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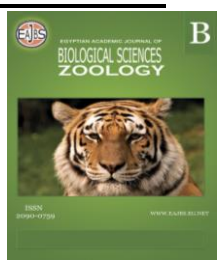

Tramadol Biological Effects, 3: Effects of *Lagenaria siceraria* Preparation and Melatonin on The Changes in Testes of Tramadol-Induced Male Mice

Abdel-Baset M. Aref
Zoology Department, Faculty of Science, South Valley University, Egypt.
E.mail*: aref322189@yahoo.com & abdelbasset.aref@sci.svu.edu.eg

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ABSTRACT

Aims: the study of the biological effects of tramadol on testes of the male mice and treatment of its side effects via *Lagenaria siceraria* or melatonin

Results: the results of the present work showed the injection of tramadol for 20 and 40 days decreased the volume of the somniferous tubules and the number and volume of nuclei of the interstitial cells of Leydig in the testes of the male mice. While stopping tramadol injection, *lagenaria siceraria* or melatonin increased the diameter of the somniferous tubules and the number and volume of nuclei of the interstitial cells of Leydig in the testes of the male mice. The present data showed the injection of tramadol for 20- and 40-days lead to a lot of the pathological features and alternate the histological architectures of testes the male mice. While stopping tramadol injection, Lagenaria Siceraria or melatonin treats the histopathological side effects of tramadol.

Conclusion: tramadol atrophy of the seminiferous tubules, and reduces the number and inhibits the cellular activities of the interstitial cells of Leydig and directly inhibits its function such as the production of testosterone hormone. Finally, tramadol inhibits germinal layer cells and the production of different reproductive cells. While stopping tramadol, *Lagenaria Siceraria*, or melatonin teats these tramadol effects.

INTRODUCTION

Yassa *et. al.* (2010) noted that tramadol administration induced a decrease in testosterone and total cholesterol and increased level of testicular levels of nitric oxide and lipid peroxidation, and decreased the antioxidant enzymes activities significantly compared with the control group. These may facilitate the damage of spermatogenic cells via the increase of reactive species. Also, tramadol is similar to Cannabis which is the most commonly abused drug in the world. Its administration led to disruption of the spermatogenic cells from the membranes. Caju *et. al.* (2012) noticed a reduction in Sertoli and Leydig cells of mature albino rats when exposed to acute and chronic doses of opiates. The testicular change occurred due to disorders in the endocrine and paracrine functions that can indirectly influence the final size of the Sertoli cell population through disordered LH, estradiol, somatotropin, somatostatin, prolactin, and GnRH (gonadotropin-releasing hormone) acted upon either on the hypothalamus or directly on pituitary glands. Ahmed and Kurkar (2014) recorded that chronic administration of

tramadol caused a decrease in sperm count, motility, and numbers of primary spermatocytes, rounded spermatid, and Leydig cells**.** They concluded that tramadol treatment affects the testicular function of adult male rats, and these effects might be through the overproduction of nitric oxide and oxidative stress induced by this drug**.** Saha *et. al.* (2011) studied that a novel protein Lagenin isolated from Lagenaria siceraria seeds possesses antitumor, immunoprotective, and antiproliferative properties.

Lagenaria siceraria was used as an antidote to certain poisons. It possesses antioxidant, anthelmintic, antibacterial, antifungal, immunomodulatory, anti-allergic, analgesic, anti-inflammatory, free radical scavenging, cytotoxic, antihyperlipidemic antidiabetic, hepatoprotective, anxiolytic, and memory-enhancing properties (Aslam and Najam, 2013). Reiter *et. al.* (2003) mentioned that melatonin is capable of prevention molecular damages caused by toxic oxygen-and nitrogen-based reactants. Melatonin functions in scavenging free radicals might be classified into four main categories: (1) as an antioxidant directly scavenges ROS (Reiter, 2000), (2) stimulates the antioxidant enzymes production and activation (Rodriguez *et. al.*, 2004) and (3) increases the efficacy of mitochondrial functions by improving MPTP, inhibition of cytochrome c release and refining of oxidative phosphorylation in the mitochondrial respiratory chain which further will decrease the peroxidation of membrane lipids (Acuna-Castroviejo *et. al.*, 2007).

MATERIALS AND METHODS

Animals:

A total number of 70 adult male Swiss albino mice were obtained from the Autoradiographic lab. of Cell Biology and Immunology, Faculty of Science, South Valley University. At the time of the experiments, the mice were each aged 90 ± 5 days and weighed 30 ± 2 g. All male mice were kept under the same conditions, an artificial light-dark cycle (12h-12h), a constant temperature (23 ± 2^{o}c), and a level of humidity (37-40%). They were supplied standard food and water *ad libitum*. Experiments of this research were conducted in a lab. achieved stability of environmental conditions, with a separation between treated animals and control ones and IACUC targets.

Chemicals:

1- **Tramadol HCl ampoules** were purchased from October Pharma S.A.E., 6 October City, Egypt) was diluted with distilled water to a concentration of 150ug/1ml.

2- **Melatonin** [Sigma-Aldrich, Co. 3050 spruce street, St. Louis, MO 63103 USA 314-771-5765] was dissolved in a few drops of absolute ethanol and diluted with distilled water to a concentration of 500 μg/ml.

3- ***Lagenaria siceraria***: special preparation of *Lagenaria siceraria,* (Gamal & Aref$_1$), were prepared by Gamal Yagteen and Abdel-baset Aref via squeezer on 6 October City, Egypt (Aref *et al.*, 2018 & Aref *et al.*, 2020 & Aref *et al.*, 2021).

Experimental Design:

2 experiments were performed differing in exposure to the various treatments.

Experiment I:

Treated animals were divided into 3 groups, including 10 males each.

Group C: animals were daily subcutaneously injected with distilled water (0.25ml/ 30g b.w.) and served as control.

Group T$_1$: animals were daily subcutaneously injected with tramadol HCl (125ug / 100g b.w.) for 20 days.

Group T$_2$: animals were daily subcutaneously injected with tramadol HCl (125ug / 100g b.w.) for 40 days.

Experiment II:

All male mice were injected subcutaneously with a daily injection of tramadol (125ug / 100g b.w) for 40 days and subdivided into 4 groups, concluding 10 males each, designated as groups T, T_S, T_L, and T_M as follows:

Group T: Animals were treated with tramadol only.

Group T_S: tramadol-treated animals were injected with a daily injection of distilled water (0.25ml / 30g b.w.) for 40 days.

Group T_L: tramadol-treated animals were treated with a daily oral dose of a special preparation of *Lagenaria siceraria* (Gamal & Aref1) (0.5 mg/ b.w.) for 40 days.

Group T_M: tramadol-treated animals were daily injected with a subcutaneous injection of Melatonin (100µg / 100g b.w.) for 40 days at 16h, 2h before the end of the light cycle for 40 days.The mice of group T in experiment II were similar to mice of group T_2 in experiment I, which were daily injected with tramadol for 40 days only before sacrifice.

All male mice were anesthetized by Chloroform and were sacrificed the day following the last injection or oral dose.

Tissues Preparation:

Tissue specimens of the testes were excised, fixed in neutral formalin 10%, dehydrated in graded series of ethanol, cleared, embedded in paraffin wax, and sectioned at 5 microns thickness. The testes sections were stained with Hematoxylin and eosin stain.

Karyometric Studies:

The measurements of the volume of nuclei of the interstitial cells of Leydig were carried out by using a camera program (LAS ZA) according to nucleus shape (rounded nuclei) and the following equation was applied: $V= 4/3\pi\ r^3$. Where: V= volume of nucleus, r = semi diameter (Lewinski *et al.*, 1984).

RESULTS

Cell Biological Changes:

1-Changes in the Somniferous Tubules in Testes of The Male Mice:

Experiment I:

In the tests of male mice of groups C, group T_1, and group T_2, values of the mean diameter of the somniferous tubules were 183, 167, and 123 respectively (Table 1).

From the quantitative point of view, the injection of tramadol for 20 and 40 days decreased the diameter of somniferous tubules by 8.7% and 32.8% respectively compared with control male mice. Also, the increase of tramadol treatment for 40 days decreased the diameter of somniferous tubules by 26% versus the tramadol treatment for 40 days (Table 1). From the cell biological point of view, tramadol has an inhibitory effect, that increased with time administration, on the diameter of the somniferous tubules in the testes of male mice. Tramadol decreased the volume of the somniferous tubules and lead to atrophy of the somniferous tubules and tests**.**

Experiment II:

In the tests of male mice of groups T_S, group T_L and group T_M, values of the mean diameter of the somniferous tubules were 152, 159, and 160 respectively (Table 1).

From the quantitative point of view, the treatments with stopping tramadol injection (T_S), *Lagenaria Siceraria* (T_L), or melatonin (T_M) for 40 days increased the diameter of the somniferous tubules by 23.6%, 29.3%, and 30.1% respectively versus mice that received tramadol for 40 days (Table 1). From the cell biological point of view, the three treatments that used to some extent counteract the effect of tramadol.

2- Changes in the Number of The Interstitial Cells of **Leydig:**

Experiment I:

In the tests of the male mice of groups C, group T_1, and group T_2, values of the mean number of the interstitial cells of Leydig were 15, 7, and 6 cells respectively (Table 1). From the quantitative point of view, the injection of tramadol for 20 and 40 days decreased the mean number of the interstitial cells of Leydig by 53.3% and 60% respectively compared with control mice (Table 1).

Cytologically, tramadol has an inhibitory effect on a number of the interstitial cells of Leydig in tests of the male mice. Tramadol inhibits, indirectly, the production of testosterone hormone

Experiment II:

In the tests of male mice of groups T_S, group T_L and group T_M, values of the mean number of the interstitial cells of Leydig were 9, 10, and 11 cells respectively (Table 1).

From the quantitative point of view, the treatments with stopping tramadol injection (T_S), *Lagenaria Siceraria* (T_L), or melatonin (T_M) for 40 days increased the mean number of the interstitial cells of Leydig by 33%, 44%, and 55% respectively compared with the mice that received tramadol for 40 days (Table 1).

From the cell biological point of view, stopping tramadol injection, *Lagenaria Siceraria*, or melatonin increased the number of the interstitial cells of Leydig and indirectly, induce the production of testosterone hormone in testes. they are to some extent counteract the effect of tramadol.

3- Changes in the Volume of Nuclei of Interstitial Cells of Leydig in Testes:

Experiment I:

In testes of the male mice of groups C, group T_1, and group T_2, values of mean volume of nuclei of the interstitial cells of Leydig were 78 μ, 44 μ, and 31 μ respectively (Table 1). Tramadol injection for 20 and 40 days decreased volume of nuclei of the interstitial cells of Leydig in testes versus control mice. While mice received tramadol for 40 days more decreased volume of nuclei of Leydig cells versus mice that received tramadol for 20 days. From the quantitative point of view, the injection of tramadol for 20 and 40 days decreased the volume of nuclei of the interstitial cells of Leydig by 43.6% and 60.3% versus control mice (Table 1). From the ell biological point of view, tramadol has an inhibitory effect, that increased with increasing of term injection tramadol, on the volume of nuclei of the interstitial cells of Leydig in testes of the male mice. Tramadol inhibits the cellular activities of the cells of Leydig and directly inhibits its function such as the production of testosterone hormone. Finally, tramadol inhibits germinal layer cells and the production of different reproductive cells.

Experiment II:

In the tests of male mice of groups T_S, group T_L and group T_M, values of mean volume of nuclei of the interstitial cells of Leydig were 64 μ, 63 μ, and 64 μ respectively (Table 1). From the quantitative point of view, the treatments with stopping tramadol injection (T_S), *Lagenaria Siceraria* (T_L), or melatonin (T_M) for 40 days increased the volume of nuclei of the interstitial cells, by approximately the same rate, by100.6%, 103%, and 106% respectively versus the male mice received tramadol for 40 days (Table 1). From the cell biological point of view, all previous treatments; stopping tramadol, *Lagenaria Siceraria*, and melatonin treat the side effects of tramadol.

Stopping tramadol, *Lagenaria Siceraria*, or melatonin increased the cellular activities of the interstitial cells of Leydig and stimulates its hormonal function such as the production of testosterone hormone.

Table 1: Mean diameter of somniferous tubules, number of interstitial cells and volume of nuclei of interstitial cells in tests of control (C) and tramadol treated male mice for 20, 40 days (T_1, T_2) of experiment **I** and tramadol treated male mice for 40 days (T), tramadol treated mice for 40 days and then treated with D.W. (T_S) or *Lagenaria Siceraria* oil (T_L) or melatonin (T_M) for 40 days in experiment **II**. Also, should is the percentage stimulation (S%) or percentage inhibition (I%) of the mean.

The seminiferous tubule of tests	Experiment I			Experiment II			
Groups / Measurements	C	T_1	T_2	T	T_S	T_L	T_M
Mean Diameter of Somniferous tubules	183	167	123	123	152	159	160
(S%) or (I%) of the mean diameter of seminiferous tubules		I=8.7%	I=32.8% I=26%		S=23.6 %	S=29.3 %	S=30.1 %
Mean number of interstitial cells of Leydig	15	7	6	6	9	10	11
(S%) or (I%) of the mean number of interstitial cells of Leydig		I=53.3 %	I=60% I=14.3%		S=33%	S=44%	S=55%
Mean volume of nuclei of interstitial cells	78 µ	44 µ	31 µ	31 µ	64 µ	63 µ	64 µ
(S%) or (I%) of the mean number of interstitial cells		I=43.6 %	I=60.3% I=29.5%		S=100.6 %	S=103%	S=106%

Histopathological Changes in Testes:

Experiment I:

The histological sections of the control testis showed normal seminiferous tubules with a normal arrangement pattern of spermatogenic cells. The intertubular tissue appears rich in interstitial cells (Fig. 1).

Testes of the mice that received tramadol for 20 days (T_1) detected atrophy of seminiferous tubules. There was intertubular edema and thickening with round cells infiltration inside tubules. Most seminiferous tubules were filled with homogenate fluid (Figs. 2 & 3).

Testes of the mice that received tramadol for 40 days (T_2) showed atrophied and inactive seminiferous tubules, in addition to vacuolation of seminiferous tubules and filled with homogenate fluids. Also, thickening and congestion of the blood vessels were noticed (Figs. 4 & 5 & 6).

Experiment II:

After stop tramadol injection (T_S), testes exhibited a moderate reduction of seminiferous tubules in a few mice. Other mice showed atrophied seminiferous tubules and spermatozoa. Thickening and edema of the interstitial tissues were observed (Figs. 7 & 8). The mice who received tramadol plus *Lagenaria Siceraria* (T_L) showed slight reduction and vacuolation of seminiferous tubules, besides minimal interstitial edema (Fig. 9). In addition, moderate distribution of spermatozoa inside seminiferous tubules was detected. While after treatment with melatonin (T_M), testes displayed a mild reduction of spermatozoa inside seminiferous tubules (Figs. 10 & 11 & 12) with slight interstitial edema and thickening.

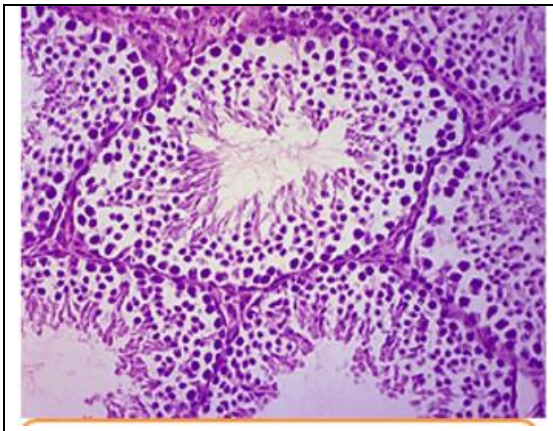

Fig.1: Photomicrograph of the testes of the control mice(C) showing normal seminiferous tubules with a normal arrangement pattern of spermatogenic cells. (H&E., X 400).

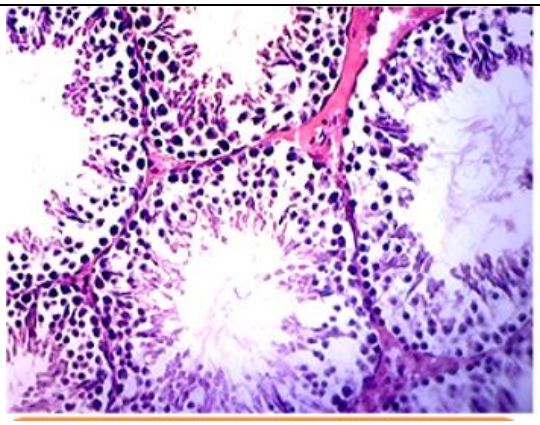

Fig.2: Photomicrograph of testes of the mice who received tramadol (T1) showing severe atrophied seminiferous tubules. (H&E., X 400).

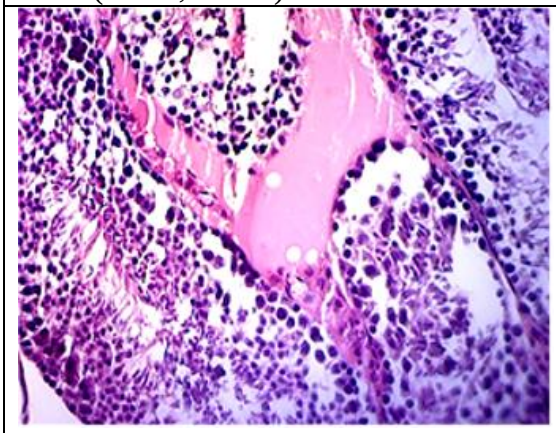

Fig.3: Photomicrograph of testes of the mice who received tramadol (T1) showing edematous and congested intertubular tissues. (H&E., X 400).

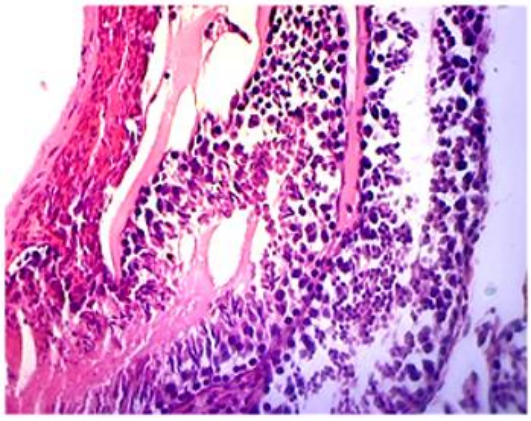

Fig.4: Photomicrograph of testes of the mice received tramadol (T2) showing vacuolation of seminiferous tubules and filled with homogenate fluids.(H&E., X 400).

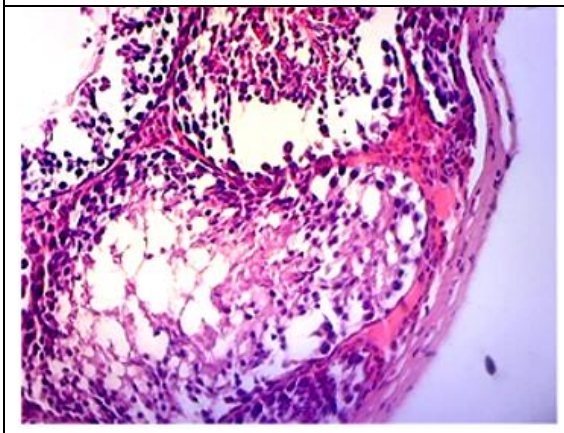

Fig.5: Photomicrograph of testes of the mice received tramadol (T2) showing seminiferous tubules filled with homogenate fluids. (H&E., X 400).

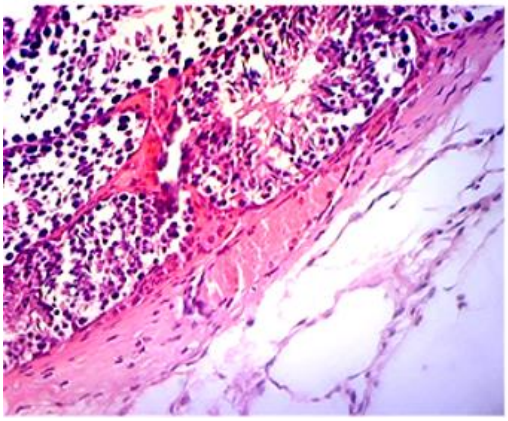

Fig.6: Photomicrograph of testes of the mice who received tramadol (T2) showing thickening and congestion of the blood vessels. (H&E., X 400).

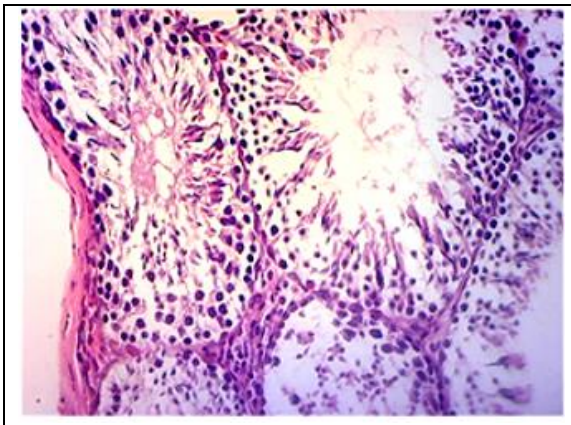

Fig.7: Photomicrograph of testes of the mice after stop tramadol injection (TS) showing moderate reduction of seminiferous tubules.(H&E., X 400)

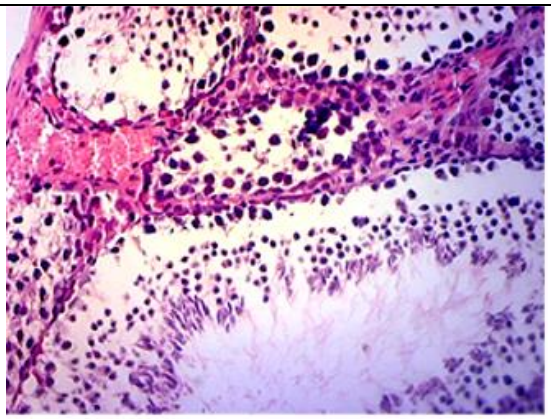

Fig.8: Photomicrograph of testes of the mice after stop tramadol injection (TS) showing thickening and edema of the interstitial tissues. (H&E., X 400)

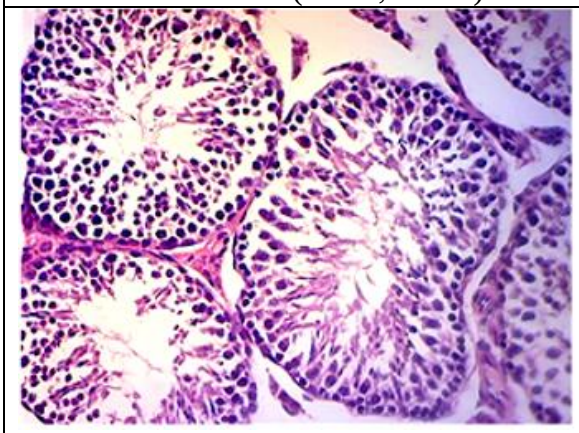

Fig.9: Photomicrograph of testes of the mice received tramadol plus Lagenaria Siceraria (TL) showing slight reduction and vacuolation of seminiferous tubules (long arrow), besides minimal interstitial edema (short arrow). (H&E., X 400).

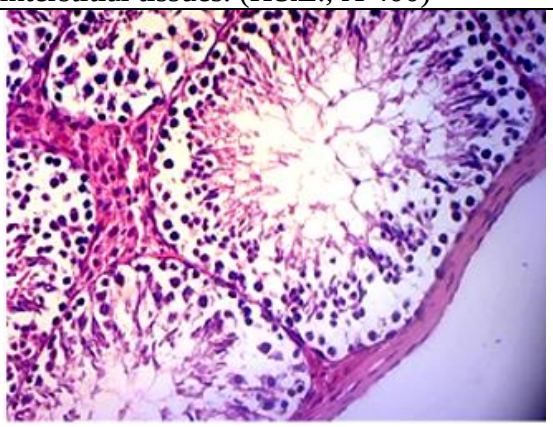

Fig.10: Photomicrograph of testes of the mice who received tramadol plus melatonin (TM) showing mild reduction of spermatozoa inside seminiferous tubules. (H&E., X 400).

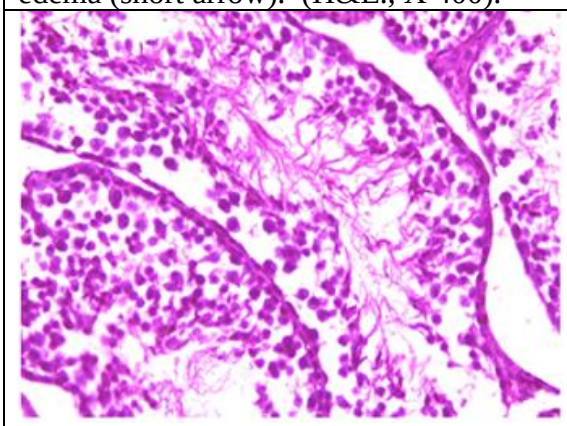

Fig.11: Photomicrograph of testes of the mice who received tramadol plus melatonin (TM) showing mild reduction of spermatozoa inside seminiferous tubules. (H&E., X 400).

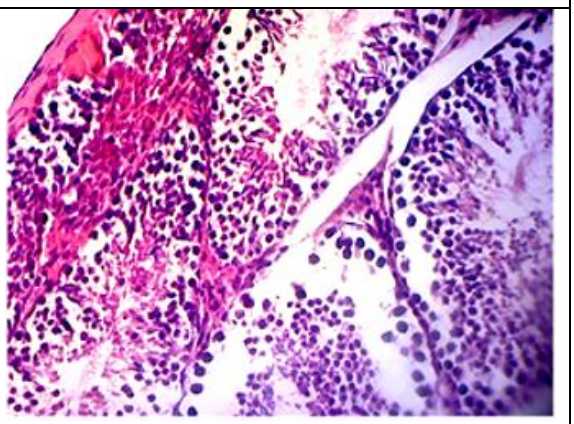

Fig.12: Photomicrograph of testes of the mice received tramadol plus melatonin (TM) showing slight interstitial edema and thickening. (H&E., X 400).

DISCUSSION

From the cell biological point of view, the results of the present work showed the injection of tramadol for 20 and 40 days decreased the volume of the somniferous tubules and the number and volume of nuclei of the interstitial cells of Leydig in the testes of the male mice. While stopping tramadol injection, *Lagenaria Siceraria* or melatonin increased the diameter of the somniferous tubules and the number and volume of nuclei of the interstitial cells of Leydig in the testes of the male mice.

From the Histopathological point of view, the present data showed the injection of tramadol for 20- and 40-days lead to a lot of the pathological features and alternate the histological architectures of testes the male mice. While stopping tramadol injection, Lagenaria Siceraria or melatonin treats the pathological side effects of tramadol.

There is little or no published literature concerning to study of the biological effect of tramadol, melatonin, and Lagenaria siceraria on the testes of the male mice, therefore may it be difficult to discuss the results of the present work with the other published works previously.

Our results indicated that Tramadol inhibits both the volume of the somniferous tubules that leads to atrophy of the somniferous tubules, the number of the interstitial cells of Leydig, and indirectly, the production of testosterone hormone. Also, Tramadol inhibits the cellular activities of the cells of Leydig and directly inhibits its function such as the production of testosterone hormone. Finally, tramadol inhibits germinal layer cells and the production of different reproductive cells. These our results agree to part with the following publications:

Ahmed and Kurkar (2014) revealed that tramadol treatment increased testicular level of nitric oxide, and lipid peroxidation and decreased the antioxidant enzymes activities; these may facilitate the damage of testicular tissues. Immunohistochemical examinations showed that tramadol increased the expression of endothelial nitric oxide synthase in testicular tissues. They concluded that tramadol treatment affects the testicular function of adult male rats, and these effects might be through the overproduction of nitric oxide and oxidative stress induced by this drug. Also, tramadol caused a decrease in sperm count, motility, and numbers of primary spermatocytes, rounded spermatid, and Leydig cells. Tramadol significantly reduced plasma levels of luteinizing hormone (LH), follicle-stimulating hormone (FSH), testosterone, and total cholesterol, but elevated prolactin and estradiol levels compared with the control group. Osadolor and Omo-Erhabor (2016) indicate that tramadol can also affect testes and ovaries causing reduced testosterone and estrogen secretions. Also, long-term use could result in opiate-induced endocrinopathy which could predispose users to cases of infertility for both males and females.

Our results showed a lot of the pathological features and alternate the histological architectures of testes of the male mice. These results are somewhat consistent with previously published data: Abou El Fatoh *et. al.* (2014) revealed that the testes undergo severe diffused testicular degeneration with numerous spermatocytes and spermatid giant cell formation (the cells might be fused to form such giant cells) without spermatogenesis after tramadol administration. The spermatocytes became mostly necrotic. Also, they found severe histopathological lesions of the testes when administrated male rats to oral doses of tramadol (tramadol HCl) suspended in saline solution. The histopathological lesions of the testes included severe diffused testicular degeneration.

Microscopic examinations revealed that tramadol led to atrophy and damage of the testicular tissue so that more degenerative changes were observed after long-term administration of tramadol. Most of the histopathological parameters returned to the

normal structure in week 12 (zari *et. al.*, 2014).

Kayaci *et. al.* (2014) showed that tramadol leads to ruptured seminiferous tubules with damaged and disorganized spermatogenic cells lifting off basal lamina and some of them are exfoliated in the lumen of the tubule. It demonstrated the pyknotic nuclei, vacuoles in the interstitial space, absence of spermatozoa, and multinucleated giant cells. Ahmed and Kurkar (2014) injected male albino rats subcutaneously with tramadol. It affected negatively the testis, spermatogenesis, sperm maturation, and led to programmed cell death and necrosis of Sertoli and germ cells. This occurred through an increase of the testicular levels of nitric oxide (eNOS) and lipid peroxidation. El Shal and Selim (2015) stated that the germinal epithelium of the seminiferous tubules of the tramadol group showed vacuolation with acidophilic materials in spermatogenic cells. Spermatids and sperms were rarely seen. Ultrastructurally, some of the spermatogenic cells were seen ruptured containing small dark condensed nuclei. The basement membrane (B.M.) was discontinuous at certain points and the collagen fibers were detected around it. Some Leydig cells were seen ruptured.

From the cell biological point of view, our results revealed that the treatment via stopping tramadol injection, Lagenaria Siceraria, or melatonin to some extent counteract the side effects of tramadol, where increased both the volume of the somniferous tubules and the number and cellular activities of the interstitial cells of Leydig that, stimulate its hormonal function such as the production of testosterone hormone. Our results reported reversibility of the changes in the cells of Leydig after withdrawal of tramadol treatment Our results showed that stopping tramadol injection, Lagenaria Siceraria, or melatonin treats the pathological side effects of tramadol and reversibility of the pathological changes in the testes after withdrawal of tramadol treatment

Conclusion

Tramadol atrophy of the seminiferous tubules, and reduces the number and inhibits the cellular activities of the cells of Leydig and directly inhibits its function such as the production of testosterone hormone. Finally, tramadol inhibits germinal layer cells and the production of different reproductive cells. While stopping tramadol, *Lagenaria Siceraria*, or melatonin teats these tramadol effects.

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