Annals of R.S.C.B., ISSN:1583-6258, Vol. 24, Issue 1, 2020, Pages. 1449 - 1463
Received 18 April 2020; Accepted 23 June 2020.

Effect of Tramadol Hydrochloride on the Testis of Adult Male Albino Rats and the Possible Ameliorating Role of L-carnitine (Histological, Immunohistochemical and Biochemical Study)

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ABSTRACT

Background: Tramadol is painkiller medication acting on the central nervous system, but its abuse has harmful effects on the testes. L-carnitine (LC) is an amino acid derivative with antioxidant properties.

Objective: the aim of this study was to investigate the ameliorative effects of L-Carnitine in rats induced testicular toxicity by Tramadol.

Materials and Methods: Fourty adult male rats divided into four experimental groups were enrolled in this study (10 rats/each) as follows: Group I (control group): received 0.1 ml of physiological saline solution. Group II (tramadol group): received Tramadol 40 mg/kg/ body weight (B.W) daily orally for 4 weeks. Group III (tramadol withdrawal group): received Tramadol 40 mg/kg.b.w daily orally then kept without treatment for another 4 weeks. Group IV (tramadol + L.carnitine group): received Tramadol 40 mg/kg body weight plus L-Carnitine350 mg/kg.bw orally daily by intragastric gavage for 4 week. Blood specimen were obtained for estimation of Serum levels of FSH, LH, prolactin, estradiol and testosterone. Rats were sacrificed, testicular specimens were prepared for assay of tissue levels of MDA, GSH and light microscopic examination and biochemical studies. Data were collected, recorded and statically analyzed.

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Results: Tramadol-treated group showed severe destruction of the testicular structure in which many seminiferous tubules were degenerated in addition to, congestion of blood vessels in the interstitium. But, in group IV (Tramadol– L-Carnitine treated group) showed more or less restoration of normal testicular structure. Serum LH, FSH and testosterone hormones levels were significantly decreased in all treated group compared to control group while serum levels of estradiol and prolactin were significantly increased in all treated group compared to control group, the L.carnitin administration showed antioxidant, antiapoptotic and antifibrotic activities .

Conclusion: Tramadol consumption resulted in testicular damage, which can be alleviated by taking L. carnitine alongside with tramadol treatment.

Keywords: Testis, Tramadol, L. carnitine, Antioxidants, infertility.

INTRODUCTION

The International Association for the Study of Pain (IASP) defines chronic pain as persistent or recurrent pain that lasts longer than three months or beyond the usual tissue healing period. (Merskey, 1986). Patients benefit greatly from chronic pain pharmacotherapies in terms of pain reduction and quality of life. However, most analgesics can cause side effects, which can be exacerbated when multiple prescription and/or over-the-counter drugs are used together (Jarernsiripornkul et al., 2019).

Chronic opioid therapy, either alone or in combination with other treatment modalities, was strongly recommended for patients with chronic non-cancer pain (CNCP) who had demonstrated medical necessity and stable pain and function improvement.(Manchikanti et al., 2017). However, due to a lack of evidence of efficacy and an increase in reports of adverse effects, the use of opioid analgesics in the long-term management of CNCP syndromes remains controversial (Campbell et al., 2018).

Tramadol is a centrally acting synthetic analgesic that was first introduced in Germany in 1977(Osterloh et al., 1978). It has a multimodal effect as a result of its dual- mode of action, which includes both opioid and non-opioid processes. As a result, it's commonly utilized as an analgesic for postoperative, cancer, or chronic neuropathic pain(Kaneko et al., 2014).Although, Tramadol has many therapeutic effects, but, it has a number of organ-related side effects including hepatotoxicity and nephrotoxicity(Elkhateeb et al., 2015).

Several studies have searched the effects of prolonged tramadol usage and addiction on the male reproductive system and sexual health, they found that it alters the sexual desire and ejaculation, as well as causing hypogonadism(El Sawy & Malak, 2015).Endogenous and exogenous opioids affect fertility and gonadal functions through acting on opioid receptors in the hypothalamus, resulting in diminished release or disruption of normal pulsatility of gonadotropin-releasing hormone secretion, as well as directly effecting the pituitary gland and testes(Vuong et al., 2010).

Preventing and treating opioid analgesic side effects and associated risks can be difficult for both patients and physicians, especially pharmacists, who play an important role in treatment optimization as part of a primary care approach to chronic pain management. (Tabeefar et al., 2020). L-carnitine (LC) is an amino acid similar to a vitamin that is utilized in the treatment of renal failure, anti-convulsive therapy, body weight regulation, and male infertility(Hegazy et al., 2020). It regulates the long chain fatty acid metabolism and

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subsequent regulation of energy production. These fatty acids are utilized as an energy source in all tissues except the brain. It possesses antioxidant properties that regulate fatty acid metabolism and prevent harmful acetyl-CoA from accumulating within cell membranes (Abd-Elrazek & Ahmed-Farid, 2018).Carnitine, which is abundant in pre-ejaculatory fluid and produced mostly in the epididymis in males, improves fertilization chances by altering sperm motility and other sperm characteristics (Yüncü et al., 2015).

So, the purpose of this study was to assess the effects of Tramadol on the testis of adult male albino rats, as well as the potential benefits of L- Carnitine.

Materials and methods

Drugs and chemicals

Tramadol was available in the form of Tramadol hydrochloride tablets (200 mg per tablet) from Hikma Pharmaceutical Co. in Giza, Egypt (Catalog Number: T712515). Each tablet is suspended in a distilled water solution of 20 mL. The daily dose of tramadol was 40 mg/kg.bw , which was equivalent to human effective therapeutic dose according to (Paget & Barnes, 1964).

L-carnitine: To obtain a concentration of 350 mg/kg bw, L-carnitine (Sigma, C0283-5G, USA) was dissolved in distilled water (Abd-Elrazek & Ahmed-Farid, 2018).

Ethical considerations:

The protocol of this research and the medical ethical committee of the Damietta Faculty of Medicine, Al Azhar University, Egypt, approved the use of animals (IRB 00012367-20-01-0016).

Animals and experimental design

Fourty adult male albino rats (weighing 120g-150g) were acquired from Mansoura University's Faculty of Pharmacy. Rats were kept in steel mesh cages (4 rats per cage) and fed on commercial standard chow and free tap water for one-week acclimation periods. Throughout the experiment, rats were fed on a well-balanced diet. The animals were randomly divided into 4 equal groups (n=10/group). The first group served as control and received 2 ml saline solution per rat daily by oral gavage. The second group, included ten rats that were orally given tramadol in a dose of 40 mg/kg body weight dissolved in saline daily for 4 successive weeks. The third group, included ten rats that were orally given tramadol in a dose of 40 mg/kg body weight dissolved in saline daily for 4 successive weeks and then kept without treatment for 4 weeks. The fourth group, included ten rats that were orally given tramadol in a dose of 40 mg/kg.bw plus L-carnitine in a dose of 350 mg/kg.bw dissolved in saline daily for 4 successive weeks. The calculated tramadol hydrochloride and L.carnitine doses were given orally to each animal using a curved needle-like oral tube that was inserted directly into the stomach (oral gavage). Rats were sedated with 4 percent isoflurane (SEDICO Pharmaceuticals Company, Cairo, Egypt) in 100 percent oxygen and then euthanized by cervical decapitation at the end of the experiment by the predetermined time for each group.

Blood collection, Serum biochemical analyses

Capillary tubes were used to obtain blood samples from the retro-orbital venous plexus. Luteinizing hormone (LH) and follicle stimulating hormone (FSH) were quantified in IU/ml,

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prolactin (PRL) in mg/ml, testosterone in pg/ml, and estradiol (E2) in pg/ml using enzyme linked immunosorbent assay (ELISA) kits according to the manufacturer's protocol.

Tissue preparation for assay of oxidative stress parameters

Right testis specimens were dissected out then minced and homogenized for biochemical study of the oxidative enzymes; reduced glutathione (GSH), and malondialdehyde (MDA) according to (Hegazy et al., 2020).

Hematoxylin and Eosin (H&E) and immunohistochemistry:

Each rat's left testis was delivered and preserved in a 10% formalin solution before being dehydrated in increasing degrees of alcohol. The specimens were embedded in paraffin blocks after being treated with xylene. Serial slices were cut using a microtome and stained with hematoxylin and eosin, Masson trichrome, and caspase-3 immunostain at a thickness of 5 um.

Morphometric analysis

The images were photographed & the percentage area density of collagen fibres& caspase-3 was measured using an Raywild E5 microscope with an Raywild M-300 digital camera with image-analyzing system (Mvi-mage program v12)**.**

Statistics

The mean and standard deviation are used to represent the data using the statistical software package SPSS for Windows (Version 21.0; SPSS Inc., Chicago, IL, USA), data were analyzed using the two-tailed Student's -test and one-way ANOVA, followed by Duncan's post hoc test for multiple group comparison. $P < 0.05$was considered statistically significant.

Results

Effects on hormonal levels :

Serum Testosterone, FSH and LH levels were significantly decreased in Tramadol treated animals compared to the control animals, while, those levels were significantly increased in both tramadol withdrawal and tramadol plus L.carnitine groups in comparison to tramadol group but they did not reach the control levels. However, Serum Estradiol and PRL levels were significantly increased in Tramadol treated animals in comparison to the control animals, while, those levels were significantly decreased in both tramadol withdrawal and tramadol plus L.carnitine groups in comparison to tramadol group but they did not reach the control levels(Table 1).

Table 1: Assay of hormonesin different groups:

Groups / Parameters	Control	Tramadol	Tramadol withdrawal	Tramadol + L. carnitine
Testosterone (mg/ml)	4.36±0.51	2.02±0.43 [a]	3.94±0.38 [a b]	4.14±0.73 [a b]
LH (IU/ml)	2.57±0.39	1.48±0.44 [a]	2.04±0.24 [a b]	2.25±0.51 [a b]
FSH (IU/ml)	2.33±0.21	0.94±0.28 [a]	1.94±0.59 [a b]	2.07±0.19 [a b]
Estradiol (pg/ml)	47.45±1.62	52.71±0.79 [a]	49.71±1.41 [a b]	48.62±1.65 [a b]
PRL (µg/ml)	7.13±1.41	13.71±1.35 [a]	10.38±1.22 [a b]	9.92±1.57 [a b]

Data are shown as mean ± SD and (n=10).

[a] Significantly different from the corresponding control group at p <0.05

[b]Significantly different from the corresponding tramadol exposed group at p <0.05

Effects on testicular tissue levels of GSH &MDA:

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Serum GSH levels were found to be significantly decreased in Tramadol treated animals compared to the control animals, while, those levels were significantly increased in both tramadol withdrawal and tramadol plus L.carnitine groups in comparison to tramadol group but they did not reach the control levels. However, Serum MDA levels were found to be significantly increased in Tramadol treated animals when compared to the control animals, while, those levels were significantly decreased in both tramadol withdrawal and tramadol plus L.carnitine groups in comparison to tramadol group but they did not reach the control levels (Table 2).

Table 2. Assay of tissue levels of MDA & GSH:

Parameters \ Groups	Control	Tramadol	Tramadol withdrawal	Tramadol + L. carnitine
GSH (µmol/g tissue)	14.37 ± 0.94	7.69 ± 0.82 [a]	11.55 ± 1.24 [a b]	12.37 ± 0.88 [a b]
MDA (nmol/g tissue)	16.43 ± 1.71	31.57 ± 4.28 [a]	21.81 ± 1.62 [a b]	20.27 ± 1.50 [a b]

Data are shown as mean ± SD and (n=10).

[a]Significantly different from the corresponding control group at p <0.05.

[b]Significantly different from the corresponding tramadol exposed group at p <0.05.

Effects on morphometric changes of area density of Collagen & caspase 3:

The Percentage are of both Collagen and capase 3 density % levels were found to be significantly increased in Tramadol treated animals in comparison to the control group, while, those levels were significantly decreased in both tramadol withdrawal and tramadol plus L.carnitine groups in comparison to tramadol group but they did not reach the control levels (Table 3).

Table 3: Assay of Morphometric Changes in different groups:

Parameters \ Groups	Control	Tramadol	Tramadol withdrawal	Tramadol + L. carnitine
Percentage are of Collagen density %	16.27 ± 1.75	45.39 ± 3.8 [a]	26.28 ± 2.74 [a b]	20.60 ± 3.19 [a b]
Caspase 3 density (mm3)	3.48 ± 0.84	18.44±2.64 [a]	9.08±2.67 [a b]	7.42±1.81 [a b]

Data are shown as mean ± SD and (n=10).

[a]Significantly different from the corresponding control group at p <0.05

[b]Significantly different from the corresponding tramadol exposed group at p <0.05

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Light microscopic results

Haematoxylin and eosin-stained sections results

Histological examination of H&E stained sections of the testes in control group revealed closely packed seminiferous tubules lined by stratified germinal epithelium. Spermatozoa were observed in the lumina of the tubules. Spermatogenic epithelium formed of; spermatogonia, primary spermatocytes, spermatids, and sperms. The tubules separated from each other by loose interstitial connective tissue containing the Leydig cells.In group II (Tramadol treated group), there was sever distortion of the testicular structure as many seminiferous tubules showed, excessive areas of vacuolations due to complete absence of spermatogenic epithelium and few remnants of the epithelial cells with irregular thick basement membrane and marked widening of the interstitial tissue containing the Leydig cells, with congested blood vessels in the interstitium. sections of testes of group III (Tramadol–withdrawal group) showed nearly the normal histological structure. Few empty spaces were still seen among spermatogenic epithelium due to epithelial disruption. Interstitial spaces with less congested blood vessels and polygonal Leydig cells. tramadol plus L.carnitin group (group IV) showing more restoration of normal shaped seminiferous tubules with regular basement membrane and less widening of the interstitial tissue containing the Leydig cells(fig.1).

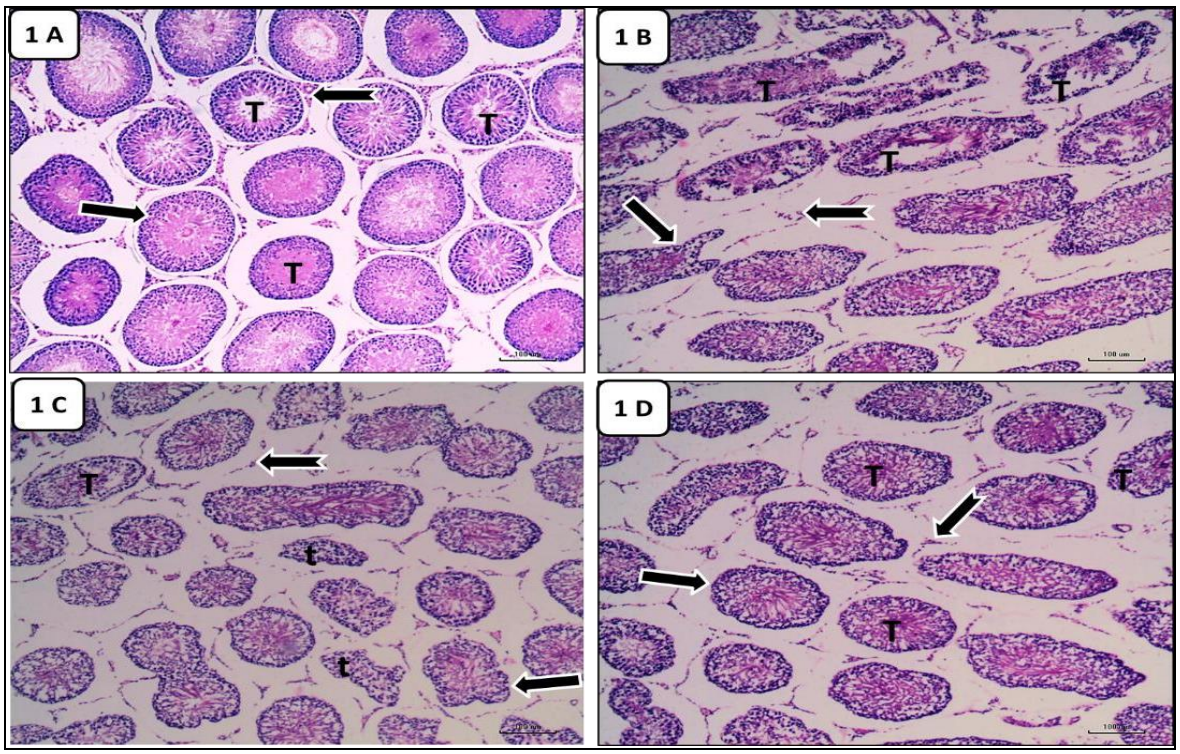

Figure 1. photomicrographs of testicular sections of lower magnification{A}: control group showing revealed closely packed seminiferous tubules lined by stratified germinal

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epithelium. Spermatozoa were observed in the lumina of the tubules. Spermatogenic epithelium formed of; spermatogonia, primary spermatocytes, spermatids, and sperms. The tubules separated from each other by regular interstitial space containing the Leydig cells (notched thick arrow).{B}: the tramadol-treated group showing excessive vacuolations in many shrinked seminiferous tubules(T) with irregular basement membrane (thick arrow) and widening of the interstitial tissue containing the Leydig cells (notched thick arrow). {C}: the tramadol withdrawal group(group III) showing less shrinked seminiferous tubules (t) with irregular basement membrane (thick arrow), less vacuolated seminiferous tubules (T) lined by spermatogenic epithelium, less widening of the interstitial tissue containing the Leydig cells (notched thick arrow). {D}: tramadol plus L.carnitin group (group IV) showing more restoration of normal shaped seminiferous tubules (T) with regular basement membrane (thick arrow) and less widening of the interstitial tissue containing the Leydig cells (notched thick arrow). A: Control group; B: Tramadol group; C: Tramadol withdrawal group; D: Tramadol +L.Carnitin group (Hx.&E. X100) Scale bars, 100 μm.

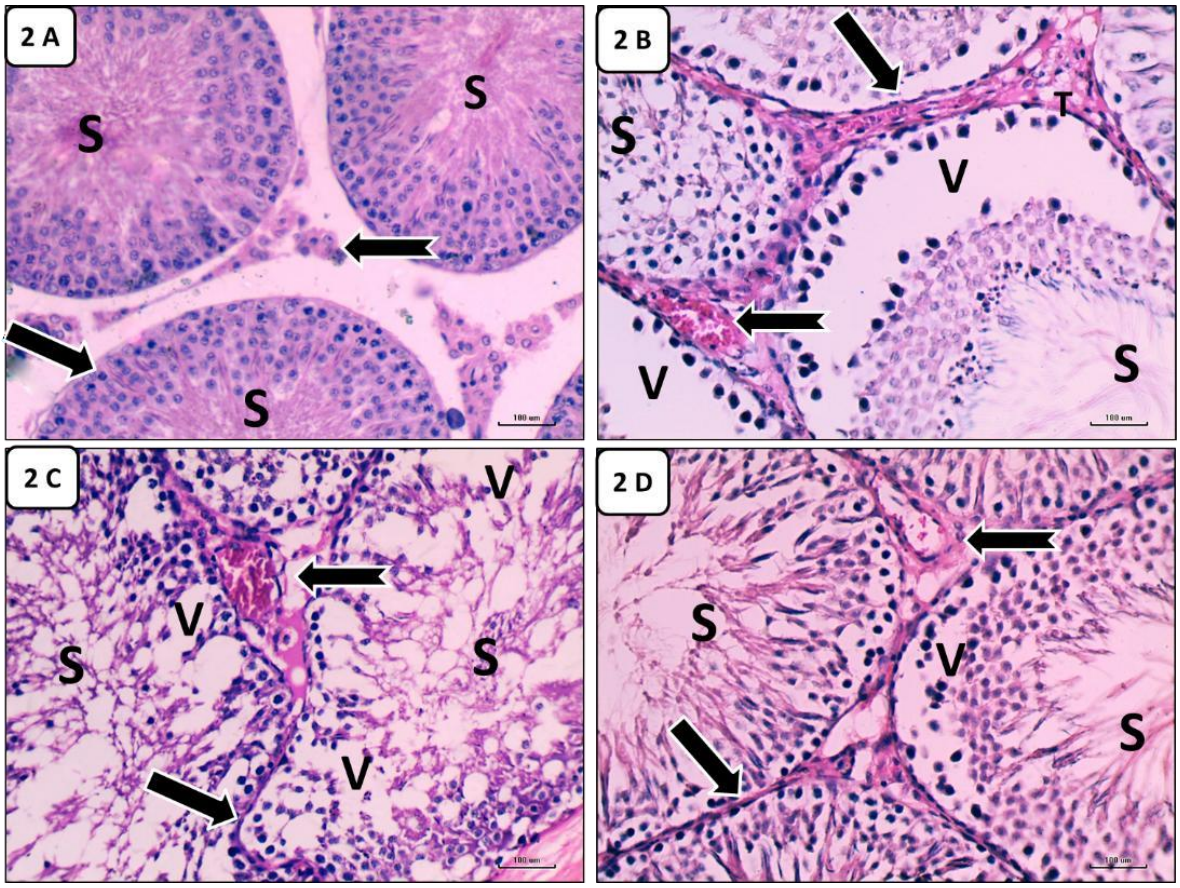

Figure 2. photomicrographs of testicular sections of higher magnification {A}: control group showing revealed closely packed seminiferous tubules lined by stratified germinal epithelium. Spermatogenic epithelium formed of; spermatogonia, primary spermatocytes, spermatids, and sperms (S) filling the tubular lumen. The tubules are separated from each other by regular interstitial space containing the Leydig cells (notched thick arrow). {B}: the tramadol-treated group showing excessive vacuolations (V) in many seminiferous tubules and sperms (S) filling the tubular lumen of few tubules with irregular thick basement membrane (thick arrow) and widening of the interstitial tissue containing the Leydig cells and congested blood vessels (notched thick arrow). {C}: the tramadol withdrawal group (group III) showing less disfigured seminiferous tubules with irregular basement membrane (thick

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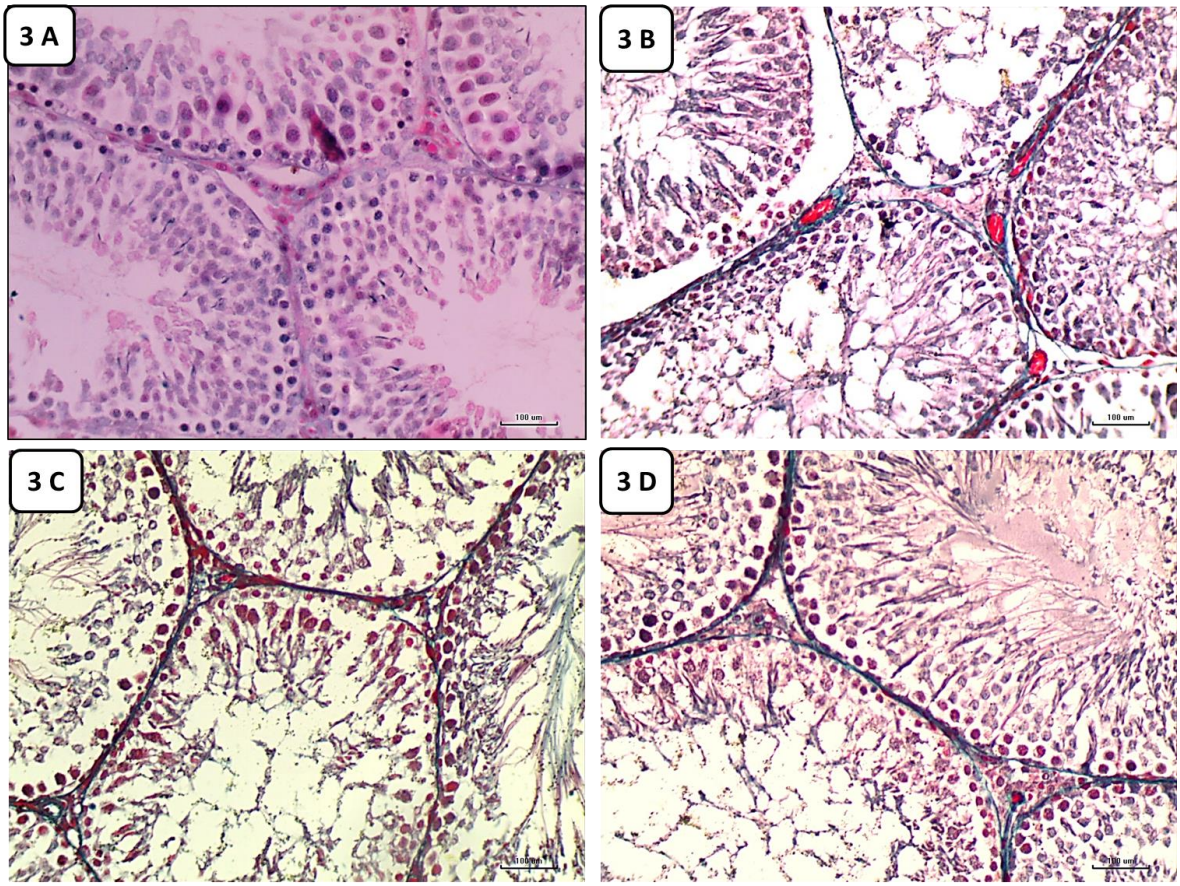
arrow), less vacuolated seminiferous tubules (V) due to desquamation of spermatogenic epithelium, and some tubules appeared with sperms filling the lumen (S) and less widening of the interstitial tissue containing the Leydig cells and congested blood vessels (notched thick arrow). {D}: tramadol plus L.carnitin group (group IV) showing more restoration of normal shaped seminiferous tubules with less vacuolations (V) and sperms (S) filling most of the tubular lumen and regular basement membrane (thick arrow) and less widening of the interstitial tissue containing the Leydig cells (notched thick arrow). A: Control group; B: Tramadol group; C: Tramadol withdrawal group; D: Tramadol + L.Carnitin group (Hx.&E. X400) Scale bars, 100 µm.

Masson trichrome stained sections:



Fi.g. 3: Photomicrographs of masson trichrome stained testicular sections showing {A}: no or weakest deposition of collagen fibres in control group. {B}; marked deposition of collagen fibres in tramadol group. {C& D}: weak deposition of collagen fibres in tramadol withdrawal group and tramadol plus L.Carnitin group. A: Control group; B: Tramadol group; C: Tramadol withdrawal group; D: Tramadol + L.Carnitin group (masson trichrome stain X400) Scale bars, 100 µm.

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Immunohistochemical assessment of caspase 3

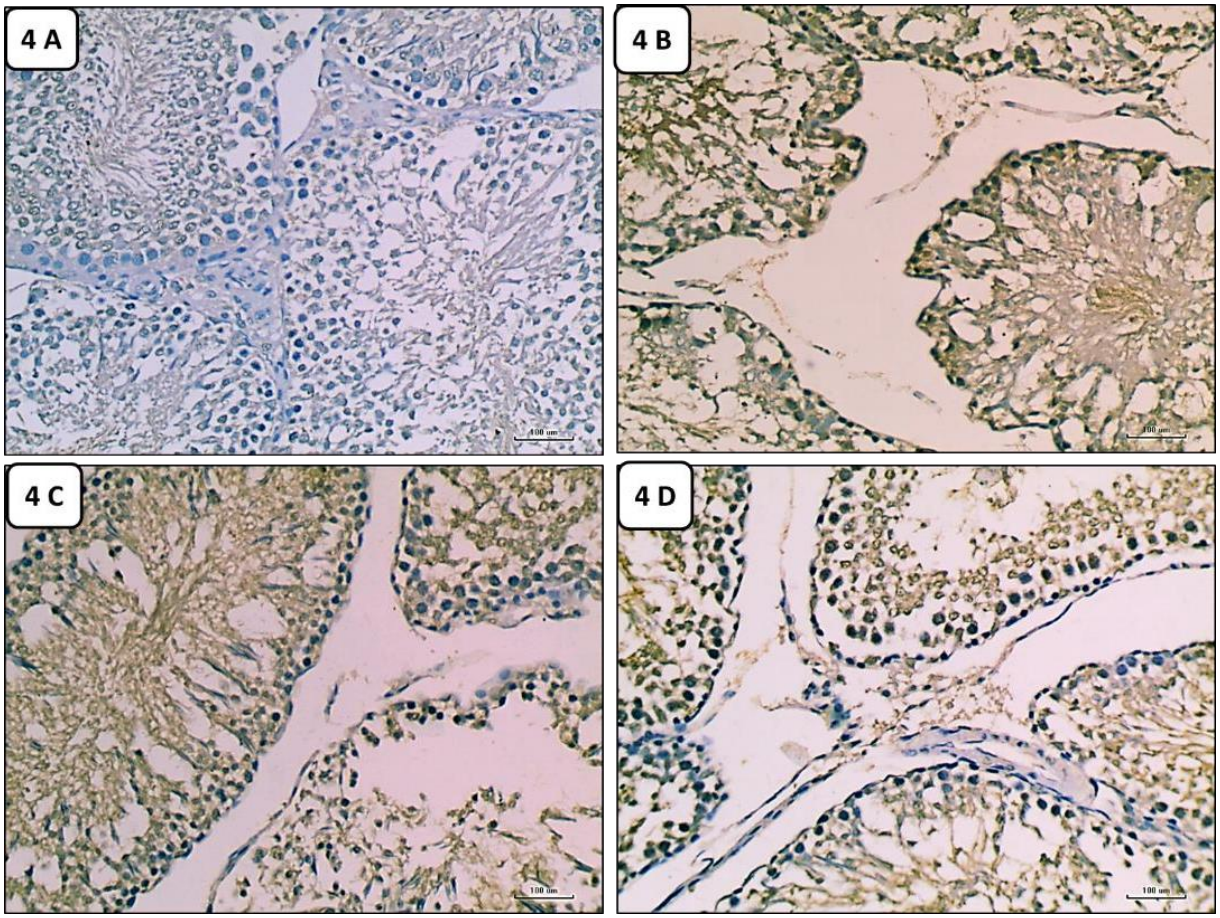

Fig.4:**Photomicrographs of Immunostained testicular sections for caspase-3** showing{A}: no or weakest expression of caspase-3 in control group. {B}; marked expression of caspase-3 in tramadol group.{C& D}:; weak expression of caspase-3 in tramadol withdrawal group and tramadol plus L.Carnitin group. A: Control group; B: Tramadol group; C: Tramadol withdrawal group; D: Tramadol + L.Carnitin group (anticaspase 3 immunostaining X400) Scale bars, 100 µm.

Discussion

Exposure to opioid analgesic medications lead to production of oxidative stress components which are the most leading causes of male infertility (Minisy et al., 2020). Tramadol is an opioid pain reliever that is commonly used to treat chronic pain and cancer. Multiple tissue toxicities have been documented in people who take tramadol multiple times a day (Abdelaleem et al., 2017; Baghishani et al., 2018).

Because tramadol, like other opioids, causes oxidative stress, which changes cell structure and leads to apoptosis(Mohamed & Mahmoud, 2019), L.carnitine was used in this study as a defensive agent in regulating the long chain fatty acid metabolism and subsequent regulation of energy production. Thus, It has an antioxidant activity and inhibits aggregation of toxic acetyl- CoA within the cell membranes (Abd-Elrazek & Ahmed-Farid, 2018). Because of its high lipid content and rapid O2 consumption, the testis is considered a vulnerable organ to lipid peroxidation. (Hassan et al., 2019).So, the goal of our research was to determine the toxicity of tramadol on testicular tissue in adults and to see if L.carnitine could protect against this toxicity..

In the present study, the histological observation of the testicular sections of tramadol treated rats revealed severe degeneration and widening of the tubular cavities, irregular basement membrane with widened interstitial spaces containing congested blood vessels, Areas of loss of germ cells, Some detached germ cells were filling the tubular lumen. This is in agreements with the findings of a recent study on animals treated with tramadol revealed distinct histological changes with abnormal appearance of the germinal epithelium. Most of the seminiferous tubules showed damage with disordered spermatogenic cells that presented with prominent multiple desquamations and vacuolar degeneration (Salah et al., 2020).

The sloughing of the germinal epithelium that involve portion or even encompass the whole circumference of the tubule may be due to toxic effect on Sertoli cell cytoskeleton(Johnson, 2014).

Also, most of those histopathological changes observed in the rat testis of tramadol group may be due to their specific harmful effects on the target organ (oxidative stress) and hormonal disproportion in the hypothalamic–hypophysial–testicular axis, probably caused by excessive tramadol intake.

Our findings demonstrated that giving tramadol (40 mg/kg) to male rats for a month increased the amount of oxidative stress, as evidenced by elevated MDA levels, but resulted in a significant reduction in GSH levels in testicular tissue. Those, were in agreement with the previous studies reported significant increase in MDA levels following tramadol consumption in some tissues and significant decrease in GSH level (Ghoneim et al., 2014; Ibrahim & Salah-Eldin, 2019; Sheweita et al., 2018).The antioxidant system's deficiency was demonstrated by a decrease in GSH levels in testicular tissue, which led to an increase in MDA levels. Because the thiol group in mitochondrial membrane proteins is oxidized, which could induce necrosis and apoptosis (Kandemir et al., 2017).

In our study we used Caspase-3 to assess the apoptosis as it represents the intrinsic pathway of apoptosis (Arisha et al., 2019), we found that tramadol induced significant increase in caspase-3 activity compared to control group which indicates the occurrence of cellular apoptosis in testicular cells. Similar to our results there were upregulation of Bax and caspase3 recorded in rat testis, cerebral cortex and lung tissues on exposure to tramadol (Awadalla & Salah-Eldin, 2016; Koohsari et al., 2020).

The testicular exposure to any toxicants can cause mitochondrial damage and decrease the expression of steroidogenic acute regulatory protein (StAR), preventing steroid secretion and steroid hormones as, Testosterone, FSH, and LH, affecting male and female reproductive activity through disturbance of the hypothalamic-pituitary-gonadal axis, which can decrease fertility or cause complete infertility (Faccio et al., 2013). In the present study we found that chronic exposure to tramadol for 4 weeks significantly decreased the Testosterone, LH, and FSH levels. In agreement to our results, a previous study confirmed that tramadol intake significantly decreased serum levels of testosterone, FSH, and LH(Salah et al., 2020). The decrease in serum levels of LH and FSH after tramadol use could be due to its effect on the central nervous system, which prevents the production and release of pituitary gonadotrophins, resulting in decreased levels of pituitary gonadotrophins, which is crucial for production and completing testicular spermatogenesis (Moeen et al., 2018).

Our study showed that rats exposed to tramadol intake had significantly increased estradiol and prolactin levels in comparison to control rats. This was similar to the results of a previous study conducted to assess some toxicological and histological changes initiated by extensive consumption of tramadol in male rats, which revealed that tramadol administration significantly increased estradiol and prolactin levels in comparison to control rats(El- Zawakry et al., 2015). The long term use of opioids had a disturbing hormonal effect on gonadotropin-releasing hormone (GnRH) by decreasing plasma FSH and LH levels and increasing levels of prolactin (Bliesener et al., 2005). When the prolactin levels increase, it directly prevent the synthesis of testosterone (Pimpinelli et al., 2006).Also, testosterone is transforming into estradiol by aromatase enzyme which explained the low testosterone and high estradiol levels(Ceccarelli et al., 2006).

As regard the fibrotic changes in the testis, our study found that the percentage of area density of collagen fibers deposition in the rat testis was increased in rat received tramadol in comparison to control group which indicating that tramadol induce testicular fibrosis. A recent study found that tramadol administration resulted in a high degree of collagen fibers deposition in the extracellular matrix surrounding the seminiferous tubules in adult rat testes, which was similar to our findings(Minisy et al., 2020).Increased collagen fibres in tramadol- treated groups could be attributed to reduced Sertoli cell activity, fibroblast activation by free radicals, or an increase in collagen formation by myoid cells, or a reduction in the extracellular matrix proteolysis rate(Amaral et al., 2010; Soliman et al., 2014).

However, simultaneous administration of L-carnitine to tramadol in this study, decreased the destruction in germ cells, repaired the morphological damage in testicular tissues, neutralized the harmful effect of ROS produced from cellular metabolic processes proving its effective antioxidant property to remove free radicals and decreased the activity of caspase-3 (anti-apoptotic activity) produced by tramadol abuse, and exhibited ameliorative effects against the damage caused by tramadol only. Numerous studies agreed with our findings in confirming the anti-apoptotic, antioxidants, antifibrotic effects of L.carnitine against different testicular toxicities induced by many drugs (Kelek et al., 2019; Mardanshahi et al., 2019; Théophile et al., 2019).

As regard tramadol withdrawal in our study, we found that, most of histological findings were nearly subsided but did not reach the normal results. This showed that apoptotic activity and oxidative stress caused by tramadol administration mostly decreased on tramadol withdrawal. Similar results reported by previous studies, they found that examination of rats after tramadol withdrawal showed marked reduction in cellular damage. Tramadol withdrawal has better histological picture than those under usage, in spite of that it is not returned back to normal, but it is associated with good future for tramadol users (Abou Elnaga et al., 2018; Ghoneim et al., 2014).

In conclusion: Our results suggest that concomitant intake of L.carnitin with several analgesic medications, particularly tramadol could have a therapeutic role in reducing the toxic effects produced on long-term exposure. To confirm this idea, more research with different analgesic medications are required.

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