ISSN: 2008-8140
E-ISSN: 2322-3618

Urmia University

VETERINARY RESEARCH FORUM

Volume 11, Number 1
March 2020

An International Quarterly Journal Published by Faculty of Veterinary Medicine, Urmia University, Urmia, I.R. Iran

ORIGINAL ARTICLE

Veterinary Research Forum. 2020; 11 (1) 35 – 42
doi: 10.30466/vrf.2018.82090.2082
Journal Homepage: vrf.iranjournals.ir

Veterinary Research Forum

The effects of exposure to fluoxetine during lactation on testicular tissue and sperm parameters in mice offspring

Mojtaba Karimipour[1], Abbas Ahmadi[2]*, Masoumeh Zirak Javanmard[1], Abbas Jafari[3], Maryam Mohebi[1], Elnaz Hosseinalipour[1]

[1] Department of Anatomy and Histology, Faculty of Medicine, Urmia University of Medical Sciences, Urmia, Iran; [2] Department of Basic Sciences, Faculty of Veterinary Medicine, Urmia University, Urmia, Iran; [3] Department of Occupational Health, School of Health, Urmia University of Medical Sciences, Urmia, Iran.

Article Info

Article history:

Received: 06 March 2018
Accepted: 25 September 2018
Available online: 15 March 2020

Keywords:

Fluoxetine
Mice
Sperm parameter
Testis

Abstract

Fluoxetine is a selective serotonin reuptake inhibitor is commonly prescribed to treat maternal depression in pregnancy and lactation. This study aimed to investigate the effects of maternal exposure to fluoxetine via lactation on testicular tissue, sperm parameters including count, motility, viability, and normal morphology and testicular oxidative stress status in male mice offspring. Ten mice dams were divided into control and experimental groups. The control group received water and the experimental group received fluoxetine (20.00 mg kg^{-1}) by gavage daily from postnatal days of 0-21. Histology of testis, sperm parameters and oxidative stress in the testicular tissue were analyzed at 80 days after birth in their male offspring (n = 8). Significant reductions in the body and testes weights were observed in animals exposed to fluoxetine. Additionally, fluoxetine exposure significantly reduced all sperm parameters, tubular diameter and epithelial height of the seminiferous tubules as well as Leydig cells number. Significant increases in the testicular malondialdehyde levels and percentage of sperm with chromatin/DNA damage were observed in mice exposed to fluoxetine compared to control. These findings suggest that maternal exposure to fluoxetine during lactation in mice has a negative effect on the testicular tissue of their offspring and impairs the spermatogenesis process which in turn can induce infertility.

Introduction

Depression is a common psychiatric illness occurs in 10.00-25.00% women worldwide and this disorder affects almost 10.00-15.00% of childbearing women.[1] Depression in women during pregnancy or postpartum can be dangerous to both mother and the fetus.[2] A previous study revealed adverse effects of maternal postpartum depression on cognitive, motor and emotional development of the newborn.[3] A study found that chronic maternal depression in the first year postnatal is associated with delayed psychomotor development in infants.[4] Moreover, untreated maternal depression is also associated with risks for mother including mother and child relationship impairment, self-neglect, decreased tendency with prenatal and postnatal care and increased risk for drug and alcohol abuse.[5,6] Thus, it is important to start effective drug therapy in the women suffering from depression disorder.[7, 8]

Selective serotonin reuptake inhibitors (SSRIs) such as sertraline, fluvoxamine, and fluoxetine (FLX) are the first-line antidepressant drugs frequently used and prescribed in this condition treatment. The SSRIs are also used to treat postpartum dysthymia, panic disorder and obsessive-compulsive disorders.[7,9]

Among SSRIs, FLX or Prozac® is the second more frequently prescribed drug to treat maternal depression during pregnancy and lactation.[10] In humans, FLX is metabolized to norfluoxetine which has a plasma half-life of 15 days and antidepressant properties similar to FLX.[11]

The FLX and its main metabolite, norfluoxetine, are highly soluble in lipids, can reach embryo through the placental barrier and are also secreted in breast milk during lactation.[12] Thus, both embryos and newborns are

***Correspondence:**

Abbas Ahmadi. DVM, PhD
Department of Basic Sciences, Faculty of Veterinary Medicine, Urmia University, Urmia, Iran
E-mail: a.ahmady@urmia.ac.ir

exposed to this drug in depressive mothers undertaking pharmacotherapy. Male factors, directly and indirectly, are responsible for approximately 50.00% of infertility among couples.[9] Various factors such as drug usage may affect the male reproductive system and result in infertility. Anti-depressant drugs are extensively used because depression is the most common psychiatric illness diagnosed by clinicians and this condition is observed more frequently at reproductive age.[13] Considering SSRIs are the first choice in the treatment of this disorder, so many patients especially mothers during gestation and lactation are exposed to these drugs.

Studies have documented the adverse effects of FLX on the male reproductive system in human offspring whose mothers have been treated with SSRIs.[14,15] The administration of SSRIs induces changes in the hypo-thalamic-pituitary-adrenal system in adolescent rats and decreases the levels of luteinizing hormone (LH), follicle-stimulating hormone (FSH) and testosterone hormones in blood serum.[16,17] In other studies, it has been indicated that SSRIs exposure in rats induces maternal and fetal toxicity and decreases male sexual behaviors.[18,19] In a study done on rats, FLX impaired spermatogenesis, decreased sperm motility and density and also diminished levels of FSH and testosterone hormones as well as reproductive organs weight.[20]

Furthermore, it has been reported that an increase in cerebral serotonin levels in adult rats treated with hydroxytryptophan inhibits the release of gonadotropin-releasing hormones (GnRH) in the hypothalamus, impairs FSH and LH secretion and ultimately causes the spermatogenesis process deficiency.[21] In addition, supplementation of rats with FLX inhibited GnRH secretion by the hypothalamus in male offspring. Thus, considering that both pre- and postnatal periods are important for testicular development, any gonadotropin changes during these times may affect testicular development.[1] However, little is known about the potentiality of the damaging effects of FLX exposure during lactation on testicular tissue and spermatogenesis process. Therefore, the current study was done to investigate the effects of maternal FLX exposure during only the lactation period on testicular tissue alterations and sperm quality and quantity along with sperm DNA damage of male mice offspring in adulthood. We also tested oxidative stress in testicular tissue of these mice.

Materials and Methods

Animals and experimental design. Ten pregnant mice (12 weeks old) were used in this study. The mice were separately kept in an individual cage under a controlled environment at 22.00 ˚C and a 12-hr light-dark cycle. Animals had free access to standard pellet food and water *ad libitum*.

The experimental protocol was approved by the Urmia University of Medical Sciences Ethics Committee (Ir.UMSU.rec.1395.322).

After delivery, the maternal mice were treated daily with FLX (20 mg kg^{-1}) by gavage during the first 21 days postnatal (weaning period) as an experimental group or with deionized water as a control group. The FLX chloride was obtained from Shahre Darou Co., (Tehran, Iran).

The dose of FLX in humans is 20.00 - 80.00 mg daily. Considering that animals are more resistant than humans, a higher dose is usually tested in animals. Previous animal studies,[1,2] have shown that administration of 20.00 mg kg^{-1} body weight of FLX during both pregnancy and lactation has a negative effect on testicular morphology, development and also function. Therefore, in this study, we selected 20.00 mg kg^{-1} body weight of FLX during only the lactation period to evaluate its effects on the testis and sperm parameters in male mice offspring.

Offspring analysis. Eight male adult pups from each group were randomly selected at the postnatal day of 80. This time was selected according to the prior study.[22] After recording the body weight, they were euthanized with intraperitoneal injection of ketamine (300 mg kg^{-1}; Alfasan, Woerden, The Netherlands). Then, the testes were removed and weighted; the left ones were immersed in formalin 10.00% for fixation. After routine tissue processing and embedding in paraffin, the 5.00 µm sections were stained with hematoxylin and eosin.

To analyze the epithelial height and diameter of seminiferous tubules (SNTs), 10 round-shaped tubules from each mouse were randomly selected and determined. For this purpose, two diameters perpendicular to each other were measured in each SNT and their average was determined. To determine the average of the epithelial thickness of the same SNT, it was measured from the basement membrane to the luminal surface at two locations. Furthermore, the Leydig cells were counted in twelve randomly selected fields per section. The measurements were performed using a light microscope (Laborlux 12; Leitz, Wetzlar, Germany) equipped with a micrometer lens at 100×.[22,23]

Sperm parameter analysis. Sperm analysis was performed at 80th day postnatal. The caudal part of both epididymides from each mouse was removed and immediately transferred to a Petri dish containing 1.00 mL human tubal fluid medium supplemented with 4.00 mg mL^{-1} bovine serum albumin (Sigma, St. Louis, USA), pre-heated at 37.00 ˚C. Then, the epididymis was minced and incubated at 37.00 ˚C in 5.00% CO_2 in order to swim-out spermatozoa into the medium. In order to assess sperm motility, 10.00 µL of sperm solution was placed on a pre-heated Neubauer slide and then the percentage of motility was determined under the light microscope with 400×.[24] For evaluating sperm count, a 1:50 diluted sperm sample with distilled water was prepared in a microtube, then

10.00 µL of this mixture was placed on a Neubauer slide and the number of sperm counted using a light microscope with the magnification of 400×.[25]

To determine the percentage of sperm viability, 20.00 µL of sperm sample from each mouse was placed on a slide, then the same value of Eosin-Nigrosin solution was added and smears were prepared. After dying the smears at room temperature, using a light microscope with the magnification of 400×, the percentage of red dead sperms and the live ones with no color were determined.[26] The percentage of sperm with abnormal morphology for each mouse was measured using the smears stained by Eosin-Nigrosin.[27]

To evaluate the percentage of sperm with DNA damage, smears were obtained from sperm solution and then stained by acridine orange. This staining method reveals sperm DNA fragmentation as a marker of male infertility. Before staining, the smears were fixed in Carnoy fixative (methanol/acetic acid; 1:3). Following drying the slides at room temperature, they were placed in a staining solution for 5 min in dark and then examined using a fluorescent microscope with the magnification of 1000×. Green colored sperms were considered normal and the yellow-red colored ones had abnormal damaged DNA.[28]

Moreover, aniline blue staining was used to determine the percentage of sperms with nucleus maturity. This staining detects the sperm chromatin condensation (replacement of histone with protamine during spermiogenesis). Abnormal immature sperms appear dark blue and normal sperms become pale using a light microscope with the magnification of 400×.[28]

Oxidative stress evaluations. In this regard, the testicular tissue was homogenized in phosphate buffer (pH = 7.40) and centrifuged at 12,000 *g* at 4.00 ˚C for 20 min. Then, the supernatant was separated and stored at – 80 ˚C. The activities of superoxide dismutase (SOD) and glutathione peroxidase (GPx) in supernatants were determined by kits from ZellBio GmbH (Ulm, Germany) according to the manufacturer's procedure. Glutathione (GSH) levels of supernatants were determined as described previously.[29,30] In brief, 10.00 µL of supernatant was mixed with 200 µL of Tris-EDTA buffer (0.25M Tris base, 20.00 mM EDTA, pH = 8.20) and 4.00 µL of 5,5-dithiobis-2-nitrobenzoic acid (10 mM) in methanol. The yellow color appeared after 30 min incubation at 37.00 ˚C. The absorbance of the supernatant was measured against a blank at 412 nm. The results were expressed as nmol GSH mg^{-1} protein. To measure malondialdehyde (MDA) levels, the supernatant (600 µL) was mixed with 150 µL of thiobarbituric acid (0.67%; w/v), incubated in boiling water bath (95.00 ˚C) for 30 min and extracted with n-butanol. Then, the solution was cooled and centrifuged. Absorbance was recorded at 532 nm and MDA levels were expressed as nmol mg^{-1} protein. The method was calibrated with tetraethoxypropane, as standard solutions.[31]

Statistical analysis. The data were analyzed by SPSS (version 16.0; SPSS Inc., Chicago, USA) using an independent *t*-test. The data were shown as mean ± standard deviation and a p value less than 0.05 was considered significant.

Results

Effects of lactational FLX exposure on body and testis weights. Body and testis weights of offspring exposed to 20.00 mg kg^{-1} FLX were decreased on postnatal day of 80 compared to control. This reduction was statistically significant ($p < 0.05$; Table 1).

Effects of lactational FLX exposure on testicular histomorphometry. Table 1 also presents the results of SNTs diameter and their epithelial height. The means of these criteria were significantly ($p < 0.001$) lower in the FLX group in comparison with the control group. Compared to control, the number of Leydig cells also significantly ($p < 0.01$) decreased in animals exposed to FLX (Table 1).

Some morphological alterations were observed in testes of male offspring whose mothers were exposed to FLX via lactation including vacuole in SNTs epithelium, SNTs with no spermatogenesis, germinal epithelium sloughing into the lumen and many tubules with little or no sperm in the lumen (Fig. 1).

Effects of lactational FLX exposure on sperm parameters. In this study, sperm parameters were evaluated at the postnatal day of 80. Sperm count in mice offspring exposed to FLX during lactation was significantly ($p < 0.001$) reduced compared to the control group (Table 2). Furthermore, FLX caused a significant decrease in the percentage of sperm viability. As shown in Table 2, the mean percentage of sperm with shape abnormality in mice exposed to FLX was significantly increased compared to the control group.

Effects of lactational FLX exposure on sperm chromatin condensation and DNA disintegrate. Aniline blue staining test showed that on postnatal day of 80 the mean percentage of immature sperms in animals exposed to FLX was significantly ($p < 0.001$) higher than control ones. Table 2 also shows the result of the acridine orange staining test.

A significant increase in the mean number of sperm with damaged DNA was observed in mice exposed to FLX ($p < 0.001$), (Fig. 2).

Effects of lactational FLX exposure on MDA and GSH levels and SOD and GPx activities in testicular tissue. Table 3 shows the stress oxidative criteria in testicular tissue in different groups. The MDA level in the FLX group was significantly ($p < 0.05$) higher than the control group. No significant differences were observed in the average of GSH levels and SOD and GPx activities between groups.

Table 1. Body and testes weights, tubular diameter and epithelial height of seminiferous tubules and Leydig cells number in mice offspring at the postnatal day of 80 whose mothers have been exposed to water as control or FLX during lactation.

Groups	Body weight (g)	Testes weight (mg)	Tubular diameter (µm)	Epithelial height (µm)	Leydig cells number
Control	33.90± 2.27	197.50 ± 13.88	207.92 ± 6.93	87.67 ± 2.35	215.50 ± 21.3
Fluoxetine	28.59 ± 1.99 [a]	175.00 ± 20.70 [a]	183.50 ± 5.01[c]	67.00 ± 3.69 [c]	187.12 ± 13.47 [b]

Different superscripts in each row indicate significant differences between groups as follows: compared to control group at [a] $p < 0.05$, [b] $p < 0.01$ and [c] $p < 0.001$, respectively.

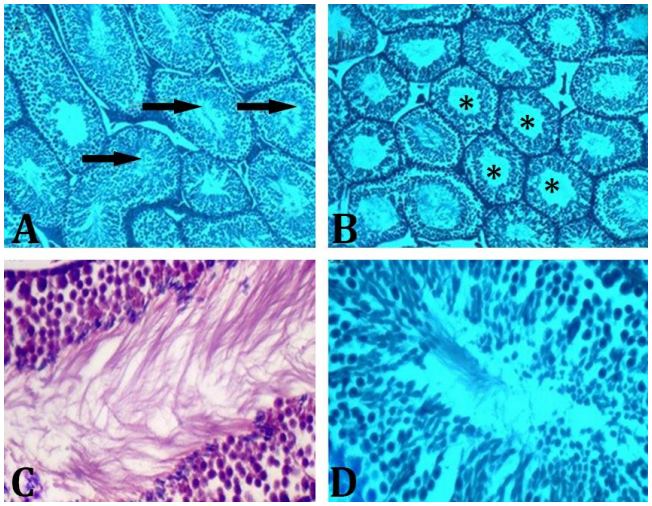

Fig. 1. Histological images of testicular tissue in the studied groups. **A** and **C)** Control group; **B** and **D)** Fluoxetine group. Arrows show seminiferous tubules and asterisks indicate seminiferous tubules (SNT) with few or no sperm in the lumen. Image C shows a SNT in the control group full of sperm and image D shows a SNT from the fluoxetine group with few sperms, (H & E; A and B :100×, C and D: 400×).

Table 2. Sperm characteristics in mice offspring at the postnatal day of 80 whose mothers have been exposed to water, as control or FLX during lactation.

Sperm parameters	Control	Fluoxetine
Count (× 10^6)	36.00 ± 6.90	18.12 ± 3.35*
Motility (%)	67.60 ± 3.87	34.25 ± 4.28*
Viability (%)	72.25 ± 3.50	41.75 ± 6.20*
Abnormal (%)	11.25 ± 5.31	26.50 ± 4.20*
Immature (%)	2.50 ± 0.57	16.20 ±0 .94*
Damaged DNA (%)	1.25 ± 0.50	11.50 ± 4.20*

* Asterisk indicates significant differences compared to control group at $p < 0.001$.

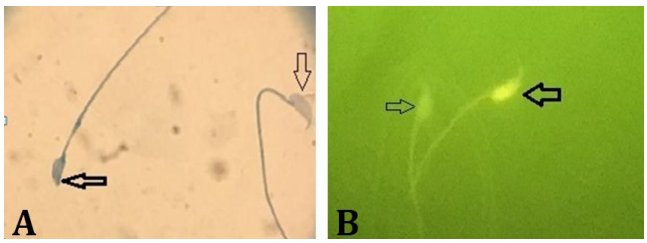

Fig. 2. Sperms stained with **A)** aniline blue and **B)** acridine orange. Thick arrow in image A shows immature sperm and in image B shows sperm with chromatin disintegrate. Thin arrows show normal sperms.

Table 3. The concentration of malondialdehyde (MDA) and glutathione (GSH) and activities of superoxide dismutase (SOD) and glutathione peroxidase (GPx) in testicular tissue of mice offspring at the postnatal day of 80 whose mothers have been exposed to water, as control or FLX during lactation.

Parameters	Control	Fluoxetine
MDA (nmol g^{-1} tissue)	22.37 ± 1.39	24.89 ± 1.71*
GSH (nmol g^{-1} tissue)	0.487 ± 0.15	0.386 ± 0.05
GPx (U mL^{-1})	401.13 ± 69.40	403.15 ± 107.30
SOD (U mL^{-1})	16.40 ± 5.60	14.81 ± 9.80

* Asterisk indicates significant differences compared to control group at $p < 0.001$.

Discussion

In the current study, we evaluated the effects of maternal exposure to FLX during the lactation period on sperm parameters and testicular tissue alterations of male mice offspring. The findings of this study revealed that maternal supplementation with FLX via lactation had detrimental effects on testicular tissue and sperm parameters at the puberty period. Moreover, the percentage of sperms with DNA damage also increased in these mice.

In the present study, male mice offspring exposed to FLX showed a significant reduction in the body weight compared to the control group as shown in Table 1. Previous studies have indicated that the administration of FLX can reduce the body weight gain of neonatal rats. According to other reports, an increase in plasma level of serotonin after FLX administration during the fetal period may reduce uterine blood flow, which is associated with the observed reduction in body weight.[32] Additionally, body weight gains significantly decreased in male offspring exposed to FLX. This body weight change could be associated with the inhibitory effect of serotonin in food ingestion, as it has been reported that FLX administration can lead to partial atrophy of intestinal villi.[33]

The results of the present study demonstrated that testis weight in offspring exposed to FLX decreased at the postnatal day of 80. These findings are in agreement with the results of a previously published study.[1] In a study by Silva *et al.*, it has indicated a 36.00% reduction in the testis weight of neonatal rats exposed to FLX.[34] The SNTs and interstitial tissue are the main components of the testicular tissue.[35] In this study, maternal exposure to FLX caused a reduction in diameter and epithelial height of SNTs. According to França *et al.*, testicular weight is

positively associated with the volume of SNTs, Sertoli cells population and sperm production.[36] Adult rats supplemented with FLX also revealed a reduction in thickness of germinal epithelium and diameter of SNTs.[37] Moreover, in another study by Vieira *et al*., it has indicated that the number of spermatozoa and epithelial height and diameter of SNTs at adulthood decrease in rat dams treated with FLX at gestation and lactation periods.[38] Findings from the current study also showed a reduction of sperm count in offspring exposed to FLX via lactation at postnatal day of 80. In this way, it could be concluded that FLX affected the continuous development of SNTs at the lactation period, resulting in a notable impairment in sexual maturity. A part of testis growth and development occurs at a neonatal period during lactation and development of different cellular populations are not simultaneously occurred and is influenced by intra- and extra-testicular factors.[39] For example, a large number of Sertoli cells population is produced on the gestational day of 18-21.[40]

Moreover, although in this study we did not record the Sertoli cells population, but decreased SNTs diameter could be associated with the reduction in the number of Sertoli cells because there is a positive correlation between the number of these cells and epithelial height and diameter of SNTs.[38,39] It was also reported that a reduction in the Sertoli cells population can lead to SNTs shrinkage.[41] On the other hand, there is a direct correlation between the number of Sertoli cells and spermatogenesis process.[42] Thus, sperm count reduction in the present study might be due to the effects of FLX on Sertoli cells number in SNTs, but yet to be determined.

Considering that sperms are the most important target cells in male reproductive toxicity for assessment of spermatogenesis process and fertility, thus one of the objectives of our study was to evaluate sperm parameters at puberty in mice offspring exposed to FLX and compare with the results obtained from control mice exposed to vehicle. Several previous studies have documented that humans and animals exposed to FLX during gestation and lactation are associated with impairment of testicular development and spermatogenesis process in adulthood. In the present study, sperm count and motility of pups whose mothers have been treated with FLX during lactation were significantly decreased in comparison with the control group. These findings suggest that maternal exposure to FLX via lactation can affect the spermatogenesis process, these results are in accordance with previous studies explaining the effects of FLX in animals being exposed during gestation until weaning and also only during adulthood.[43]

In a study by Kumar *et al*., they have indicated that FLX, sertraline, fluvoxamine, and citalopram negatively affect semen parameters and show a spermicidal activity. They have suggested that SSRIs bind to sulphyl groups in the sperm membrane and impair ATP synthesis in the sperm by interacting with phospholipids.[44]

In the present study, other sperm parameters including sperm viability and normal morphology were affected by FLX administration as presented in Table 2. Findings revealed that FLX significantly decreased the percentage of alive sperms and increased the percentage of abnormal sperms. These findings may be due to the effect of FLX on testicular development during the lactation period as stated previously. There are little data about the effects of FLX exposure on sperm viability and morphology. In agreement with our study, in a previous related study, it has been shown that FLX induces a significant increase in abnormal spermatozoa in adult mice.[43]

As stated above, FLX caused a toxic effect on SNTs epithelium. An increase in the percentage of sperm shape abnormality may reflect chromosome abnormalities in primary spermatocytes and spermatids which is associated with infertility. It has been reported that abnormality in sperm reflects point mutations in germ cells causing alterations in cell organelles involved in head and tail formation leading to sperm abnormality.[43,44]

Our study also showed that FLX via lactation increased the number of SNTs lacking lumen at the postnatal day of 22 (data are not published). This finding is in agreement with the previous study and indicates that FLX slows down the lumen formation in SNTs and results in testicular development delay.[1] It has been reported that lumen formation in SNTs reflects the maturation of Sertoli cells that produce tubular fluid and also is responsible for the blood-testis barrier (BTB).[45] For normal spermatogenesis, integrity in the BTB as created by Sertoli cell tight junctions is necessary.[46] Considering that the BTB in the mouse forms at approximately 10-16 days postnatal, [47] thus FLX exposure in this period (lactation) in the present study causes damages to Sertoli cells resulting in BTB impairment, which is accounted for interfered testicular function, but further studies are needed to clarify it.

Sperm DNA damage in mice offspring exposed to FLX via lactation is another finding obtained from this study. In accordance with this result, Safarinejad has shown that depressed patients receiving SSRIs have a significant increase in the percentage of sperm DNA damage compared to the normal controls.[17] Previous studies have shown that serotonin is able to cause DNA cleavage in the presence of copper ions through reactive oxygen species production.[48] Binding of serotonin to DNA and copper ions and formation of serotonin-Cu^{2+}-DNA is maybe the cause of sperm DNA damage by serotonin.[17]

Regarding aniline blue staining test which can evaluate sperm nucleus maturity and detect sperm chromatin defects, it can be reported that FLX exposure via lactation increased the percentage of sperm chromatin abnormality. To compare this finding with other studies, we could not

find a similar study. Thus, we are not able to explain how FLX impairs sperm nucleus maturity. This may be due to increased serotonin levels following FLX administration, but further studies are needed to explain the exact mechanism.

Leydig cells are an important cell population in testis involved in the control of Sertoli cells function. Therefore, any damage to these cells can impair spermatogenesis process.[49] In the current study, mice in the test group showed a significant reduction in the number of Leydig cells, indicating that lactational exposure to FLX can result in a significant reduction in the Leydig cells population.

Our study had limitations. We did not test endocrine hormones. We assume that the sperm quality reduction and damaged sperm DNA integrity in this study may be due to dysfunction in the hypothalamus-hypophysis-testis axis, but it requires more studies.

Oxidative stress is an imbalance between free radicals generation and antioxidant defenses being considered as a major causative factor of male fertility impairment. Male germ cells have a high content of polyunsaturated fatty acids in the plasma membrane, as well as a low preventive antioxidant capacity and these, make them susceptible to damage by free radicals.[50] Overall, the levels of the enzymatic (SOD and GPx) and non-enzymatic (GSH) anti-oxidants in the control group were greater than those of the FLX group, and the MDA (an important indicator of peroxidation of lipid) level was lower in the control group. However, there was only a significant difference in terms of the MDA level between two groups, and no statistically significant difference was observed with regard to the other oxidative stress parameters, which was probably due to sample size. The standard deviation of GSH, SOD and GPx levels were high and it seems that with an increasing number of animals, the two groups could also have significant differences in terms of these parameters. Meanwhile, in contrast to our findings, Erdemir *et al*., in a study have shown that administration of SSRIs such as sertraline, paroxetine, and FLX in adult rats cannot significantly increase the MDA levels of blood serum.[9] This may be due to the difference in the timing of drug exposure.

In conclusion, our findings indicated that exposure to 20.00 mg kg^{-1} FLX in lactation period decreased body and testicular weight and induced histomorphological changes in the testes of mice in adulthood. In addition, a reduction in the sperm parameters and an increase in the levels of MDA and the percentage of sperm DNA damage with testicular tissue alterations were observed in these mice.

Acknowledgments

This work was supported by a grant from Deputy of Research, Urmia University of Medical Sciences, Urmia, Iran. We also would like to thank Shahre Darou Co. (Tehran, Iran) for providing fluoxetine for us as a gift.

Conflict of interest

There are no conflicts of interest to declare by authors.

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