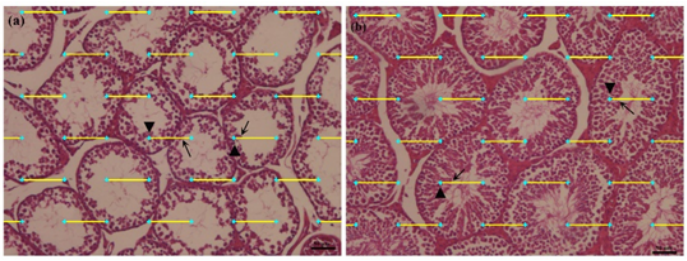

Fig. 4. Estimation of the surface density of the germinal epithelium using test lines in 30- (a) and 60-day old (b) mice. The total number of points hitting the germinal epithelium (arrowheads) and the number of intersections of the test line with the inner surface of the germinal epithelium (arrows) were counted.

2.8. Height of germinal layer

The height of the germinal layer of seminiferous tubules was estimated using the following formula (Nyengaard, 1999): H= Vv (germinal layer) / Sv (germinal layer) where Vv (germinal layer) and Sv (germinal layer) are the volume density and surface density of the germinal layer, respectively.

2.9. Statistical analysis

The data were expressed as mean ± standard deviation. The comparisons between groups were evaluated using the one-way analysis of variance (ANOVA) followed by the Tukey's post-hoc test. A $p < 0.05$ was considered as significant.

3. Results

3.1. Total volume of testis, seminiferous tubules and interstitial tissue

30-day old offspring

Mice in the lactation group had a lower testicular volume than those in the control group (52.5 ± 17 mm^3 vs 95 ± 5.7 mm^3, respectively) (P<0.05). A significant decrease was observed in the total volume of seminiferous tubules of the pre-pregnancy (56.3 ± 8.3 mm^3) and lactation groups (38 ± 13.1 mm^3), in comparison with the control group (75.5 ± 5.9 mm^3) (P<0.05). The mean volume of interstitial tissue in the lactation group was significantly decreased (9.5 ± 2.8 mm^3 in lactation group vs 16.3 ± 0.9 mm^3 in control group) (P<0.05) (Figure 5).

60-day old offspring

The total volume of the testis was found to be considerably lower (P<0.05) in the pre-pregnancy (106.7 ± 15.2 mm^3), pregnancy (103.3 ± 11.5 mm^3) and lactation (97.5 ± 9.5 mm^3) experimental groups, when compared with the control group (140 ± 10 mm^3). Similarly, reductions occurred in the total volume of seminiferous tubules in the pre-pregnancy (85.6 ± 10.8 mm^3), pregnancy (82.3 ± 13.2 mm^3) and lactation (73.8 ± 12.1 mm^3) groups, in comparison with the control group (117.8 ± 12.4 mm^3) (P<0.05).

However, there was no significant difference (P<0.05) in the volume of interstitial tissue between all experimental groups and the control group.

3.2. Volume, height and surface area of the germinal layer of seminiferous tubules

30-day old offspring

Observations revealed that the mean volume of the germinal

layer in seminiferous tubules demonstrated a significant decrease in the lactation group (28.1 ± 9.9 mm³), in comparison with the control group (61.1 ± 3.2 mm³) (P<0.05). Likewise, the mean height of the germinal layer was remarkably lower (P<0.05) in the lactation group (35.42 ± 5.07 µm), when compared with the control group (42.58 ± 2.57 µm). In addition, results showed a lower surface area for the germinal layer (P<0.05) in the lactation group (4.26 ± 1.66 cm²), in comparison with the control group (9.30 ± 1.23 cm²) (Figure 6).

60-day old offspring

A reduction in the mean total volume of the germinal layer was detected in the second (71.1 ± 11.8 mm³), third (69.4 ± 12.5 mm³) and fourth (58.7 ± 11.6 mm³) experimental groups, when compared with the control group (103 ± 10.6 mm³) (P<0.05). The height of the germinal layer was equivalent between the experimental groups and the control group. However, the surface area of the germinal layer in the fourth group (7.57 ± 1.90 cm²) resulted considerably lower (P<0.05) than that in the control group (13.65 ± 1.47 cm²).

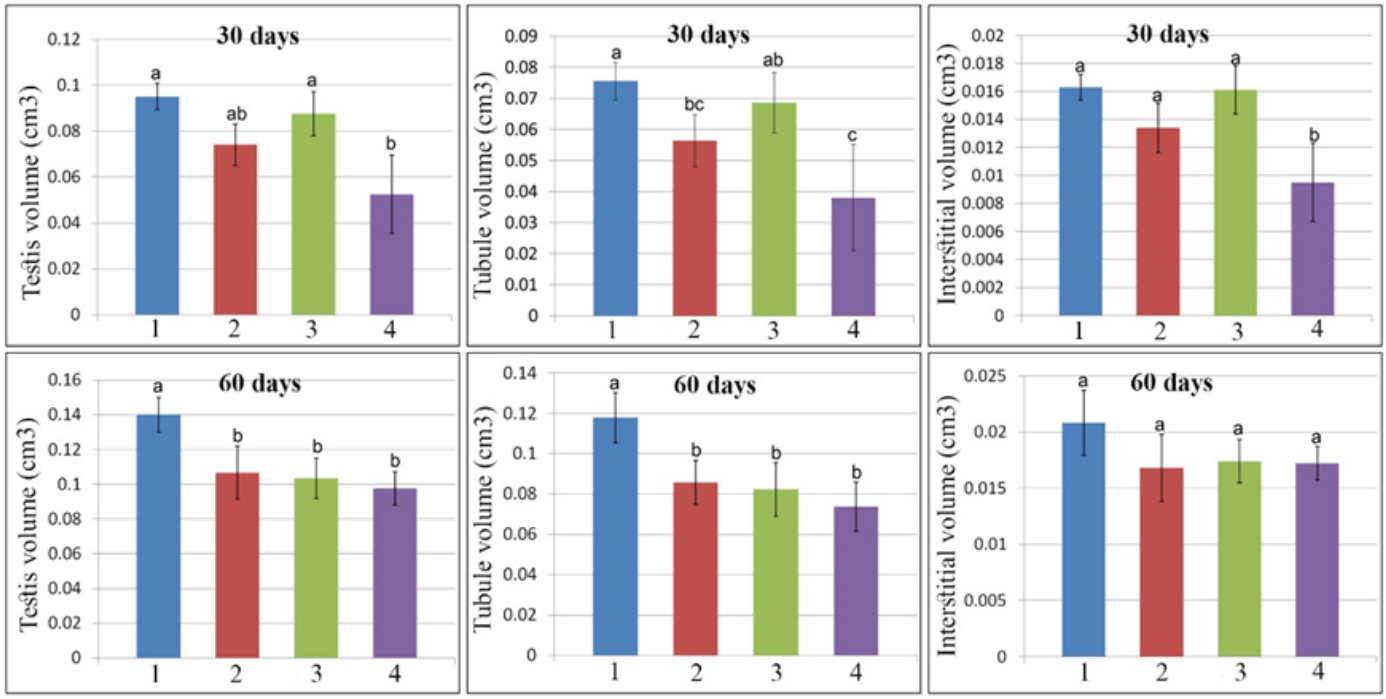

Fig. 5. Comparison of testicular volume and the volume of seminiferous tubules and interstitial tissue among groups 1 (control), 2 (pre-pregnancy), 3 (pregnancy) and 4 (lactation) in 30- and 60-day old mice

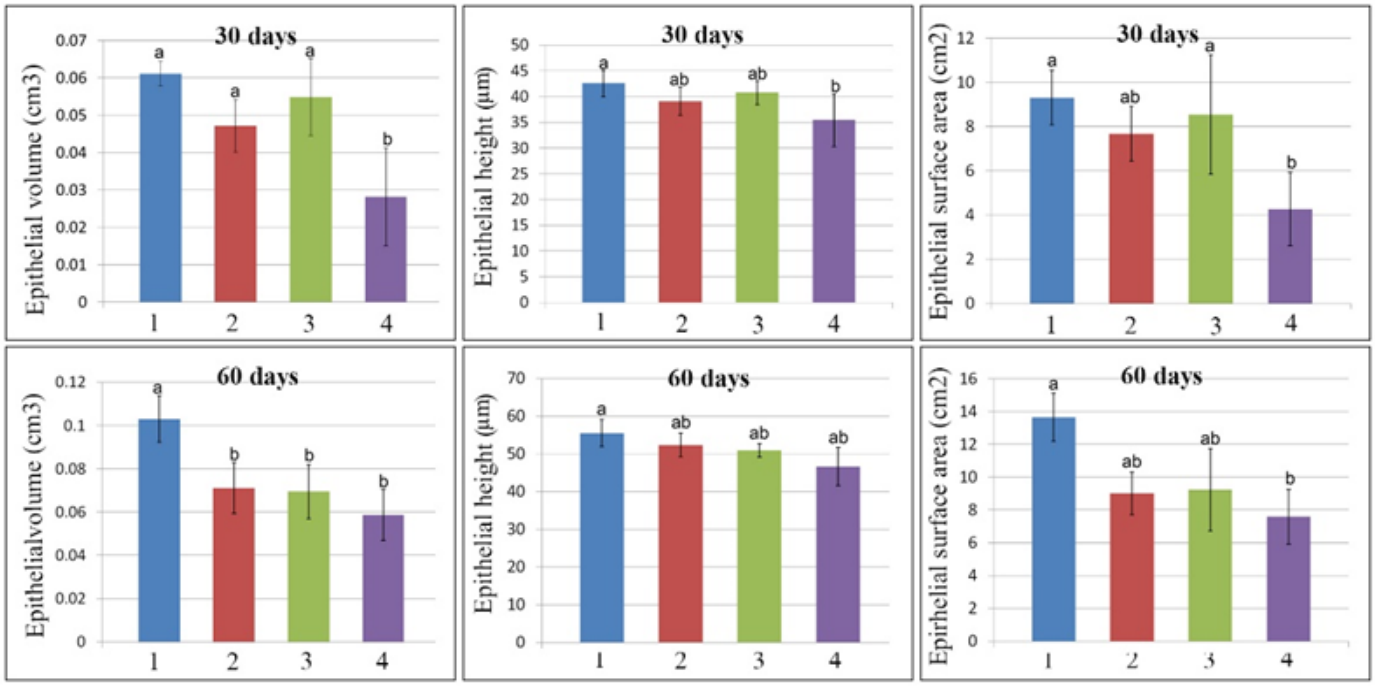

Fig. 6. Comparison of volume, height and surface area of the germinal epithelium among groups 1 (control), 2 (pre-pregnancy), 3 (pregnancy) and 4 (lactation) in 30- and 60-day old mice

3.2. Diameter and length of seminiferous tubules

30-day old offspring

Our findings revealed that the mean diameter of seminiferous tubules in the third (152.90 ± 6.06 µm) and fourth (141.91 ± 5.62 µm) groups were significantly reduced (P<0.05) in comparison with the control group (168.81 ± 6.14 µm), and that the length of seminiferous tubules in the second (168.26 ± 21.76 cm) and fourth (122.57 ± 21.78 cm) groups was considerably shorter than that measured in the control group (227.32 ± 24.92 cm) (P<0.05) (Figure 7).

60-day old offspring

The mean diameter of the seminiferous tubules was observed to be much lower (P<0.05) in the second (201.29 ± 9.84 µm), third (199.01 ± 7.84 µm) and fourth (189.28 ± 26.4 µm) groups, when compared with the control group (218.63 ± 9.52 µm). Also, the length of the seminiferous tubules was substantially lower (P<0.05) in the second (256.97 ± 47.55 cm), third (247.46 ± 24.75 cm) and fourth (225.00 ± 16.13 cm) experimental groups, in comparison with the control group (339.56 ± 34.77 cm).

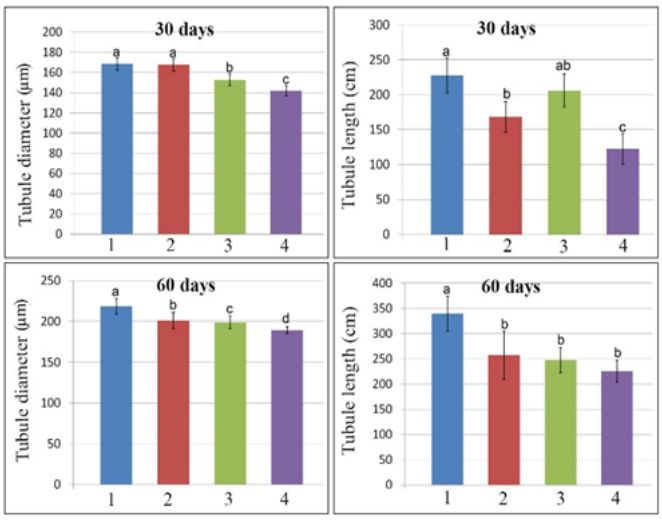

Fig. 7. Comparison of the diameter and length of seminiferous tubules among groups 1 (control), 2 (pre-pregnancy), 3 (pregnancy) and 4 (lactation) in 30- and 60-day old mice

4. Discussion

Our findings indicated that the mean volume of the testes of 30-day old offspring significantly decreased in the lactation group, in comparison with the control group (P<0.05). In addition, the testicular volume of 60-day old offspring in all experimental groups resulted considerably lower, when compared with the control group (P<0.05). It is possible that anatomical changes such as a decrease in size and volume, dependent on the dosage and treatment period, occurred as a result of the production of free radicals in the extremely sensitive testicular cells, resulting in the apoptosis of these cells (Aitken et al., 2008). In addition, a positive correlation has been detected between testicular volume and the number of germinal cells (yang et al., 2004). Therefore, the decrease in volume may be the sign of an attack on germinal cells by the chemotherapy agent Ox.

The mean volume of seminiferous tubules was observed to be considerably lower for the testes of 30-day old offspring in the pre-pregnancy and lactation groups and also in 60-day old offspring in all experimental groups, in comparison with the control group (P<0.05). The decrease in the volume of seminiferous tubules can be linked to a reduction in the number of germinal cells and to an increment in the apoptosis of these cells through the inhibition of calcium pumps in the endoplasmic reticulum (Gong et al., 2009; Choi et al., 2011). Furthermore, the mean total volume of testicular interstitial tissue was found to be lower than that of the control group (P<0.05) only in the testes of the 30-day old offspring belonging to the lactation group only. The absence of differences in the volume of interstitial tissue was also witnessed in the 60-day old offspring in all experimental and control groups. It is known that the administration of cisplatin increases testicular interstitial tissue by causing an edema in interstitial spaces through the prevention of calcium diffusion into the smooth muscles of blood vessels and the subsequent dilatation of the blood vessels themselves (Seethalakshmi et al., 1992; Lirdi et al., 2008). However, this did not happen in this study, and even the volume of interstitial tissue was substantially lower in the 30-day old lactation group, compared with the control group. This result can be connected to a severe reduction in total testicular volume in this group, in comparison with the control group, to such an extent that it also led to a decrease in the volume of seminiferous tubules and interstitial tissue. There may be a possibility that the amount of Ox and / or duration of the treatment period were not sufficient to cause changes in the diameter of blood vessels and, as a consequence, to cause an edema in the interstitial tissue. In support of the results from this study, we can refer to a study by Dehghani et al. (2013) in which a decrease in total testicular volume, as well as in the volume of tubules and interstitial tissue was reported in rats being administered Busulfan.

In this study, the mean height of the germinal layer of the seminiferous tubules was drastically lower in the 30-day old offspring belonging to the lactation group, in comparison with the control group (P<0.05). A reduction in the volume of the germinal layer of seminiferous tubules in 30-day old offspring was only detected in the lactation group. Our findings revealed that the mean values of these factors decreased significantly in the 60-day old offspring in all experimental groups, when compared with the control group (P<0.05). Moreover, the mean surface area of the germinal layer of the testicular seminiferous tubules in the 30- and 60-day old offspring belonging to the lactation groups was considerably inferior than that measured in the control group (P<0.05). The decrement of these factors may be due to a reduction in the number of germ cells in the germinal layer of the seminiferous tubules and to an early detachment of immature spermatids into the lumen caused by a damage to the connections between Sertoli cells (Varghese et al., 2008). The seminiferous

germinal layer and its cytoarchitecture are important for spermatogenesis (Azu et al., 2014). Considering the oxidative effect of Ox (Jamieson et al., 1999), the decrease in the number of spermatogenesis epithelial layers can be the result of a reduction in the divisions of spermatogonia B caused by an elongation of the G1 phase (Jedlinska-krakowska et al., 2006). In support of our findings, a reduction in the thickness of the germinal epithelium has also been reported, in response to a Cisplatin treatment, as the result of decreased cellular divisions (Mohammadnejad et al., 2012) and oxidative stress (Atessahin et al., 2006). Another study indicated a decrease in tubular diameter and epithelial height as the result of Di-n-butyl phthalate (DnBP) administration, due to the loss of cells in the germinal layer (Zhou et al., 2010; Zhang et al., 2014).

In the present study, it has been observed that the diameter of seminiferous tubules in the testes of 30-day old offspring in the pregnancy and lactation groups was reduced in comparison with that measured in the control group ($P<0.05$). Moreover, the diameter of the seminiferous tubules in the testes of 60-day old offspring decreased significantly in all experimental groups, when compared with the control group ($P<0.05$). There is generally a positive correlation between the diameter of seminiferous tubules and the spermatogenic activity of the testes (Sinha-Hikim et al., 1989; Franca et al., 1998; de Souza Predes et al., 2010). The decrease in the diameter of seminiferous tubules can indicate a reduction in the number of Sertoli cells and the disruption of the process of spermatogenesis (Kovačevič et al., 2006). Likewise, Cisplatin and Doxorubicin can cause a decrease in the diameter of seminiferous tubules in prepubertal testes (Smart et al., 2018). Cyclophosphamide and Decapeptyl in mice (Niakani et al., 2013), and Doxorubicin (Silva et al., 2018) in rats, have been reported to reduce the thickness of the epithelium and the diameter of seminiferous tubules.

The length of seminiferous tubules significantly decreased ($P<0.05$) in the 30-day old mice belonging to the pregnancy and lactation groups and in the 60-day old offspring in all experimental groups, in comparison with the control group. The length of seminiferous tubules is dependent on three testicular structures, specifically testicular size, diameter, and volume density of seminiferous tubules (Franca et al 1998). Therefore, the evaluation of tubular length is necessary, given the fact that a reduction in the length and diameter of the tubules may cause atrophic changes and a decrease in tubular volume (Noorafshan et al., 2011). From a recent study emerged how the proliferation of Sertoli cells and spermatogenic cells occurring during puberty increases the diameter and length of the seminiferous tubules leading subsequently to an increase in testicular volume (Pereira et al., 2020). Therefore, the decrease in diameter and length of seminiferous tubules occurring in most 30-day old groups and in all 60-day old ones is presumed to be the result of the cessation of the above-mentioned cellular proliferation process.

Having acknowledged the ability of Oxaliplatin to pass through the placental blood barrier and to also pass into the milk, this study confirms the effect of the administration of this drug on male offspring testes following the perinatal period. This ameliorating effect appeared more evident when Ox was administered during lactation since the sampling and evaluation of the testes occurred temporally closer to this experimental phase. Considering the fact that this drug is used in the perinatal stage, precautions must be taken in order to preserve the fertility of male offspring.

Conflict of interest statement

There is no any conflict of interest. The experiment was in accordance with the standard guide for the care and use of laboratory animals (Faculty of veterinary medicine, University of Tehran, Tehran, Iran. Ethical number: 6067543).

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