A Study on the effect of different doses of pregabalin on the testis in adult male albino rat

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Abstract-
Background: Pregabalin (PGB) is an anti-convulsant drug that is indicated in epilepsy, neuropathic pain and anxiety. Pregabalin is an analogue to the neurotransmitter gamma amino butyric acid (GABA). Its main site of action is in the peripheral and central nervous systems. The present study aimed to evaluate the effect of different doses of pregabalin on the Testis in adult male rats.
Methods: 30 male albino rats were divided into 3 groups with 10 rats in each group. Group I (control group). Group II animals received pregabalin orally in a dose of 600 mg/kg/day for 4 weeks. Group III animals received pregabalin orally in a dose of 1200 mg/kg/day for 4 weeks. At the end of the experiment, Blood samples were taken and concentrations of testosterone, FSH and LH were measured by Radioimmunoassay method. Then, the testes were processed for histopathological study.
Results: The results showed that administration of PGB at a dose of 1200 mg/kg reduced serum testosterone level while it increased FSH and LH levels ($p<0.05$). It also caused a decrease in sperm motility, count and percent of viability and an increase in sperm abnormalities ($p<0.05$). Histological examination of the testes showed reduction in the number of spermatogenic and Leydig cells.
Conclusion: PGB has toxic effects on the testes when given at a dose of 1200 mg/kg as indicated by decreased concentration of testosterone, increased FSH and LH levels, a decrease in sperm motility, count and percent of viability, an increase in sperm abnormalities and reduced number of spermatogenic and leydig cells.

***Keywords*: Pregabalin, Testis, Histopathology, Testosterone, FSH, LH, Sperm parameters.**

1. Introduction

Epilepsy is the most common chronic neurological disorder in the world. The lost natural balance between excitement and inhibition in the central nervous system causes convulsions (Asadi-Pooya et al. 2012). Antiepileptic drugs create a natural balance between excitatory and inhibitory postsynaptic potential through different mechanisms (Deckers et al. 2000).

Pregabalin (PGB) is an antiepileptic drug with potent analgesic activity. PGB is an analogue of gamma-aminobutyric acid (GABA), but it binds to voltage-gated calcium channels (Field et al. 2006). Its main site of action is in the peripheral and central nervous systems (Sills 2006). PGB was displayed for the first time on the market by Pfizer under the trade name Lyrica. It was approved by the Food & Drug Administration (FDA) in 2004 (Al-Zubaidi et al. 2015).

PGB is prescribed for the treatment of neuropathic pain, post-herpetic neuralgia, seizures, fibromyalgia, and generalized anxiety disorder (Baldwin et al. 2013; Evoy et al. 2017). The broad therapeutic effects of PGB such as reducing neuropathic pain and anxiety and controlling epilepsy come from its ability to reduce the release of several excitatory neurotransmitters (like substance P and glutamate) and peptides like calcitonin gene-related peptides (Foroutan and Nikvarz 2016). With this action the existing neurons in the central nervous system that have been overstimulated return to their normal position (Baidya et al. 2011).

In pre-clinical animal studies, Pregabalin (PGB) was associated with reversible effects on sperm parameters (decreased sperm count and motility, and increased sperm abnormalities) and reproductive function (reduced fertility and increased pre-implantation embryo loss) (Ding et al. 2017; Etemad, et al. 2013). Some clinical studies have shown that pregabalin changes sexual desire, causes erectile dysfunction, and delayed ejaculation (Bucur and Jeczmien 2011; Calabro and Bramanti 2010). Unlikely other clinical trials found that treatment with PGB 600 mg/day for 12 weeks did not significantly affect spermatogenesis or serum levels of FSH and testosterone in healthy males (Sikka et al., 2015).

Hence, this work was performed to evaluate the effect of different doses of pregabalin on the testis in adult male albino rats.

2. Methods

2.1. *Animals*

The present study was carried out on 30 healthy adult male albino rats weighing from 200-250 g. They were purchased from the animal house of Assiut Faculty of Medicine, Assiut University, Egypt. The rats were housed in polypropylene cages under standard lightening in a temperature-controlled room (25 ± 2◦C) and had free access to laboratory food and water throughout the experiment. They were acclimatized to their environment for at least two weeks before starting the experiment. All animal procedures were approved by the local Institutional Animal Ethical Committee of faculty of medicine, NBU, KSA.

2.2. *Experimental design*

After the acclimatization period, rats were randomly divided into three groups (ten rats in each) as follows:

Group I (Control group): Received distilled water via oral gavage daily for 4 weeks.

Group II: Received pregabalin dissolved in distilled water via oral gavage (Sigma-Aldrich Chemical Co. St. Louis, MO, USA) at a dose of 600 mg/kg/day for 4 weeks.

Group III: Received pregabalin dissolved in distilled water via oral gavage at a dose of 1200 mg/kg/day for 4 weeks.

Twenty-four hours after the last drug regimen, the rats were anesthetized with ether and about 5 ml of blood was taken from the heart of each rat and poured in test tubes. Blood samples were centrifuged for 15 minutes at 3000 rpm to separate the serum. The samples were kept at -20°C for the measurement of serum concentrations of FSH, LH and testosterone. Measuring hormones was performed using radioimmunoassay (RIA).

The animals were then sacrificed by decapitation, and opening the abdomen and scrotum of the rats was done to extract both testicles and epididymes. The testicles were weighed then sampled for histopathological studies.

2.3. *Serum hormonal assay*

Testosterone, luteinizing hormone (LH) and follicle-stimulating hormone (FSH) levels were measured by radioimmunoassay using commercial kit (AccuBind ELISA Kits, California, USA). Analyses were carried out according to the manufacturer's instructions.

2.4. *Semen analysis*

The caudal part of the epididymis was cut and immersed in a Petri dish containing 2 ml of normal saline (0.9% NaCl) at 37ºC. The tissue is left for 30-60 seconds to let sperms leak from the tubules (the solution will tint whitish/grayish, similar to the diluted semen), and the resulted fluid will be handled exactly as the semen. Collect the fluid only into an Eppendorf tube.

Sperm count:

0.5 ml of the semen was added to 1 ml of the semen diluting fluid (sodium bicarbonate 5 g, formalin 1 ml, distilled water 99.0 ml) and subsequently mixed well. One drop of sperm suspension was loaded into hemocytometer chamber. and the sperms were allowed to settle by keeping the hemocytometer in a humid place for 10 minutes. The number of spermatozoa in the squares of the hemocytometer was counted under microscope. [The number of spermatozoa per ml = No. of sperms X dilution factor X depth factor /No. of areas counted].

Sperm motility:

Mixing one drop of semen with two drops of warm 2.9% sodium citrate on a pre-warmed slide, the slide was then covered with a warm cover slip and examined under the microscope using X400 magnification. Ten fields of the microscope were randomly selected and the sperm motility of 10 sperms was assessed on each field. Therefore, the motility of 100 sperms was assessed randomly. The percentage of motile sperms was defined as the number of motile sperms divided by the total number of counted sperms (i.e., 100) (Oyedeji et al. 2013).

Sperm viability (Life/dead ratio):

Mixing one drop of semen with two drops of warm Eosin/Nigrosin stain on a pre-warmed slide, a uniform smear was then made and dried with air; the stained slide was immediately examined under the microscope using X400 magnification. The live sperm cells were unstained while the dead sperm cells absorbed the stain. The stained and unstained sperm were counted and the percentage was calculated (Oyedeji et al. 2013).

Sperm morphology:

Mixing one drop of semen with two drops of warm Eosin/Nigrosin stain on a pre-warmed slide, a uniform smear was then made and dried with air; the stained slide was immediately examined under the microscope using X400 magnification. Five fields of the microscope were randomly selected and the number of abnormal spermatozoa were evaluated from the total number of spermatozoa in the five fields; the number of abnormal spermatozoa were expressed as a percentage of the total number of spermatozoa (Oyedeji et al. 2013).

2.5. *Histological examination*

The testis from each animal is kept in 10% of neutral buffered formalin for 24 h. It is then processed and embedded in paraffin wax and sections of 4 µm thickness were taken using a microtome. These sections were stained with

hematoxylin and eosin (H&E) and are examined under a light microscope, to detect histological changes (Bancroft and Layton 2012).

2.6. *Statistical Analysis*

All analyses were performed using the software Statistical Package for Social Sciences version 17 (SPSS Inc, Chicago, IL, USA). Data were presented as the mean ± standard deviation (SD). Comparisons between two groups were analyzed by unpaired Student "t" test. The probability of chance (*P* value) < 0.05 was considered statistically significant.

3. Results

None of the experimental rats died during the experiment period (4 weeks).

3.1. *Evaluation of testicular weight*

At the end of the experimental period, the testis weights in group III (PGB given at a dose of 1200 mg/kg) were significantly lower than the control group. In group II, no significant difference was detected **(Table 1)**.

3.2. *Serum hormonal assay*

There was a decrease in serum concentration of testosterone hormone in PGB-treated rats compared with normal rats. This decrease was significant in group III. There was an increase in serum concentration of FSH and LH hormones in PGB-treated rats compared with normal rats. This increase was also significant in group III. (**Table 2**).

3.3. *Semen analysis*

There was a decrease in sperm motility, count and percent of viability and an increase in sperm abnormalities in PGB-treated rats compared with normal rats. These changes were significant in group III (**Table 3**).

3.4. *Histological results*

The testis of control rats of H&E-stained sections showed seminiferous tubules surrounded by connective tissue, containing many rounded or polygonal interstitial cells of leydig. Immediately surrounding each tubule were flattened myoid cells. Inside the tubule, there was Sertoli cells, and germ cells of the spermatogenic lineage. Prominent among the latter were spermatogonia, located near the basement membrane, and primary spermatocytes closer to the lumen of the tubule. Sperm were located in the center of the tubule (**Fig. 1**).

In group II (rats receiving PGB at a dose of 600 mg/kg), H&E-stained sections showed a histological structure which was more or less similar to normal. There was no evidence of adverse effects on the testicular tissue caused by the drug (**Fig. 2**).

In group III (rats receiving PGB at a dose of 1200 mg/kg), H&E-stained sections showed loss of continuity of the chain of spermatogenesis in some seminiferous tubules (**Fig. 3A**). Other tubules showed predominance of the spermatogonia on the expense of the other spermatogenic cells (**Fig. 3B**). Some tubules showed loss of sperms in its center (**Fig. 3C**). The interstitium showed decreased number of the interstitial cells of leydig (**Fig. 3D**).

4. Discussion

The period of spermatogenesis is considered as a period of intense cellular transformation which is regarded to be highly susceptible to external insults. Various germ cell stages are affected differentially by exposure to injurious agents. Spermatogonia have been recognized as target cells for ionizing radiation, while chemical agents are more considered as spermatocyte toxins (Griswold 2016). Gabapentinoids (e.g., pregabalin and gabapentin) are widely used in neurology, psychiatry, and primary healthcare but are increasingly being reported as possessing a potential for misuse (Liliana et al. 2015).

In this research, we aimed to evaluate the effect of different doses of pregabalin on the Testis in adult male rats. According to previous studies, the maximum tolerable dose in humans is 600 mg/day. So, we used 600 mg/kg in rats as the low dose which was also tolerable and didn't cause side effects. Then we doubled the dose to see the difference.

The present study revealed that administration of PGB at a dose of 1200 mg/kg caused a significant decrease in testicular weight compared with normal rats. This decrease in testicular weight could be attributed to the impairment of spermatogenesis with reduced number of spermatogenic and Leydig cells. This result was supported by Kamel and Khalifa (2015) who reported a significant decrease in the weights of the testis, seminal vesicles and prostate glands after treatment with pregabalin 20 mg/kg for 65 consecutive days. On the contrary, another study stated that testicular weight did not show significant differences between groups (Hareedy et al. 2020)

In the present study, administration of PGB at a dose of 1200 mg/kg produced a significant decrease in serum testosterone level and a significant increase in FSH and LH levels compared with normal rats. These results agreed with Hareedy et al. (2020) who found that administration of PGB for 2 months led to a significant decrease in testosterone levels and a significant increase in the levels of both FSH and LH compared to the control group. Another study reported that Pregabalin reduced serum testosterone level while it increased FSH and LH levels when given orally for 28 days (Bostanian et al. 2016). A third study showed decreased levels of both testosterone and LH hormones, while plasma concentration of FSH was not affected when pregabalin 20 mg/kg was given for 65 consecutive days (Kamel MA and Khalifa 2015).

Many theories interpreted the cause of testosterone reduction. One of these theories is that PGB increases prolactin by reducing the release of dopamine in the synaptic cleft. Prolactin through increasing nitric oxide inhibits the conversion of cholesterol to pregnenolone which reduces testosterone (Mokhtari et al. 2007). Another theory declared that PGB by reducing serotonin release, increases glutamate decarboxylase enzyme activity which increases the synthesis of GABA. GABA inhibits ACTH hormone secretion by the anterior pituitary. By reducing ACTH, adrenal gland cortex activity to produce steroids is reduced, thus testosterone level is reduced (Soltani et al. 2009; Guyton and Hall 2006). Low testosterone secretion based on negative feedback allows the hypothalamus to release large amounts of GnRH resulting in increased LH and FSH secretion from the anterior pituitary gland. Reduced testosterone levels may have a negative feedback effect on the pituitary gland directly, and this pituitary feedback increases LH secretion exclusively (Modaresi and Amiri 2011).

The present study revealed that administration of PGB at a dose of 1200 mg/kg caused a significant decrease in sperm motility, count, and percent of viability and a significant increase in sperm abnormalities compared with normal rats. Pregabalin by increasing prolactin secretion causes a reduction in the activity and division in the epithelial cells of the testes, therefore reduced number of Spermatogenic cells with PGB treatment is expected (Bostanian et al. 2016). These results agreed with Kamel and Khalifa (2015) who denoted a significant decrease in sperm motility, count, and percent of viability and a significant increase in sperm abnormalities with oral administration of pregabalin 20 mg/kg for 65 consecutive days. Another study found a significant decrease in sperm count, motility, and morphologically normal sperms and a significant increase in sperm agglutination when exposed to PGB 600 mg/Kg daily for 35 days (Al-Zubaidi et al. 2015). In contrast, in controlled trials to assess the effect of PGB on sperm motility in healthy men who were exposed to PGB 600 mg/kg for three months (one complete sperm cycle in humans), PGB did not exhibit significant detrimental effects on the reproductive functions of healthy male subjects, as measured by semen analysis. This makes PGB a rather safe drug for human usage regarding its effect on spermatogenesis (Sikka et al. 2015).

Histological examination of PGB-treated rats revealed that it has many adverse effects on the testis structure when given at a dose of 1200 mg/kg. The sections of the testis stained with H&E showed a reduced number of primary spermatocytes, spermatids, and sperms in the seminiferous tubules with predominance of the undifferentiated spermatogonia in some tubules. The sections also showed a reduced number of leydig cells. The findings of the present study were supported by another study which revealed degenerated seminiferous tubules in the PGB-treated group. Some seminiferous tubules (about 30%) show late spermatid while most of the tubules showed spermatogenesis at the level of early spermatid (Hareedy et al. 2020). A third study reported a reduced number of spermatogonia, primary spermatocytes, and spermatids with destruction and loss of the interstitial cells in the groups treated with PGB (Bostanian et al. 2016). Al-Zubaidi et al. (2015) noticed that the spermatogonia overpopulate most of the other spermatogenic cells. Thus, there was a relative prevalence of primitive germ cells at the expense of more mature ones.

5. Conclusions

It could be concluded that PGB had toxic effects on the testis of rats when given at a dose of 1200 mg/kg/day for 4 weeks. These were in the form of decreased concentration of testosterone, increased FSH and LH levels, a decrease in sperm motility, count and percent of viability, an increase in sperm abnormalities and reduced number of spermatogenic and leydig cells. Further investigation on humans is recommended for better assessment of the adverse effects of PGB.

List of Abbreviations

PGB	Pregabalin

Declarations

Ethics approval and consent to participate:

The manuscript doesn't involve human participants, human data or human tissue.

Consent for publication:

The manuscript doesn't contain any individual person's data in any form.

Availability of data and material:

All data needed is discussed in details in the manuscript.

Competing interests:

The author declares that there are no conflicts of interest related to the subject matter or materials discussed in this article.

Authors' contributions:

I am the only author.

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Table 1. Testicular weights of rats in the different studied groups.

Parameters	Group I (Control)	Group II (PGB 600 mg/kg)	Group III (PGB 1200 mg/kg)
Testis weight (g)			
	2.85±0.013	2.83±0.012	2.15±0.025[a]

Data is expressed as mean ± standard deviation. Results were statistically analyzed by using Student's *t* test at P < 0.05.

[a]*p* < 0.001 compared with the control group (group I).

Table 2. Serum levels of testosterone, FSH and LH hormones in the different studied groups.

Parameters	Group I (Control)	Group II (PGB 600 mg/kg)	Group III (PGB 1200 mg/kg)
Testosterone (ng/ml)	4.82±1.08	4.75±0.005	2.43±1.29[a]
FSH (mIU/ml)	8.69±0.04	9.17±1.09	13.44±0.35[a]
LH (mIU/ml)	5.07±0.012	5.16±1.03	7.21±0.002[a]

Data is expressed as mean ± standard deviation. Results were statistically analyzed by using Student's t test at P < 0.05.

[a]p < 0.001 compared with the control group (group I).

Table 3. Sperm parameters in the different studied groups.

Parameters	Group I (Control)	Group II (PGB 600 mg/kg)	Group III (PGB 1200 mg/kg)
Sperm count (x10^6/ml)	104.7±8.8	99.4±2.05	63.3±6.29[a]
motility (%)	91.3±2.8	89.2±1.09	48.44±1.6[a]
Viability (%)	94.6±3.45	90.23±1.03	62.81±2.13[a]
Abnormalities (%)	2.43±0.13	2.52±0.24	9.64±0.15[a]

Data is expressed as mean ± standard deviation. Results were statistically analyzed by using Student's t test at P < 0.05.

[a]p < 0.001 compared with the control group (group I).

Figure legends:

Fig. 1: Photomicrograph of normal architecture of the testis in the control group showing seminiferous tubules surrounded by connective tissue, containing many rounded or polygonal interstitial cells of leydig (L). Immediately surrounding each tubule were flattened myoid cells (M). Inside the tubule, a unique seminiferous epithelium composed of columnar Sertoli cells (SC), and germ cells of the spermatogenic lineage is seen. Prominent among the germ cells are spermatogonia (SG), located near the basement membrane, and primary spermatocytes (PS) closer to the lumen of the tubule. Sperm are located in the center of the tubule (S) (H&E, x400).

Fig. 2: Photomicrograph of testis in rats treated with PGB at a dose of 600 mg/kg showing normal looking testicular tissue more or less similar to normal. (H&E, x400).

Fig. 3: (A-D) Photomicrographs of testis in rats treated with PGB at a dose of 1200 mg/kg. (A) loss of continuity of the chain of spermatogenesis is seen in some seminiferous tubules. (B) some tubules show predominance of the spermatogonia on the expense of the other spermatogenic cells. (C) Some tubules show loss of sperms in its center (**). (D) The interstitium shows destruction and loss of the leydig cells (L) (H&E, x400).

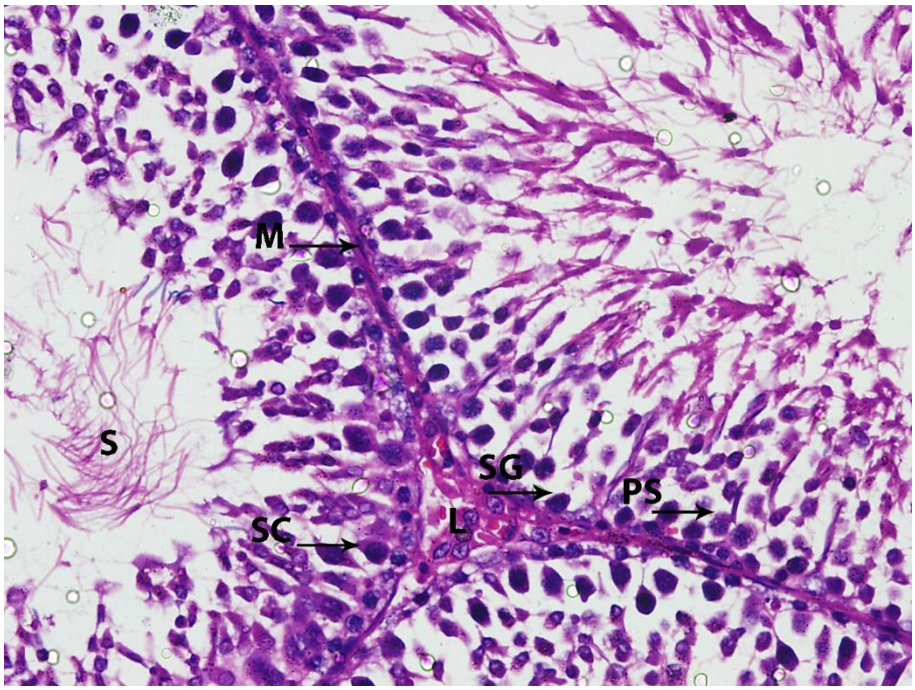

Figure 1

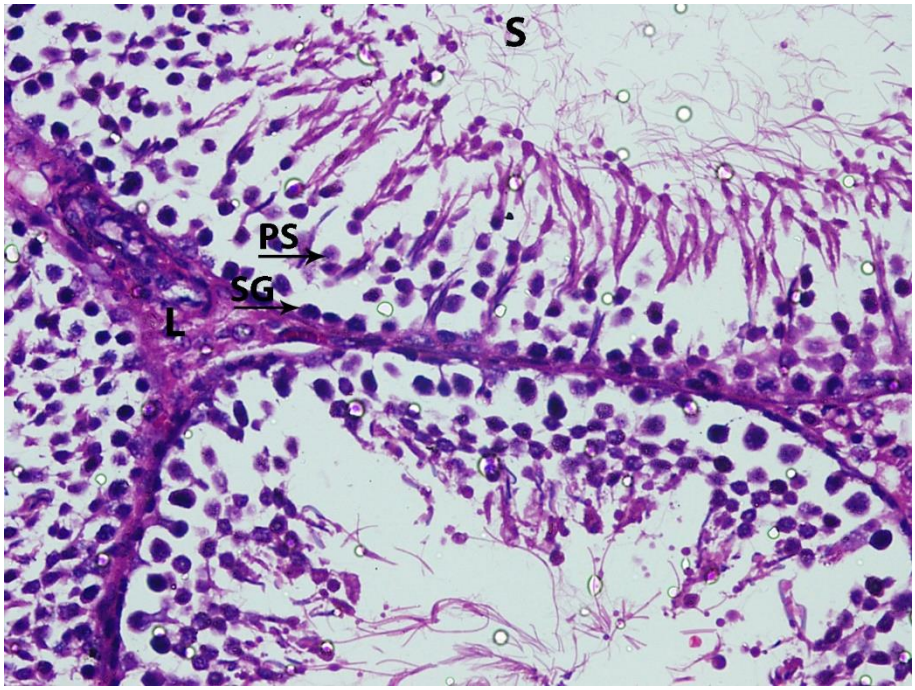

Figure 2

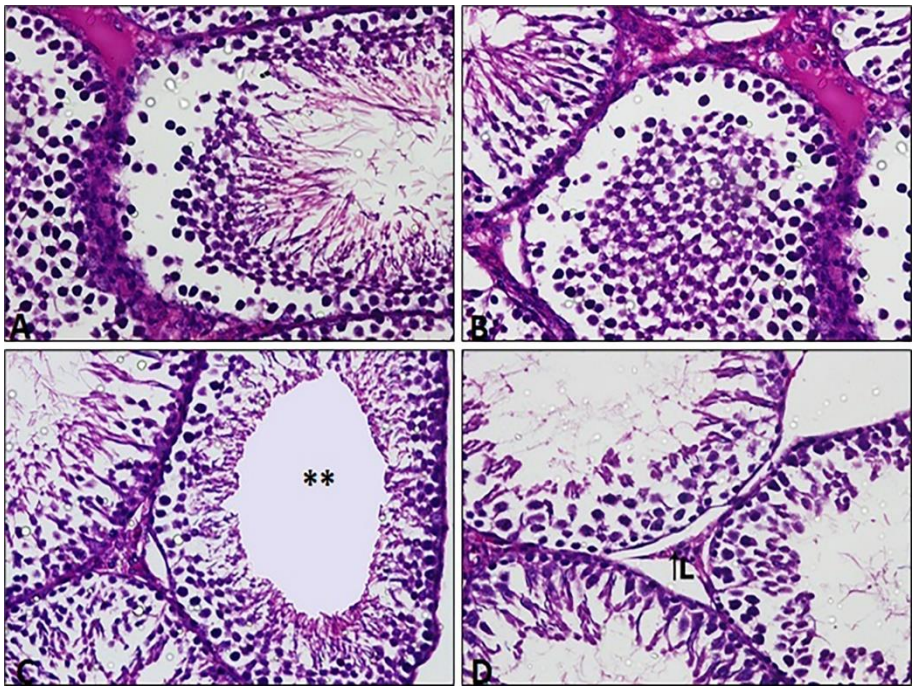

Figure 3