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Original Article

Experimental study of chronic toxicity of tramadol on liver, kidney and brain in albino rats (biochemical and histopathological study)

Nagla Ahmed El-Nabarawy[1,*], Sherien S. Ghaleb[2], Amr R. Zaki[2], Samia El Naggar[3] and Nermeen N. Welson[2]

[1]National Egyptian Center of Environmental and Toxicological Research, Faculty of Medicine, Cairo University, Egypt; [2]Forensic Medicine and Clinical Toxicology Department, Beni-Suef University, Egypt; [3]Pathology Department, Beni-Suef University, Egypt.

ABSTRACT

Tramadol has become the most prescribed opioid worldwide. In this study, we investigate the chronic tramadol toxicity on liver, kidney and brain through analysis of the biochemical, histopathological and immunohistochemical findings in albino rats. This work included two groups; the first group served as control (n = 20 rats) and received saline solution only orally along the study. The second group received oral doses of tramadol HCl (n = 20 rats) suspended in saline solution equal to 60 mg/kg/daily for 90 days. After sacrifice biochemical evaluation of liver function (ALT: alanine transaminase, AST: aspartate transaminase, LDH: Lactate dehydrogenase), kidney function (BUN: blood urea nitrogen, creatinine), oxidative stress markers (Malondialdehyde (MDA) in liver and kidney) and total antioxidant capacity (TAC) was carried out followed by histopathological and immunohistochemical examination of liver, kidney and brain. Highly significant elevation in serum ALT, AST and LDH in the tramadol group was detected in comparison with the control group. Moreover, there was highly significant elevation of MDA in liver and kidney and serum TAC in the tramadol group compared to the control. Histopathological examination of the liver, kidney and brain showed various changes in Bcl2 expression. It was decreased in the organs of the tramadol group and was more obvious in brain. Chronic usage of tramadol has toxic effects on liver and kidney as indicated by biochemical elevation of enzymes and histopathological changes. Moreover, tramadol mainly affected the brain as the histopathology showed marked hypercellularity, disarrangement of nerve cells and irregular shrunken neurons. Tramadol also induced apoptosis as indicated by the decrease in the protective anti apoptotic antigen Bcl2.

KEYWORDS: tramadol, opioids, experimental toxicity, hepatotoxicity, nephrotoxicity, neurotoxicity.

INTRODUCTION

For moderate to severe pain tramadol is commonly prescribed at a dose of 200 mg/day [1]. It is composed of a racemate of the two enantiomers (+)-tramadol and (-)-tramadol and is converted to N- and O-demethylated metabolites by cytochrome P4502B6 (CYP2B6)/CYP3A4 and CYP2D6, respectively. Tramadol is a synthetic opioid which acts through three mechanisms: mu-opioid binding (through its metabolite O-desmethyltramadol), serotonin reuptake inhibition (through (+) -tramadol) and nor-epinephrine reuptake inhibition (through (-)-tramadol) [2]. Also Tramadol has NMDA-type antagonist effects, which has given it a potential application in neuropathic pain states [3].

Like other opioids, tramadol has side effects such as headache, nausea, dizziness, somnolence, constipation,

*Corresponding author: n.a.nabarawy@gmail.com

sweating, pruritus, and central nervous system stimulation [4]. Chronic usage of tramadol might lead to the accumulation of toxic metabolites in the body and increase the risk for pharmacokinetic interactions [5].

Tramadol in Egypt is easily accessible and is provided at cheap cost despite being scheduled [6].

The objective of this work is to study the chronic tramadol toxicity on liver, kidney and brain through analysis of the biochemical, histopathological and immunohistochemical findings in albino rats.

MATERIALS AND METHODS

Drug

Tamol X (tramadol HCl), 225mg tablets, alpha international company, India. Its chemical name is (±) cis-2 [(dimethylamino) methyl]-1-(3-methoxy phenyl) cyclohexanol hydrochloride.

Animals

Forty male albino rats weighing 100-120 g were housed for 10 days before study; they were given normal rat chow and water throughout the experiment. The animals were grouped and housed in cages (5 in each cage). The study was conducted in the animal house of the faculty of sciences, Beni Sueif University.

Experimental treatment

This study was approved by the Institutional Animal Care and Use Committee (CU-IACUC), Faculty of Medicine, Cairo University (CU/III/F/45/19). The animals were randomly divided into two equal groups (n = 20/group). The first group served as control and was administered saline solution only throughout the study. The second group was administered oral doses of tramadol (tramadol HCl) equal to 60 mg/kg/daily (which is 1/5 LD50) for 90 days by gavage. The calculated tramadol hydrochloride doses were given orally to each animal by a gavage process. At the end of the experimental period and under anesthesia all control and treated rats were sacrificed 24 hours after the last dose.

Physical parameters

Mortality and body weight were recorded during treatment of all groups of animals. Necropsy was intended to be done if animals died during the course of treatment.

Biochemical parameters

Blood samples were collected for serum preparation, and sera were separated and preserved at -20 °C till use for analysis of liver function (ALT, AST, LDH), kidney function (BUN, creatinine) and total antioxidant capacity. Kits for TAC were obtained from Biodiagnostic Company, Giza, Egypt and TAC was measured according to the manufacturer instructions.

For MDA analysis, the liver and kidney specimens were collected, frozen on ice and stored until use for experiments after preparation of the tissue homogenate.

Histopathological examination

Liver, kidney and brain specimens were isolated and fixed in 10% buffered formalin for histopathological examination. Then specimens were dehydrated in a graded alcohol series. After xylene treatment, the specimens were embedded in paraffin blocks. Five-micron thick sections were cut and stained with hematoxylin and eosin (H&E).

Immunohistochemical examination

Immunohistochemistry (IHC) staining was performed on formalin-fixed, paraffin-embedded materials that were sectioned at 5 μm thickness and placed onto positive charged slides. AntiBcl2 rat polyclonal antibodies dye was used to stain Bcl2 antigens in the tramadol and control groups to detect the extent of this antigen expression change.

Statistical analysis

Data were collected and represented as mean ± SD. Statistical data was analyzed by Student t's test and Analysis of variance (ANOVA) test, between control and tramadol group. $p < 0.05$ was considered statistically significant.

RESULTS

There was highly significant elevation in serum ALT (49.9 ± 7 vs. 23 ± 3.7), AST (122 ± 38.7 vs. 60.6 ± 8) and LDH (915.1 ± 188.4 vs. 641 ± 59.7) in the tramadol group in comparison with the control group; $P < 0.001$ as shown in Table 1.

There was insignificant elevation of BUN (21.1 ± 4.8 vs. 19.3 ± 3) and serum creatinine (0.43 ± 0.09 vs. 0.39 ± 0.07) in the tramadol group in comparison to the control; P value > 0.05 as shown in Table 2.

There was highly significant elevation of liver MDA (136.8 ± 21.7 vs. 28.2 ± 4.6), kidney MDA (83.95 ± 11.89 vs. 26.2 ± 4.6) and serum TAC (2.02 ± 0.16 vs. 2.39 ± 0.05) in the tramadol group in comparison to the control, P value < 0.01. No mortalities were observed during the experiment as shown in Table 3.

Histopatholgical examination of the liver showed sinusoidal dilatation, congestion, and hydropic degeneration (ballooning) in most of the rats in the perivenular region (zone 3). Degeneration had proceeded to midzonal region (zone 2) in some rats and perivenular necrosis and haemorrhge were also found. Hydropic degeneration was found in 70%, haemorrhage in 30%, congestion in 70%, cytolysis in 50%, apoptotic bodies in 50%, granuloma formation in 20%, perivenular necrosis in 20% and focal microvesicular steatosis in 30% as shown in Table 4 and Figure 1.

Histopathological examination of the kidney showed infiltration of interstitial mononuclear cell in some rats with minimal vacuolization. No glomerular damage was found in all examined rats. Tubular epithelial vacuolization was found in 10%, interstitial cellular infiltration in 30%, focal necrosis in 20%, glomerular hemorrhage in 20% and degenerated tubules in 20% as shown in Table 5 and Figure 2.

In the tramadol-treated group, brain showed marked disorganization of the cortical layers and hypercellularity and increased apoptotic cells.

Table 1. Effect of chronic tramadol toxicity on liver enzymes (60 mg/kg for 90 days).

	Group	Mean	St. Deviation	Range	P value	t	F
ALT	Control	23	3.7	17- 28	<0.0001	10.2	3.7
	Tramadol	47.9	7	40- 68			
AST	Control	60.6	8	48- 73	<0.0001	6.5	12.8
	Tramadol	122	38.7	80- 166			
LDH	Control	641	59.7	559.5- 747.9	0.0002	4.4	10
	Tramadol	915.1	188.4	506.2- 1163.5			

Table 2. Effect of chronic tramadol toxicity (60 mg/kg for 90 days) on renal function.

	Group	Mean	St. Deviation	Range	P value	t	F
BUN	Control	19.3	3	15- 25	0.3	1.06	2.5
	Tramadol	21.1	4.8	15- 32			
Creat.	Control	0.39	0.07	0.29- 0.55	0.36	0.94	1.453
	Tramadol	0.43	0.09	0.23- 0.60			

Table 3. Effect of chronic tramadol toxicity (60 mg/kg for 90 days) on oxidative stress markers and TAC.

	Group	Mean	St. Deviation	Range	P value	T	F
Liver MDA	Control	28.2	4.6	20- 35	<0.0001	15.47	21.9
	Tramadol	136.8	21.7	107.36- 179.85			
Kidney MDA	Control	26.2	4.6	18- 33	<0.0001	14.5	6.58
	Tramadol	83.95	11.89	65.79- 110.96			
TAC	Control	2.39	0.05	2.31- 2.46	<0.0001	7.02	11
	Tramadol	2.02	0.16	1.8- 2026			

Table 4. Liver histopathological changes in the tramadol-treated group.

Histopathological changes	%
Hydropic degeneration	70
Haemorrhage	30
Congestion	70
Cytolysis	50
Apoptotic bodies	50
Granuloma	20
Perivenular necrosis	20
Focal microvesicular steatosis	30

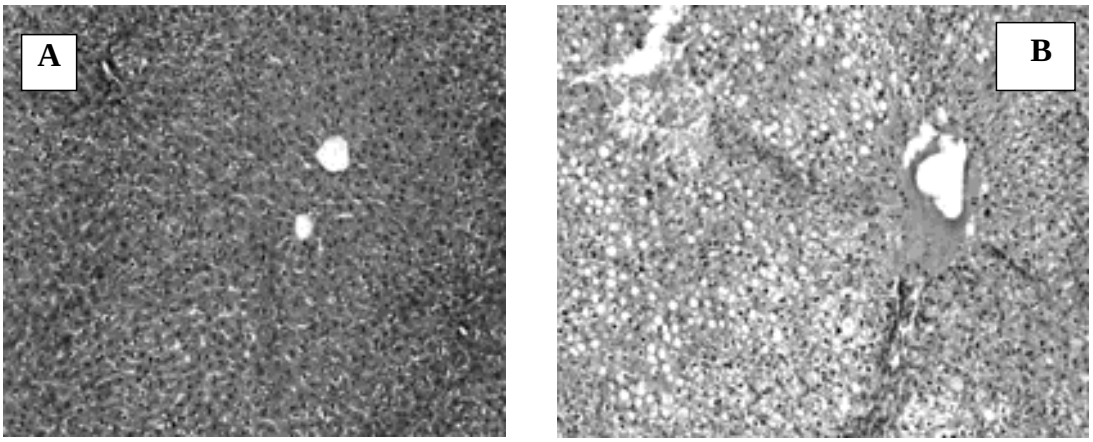

Figure 1. Photomicrograph of cross section of liver of the control group (**A**) & the tramadol group (**B**) showing congestion with hemorrhage and sinusoidal dilatation (H&E × 200).

Table 5. Kidney histopathological changes in the tramadol-treated group.

Hisstopathological changes	%
Tubular epithelial vacuolization	10
Interstitial cellular infiltration	30
Focal necrosis	20
Glomerular haemorrhage	20
Degenerated tubules	20

Pyramidal cells appeared irregular, darkly stained with pyknotic nuclei and surrounded by haloes and some of them were shrunken and showed marked cytoplasmic vacuolization. Others appeared with faintly stained cytoplasm and nuclei. There were areas of haemorrhage and dilated blood vessels. Hypercellularity was found in 64%, apoptotic cells were found in 70%, inflammatory cellular infiltration in 45%, congested blood vessels in 50%, haemorrhage in 55%, degenerated neurons in 55% and cytoplasmic vacuolization in 50% as shown in Table 6 and Figure 3.

Immunohistochemical study of the expression of the Bcl2 antigen in the rats' livers, kidneys and brains showed decrease in the reactivity of the tramadol group in comparison to the control group. The tramadol-treated group is less positive than the control group as shown in Figure 4.

DISCUSSION

In this study, there was significant increase in liver enzymes (ALT, AST, LDH) in the tramadol

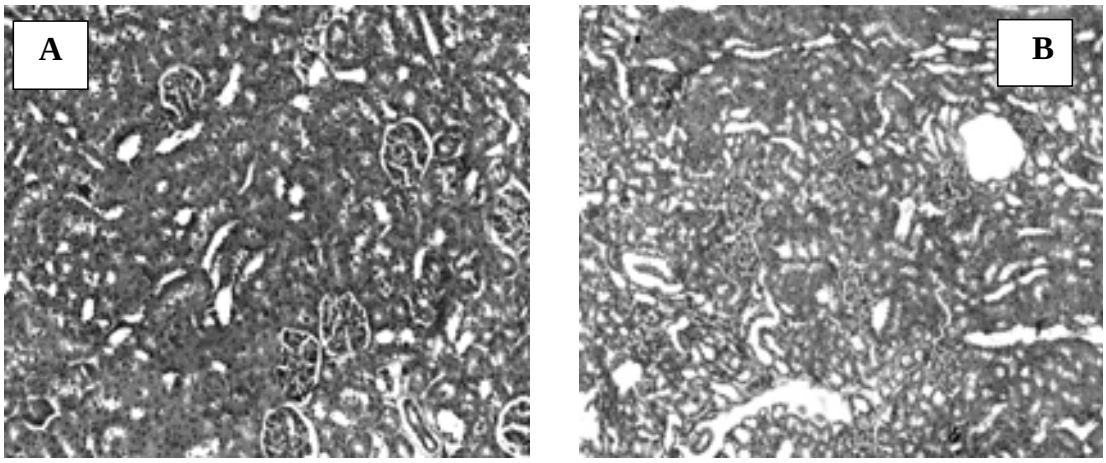

Figure 2. Photomicrograph of cross section of kidney of the control group (**A**) & the tramadol group (**B**) showing interstitial cellular infiltration & tubular atrophy (H&E × 200).

Table 6. Brain histopathological changes in the tramadol-treated group.

Hisstopathological changes	**%**
Hypercellularity	65
Apoptotic cells	70
Inflammatory cellular infiltration	45
Congested blood vessels	50
Haemorrhage	55
Degenerated neurons	55
Cytoplasmic vacuolization	50

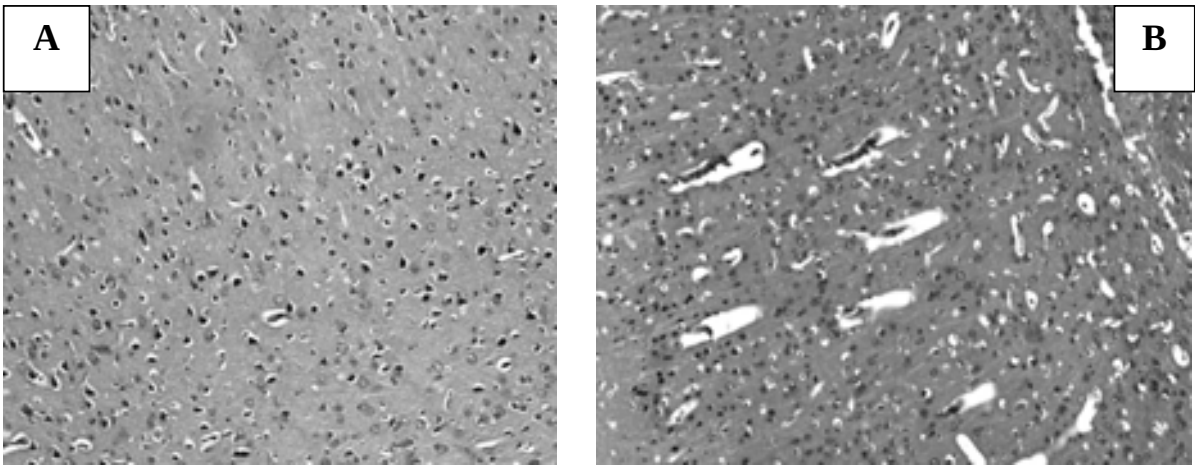

Figure 3. Photomicrograph of cross section of brain of the control group (**A**) & the tramadol group (**B**) showing hypercellularity, and irregular darkly stained neurocytes with pyknotic nuclei surrounded by haloes (H&E × 200).

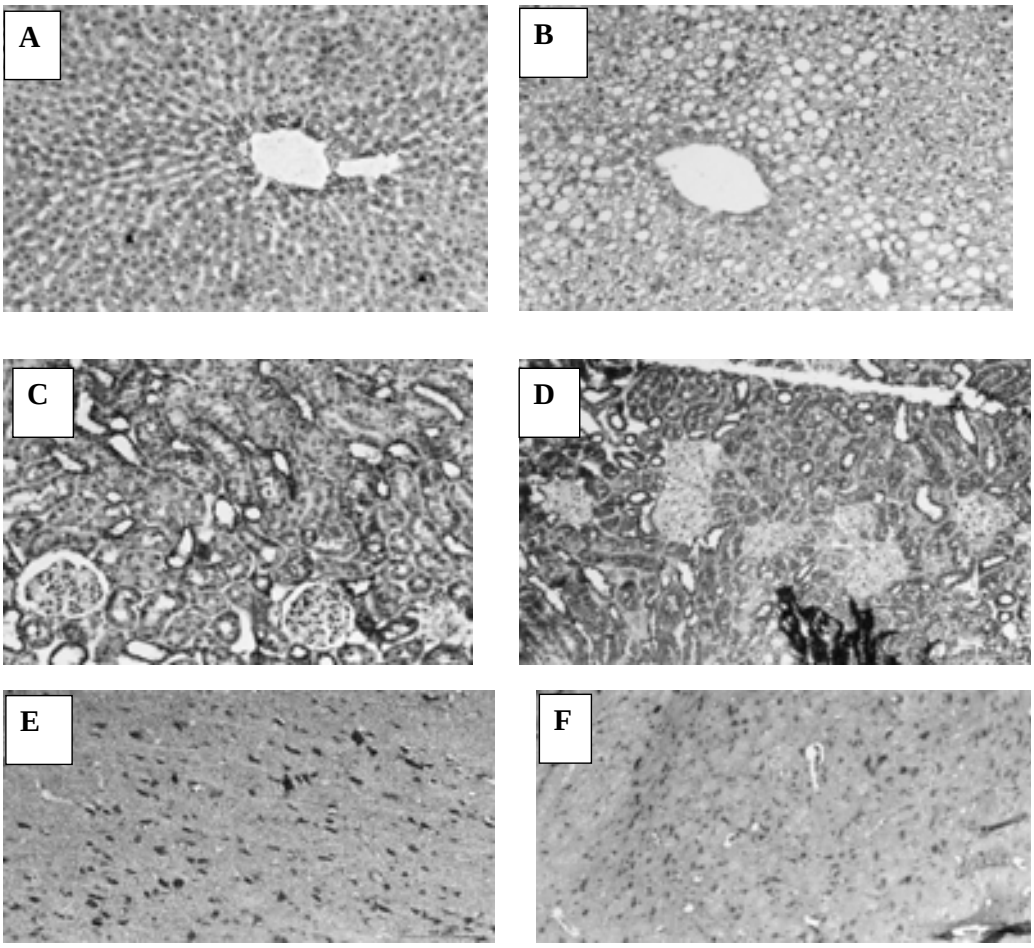

Figure 4. Bcl2 IHC (Immnunohistochemistry) brown staining of the liver of the control group showing marked positive immunostained hepatocytes meaning normal Bcl-2 expression (**A**) in comparison with less positive immunostained hepatocytes of the tramadol group (**B**) meaning decreased Bcl-2 expression. Normal Bcl2 expression in kidney of control group (**C**) compared with less expression in kidney of tramadol group (**D**). Bcl2 expression in brain of control group (E) showed more positive staining than brain of the tramadol group (**F**) meaning less Bcl2 expression. (× 200).

group (treated with 60 mg/kg/day for 90 days) in comparison with the control group.

Elevation of serum glutamic pyruvic transaminase [SGPT] and serum glutamic oxaloacetate transaminase [SGOT] enzymes occurred during liver damage. Their elevation also helps in detecting hepatocellular necrosis [7]. Also elevation of the level of Lactate dehydrogenase [LDH] enzyme was detected during liver damage and estimation of this enzyme is a more specific test for detecting liver abnormalities [8].

In accordance with these data, Saleem *et al*., 2014 [9] stated that the serum ALT and AST activity significantly increased in experimental groups administered with different doses of tramadol (12.5 mg/kg, 25 mg/kg and 50 mg/kg) IM for 40 days compared to control group. They explained this increase in the level of serum liver enzymes, as indicative of the malfunctioning and damage of liver tissues.

Also, Youssef and Zidan, 2015 [10] found that there was a significant increase in serum AST, ALT, ALP and bilirubin in the tramadol group receiving dependent doses of tramadol orally in an increasing pattern according to Paget equation till 60 days, compared with the control group.

And also, Hafez *et al*., 2015 [11] stated that there was significant increase in serum ALT, AST, ALP in the groups of rats treated with 12.5, 25, 50 mg/kg/day of tramadol hydrochloride intramuscularly for two weeks.

Elwy and Tabl, 2014 [12] reported that there was a significant increase in the AST, ALT, LDH blood levels in the tramadol group (treated with 37.5 mg\kg t.d.s for one month) compared to the control.

Our results also coincide with Elmanama *et al*. 2015 [13] who worked on a total of 100 specimens (50 tramadol abusers and 50, of matching age and sex, as control). ALT results were significantly higher in tramadol abusers (72%) against the control group (0%). Abnormal AST and LDH in 50% of tramadol abusers were found versus the control group which recorded 16% only. On the other hand, there was no significant difference between the two groups in ALP, direct bilirubin and total bilirubin.

Moreover, Atici *et al*., 2005 [14] found that in the tramadol group there was a significant increase in ALT level ($P < 0.05$) but AST and LDH levels were not different between the tramadol and the control groups. This tramadol group received doses of 20, 40 and 80 mg/kg/day on the first, second and third ten days of the study, respectively.

The assessment of serum creatinine and blood urea nitrogen (BUN) is highly important and considered standard in the determination of renal functions in the clinical setting. The serum creatinine level is not largely affected by extra-renal factors like generation, intake and metabolism, compared to BUN level. Moreover it helps to assess the glomerular filtration rate (GFR) [15].

In this study, there was no significant increase in the kidney function, BUN and creatinine, in the tramadol group (treated with 60 mg\kg for 90 days) in comparison with the control group.

In accordance with these results Atici *et al*., 2005 [14] mentioned that no significant differences in BUN and creatinine levels were found in both groups. This normal kidney function may indicate that tramadol is a safer drug in comparison to morphine.

Hafez *et al*., 2015 [11] found that in blood urea nitrogen (BUN) there was a significant difference between the control group and other groups induced by tramadol administration which indicates nephrotoxicity. On the other hand, there was no significant difference in serum creatinine (SC) between all groups. This may indicate that the tramadol-induced renal toxicity takes longer time to occur compared to opiods and thus it is safer, as reported by Elmanama *et al*., 2015 [13] and Hilaire, 2014 [16].

In our study there was a significant increase in liver MDA, kidney MDA and significant decrease in TAC between the tramadol and the control groups.

In accordance with these results, Elkhateeb *et al*., 2015 [17] reported that a significant increase in the MDA level was found in tramadol-treated group (30 mg\kg for 30 days) which also showed a highly significant decrease in the glutathione peroxidase level in comparison to the control group ($p < 0.001$).

Also Awadalla and Salah-Eldin, 2015 [18] showed that rats treated with tramadol (40 mg\kg for 20 days) lead to significant increase of oxidative stress enzymes (MDA), and significant decrease in the levels of antioxidant enzymes (SOD, GSH and CAT) in liver and kidney tissues.

This significant decrease in serum TAC which is paralleled with an increase in MDA level may be attributed to the enhanced lipid peroxidation leading to tissue damage and failure of antioxidant defense mechanism. The decrease in TAC could be attributed to the fact that antioxidant enzymes were found to contain a transition metal as a cofactor. Tramadol may interact with the metals of these enzymes resulting in the inhibition of TAC [19].

In this study there were significant diffuse changes in the liver histopathlogy of the tramadol-treated group compared to the control group. These changes were mainly sinusoidal dilatation, congestion, and hydropic degeneration (ballooning) in most of the rats in the perivenular region (zone 3). Degeneration had proceeded to midzonal region (zone 2) in some rats and perivenular necrosis and haemorrhge were also found. Hydropic degeneration was found in 70%, haemorrhage in 30%, congestion in 70%, cytolysis in 50%, apoptotic bodies in 50%, granuloma formation in 20%, perivenular necrosis in 20% and focal microvesicular steatosis in 30%.

In accordance with these findings, Saleem *et al*., 2014 [9] stated that there was necrosis, vacuolization, haemorrhage, and cytolysis, mostly perivenular with central vein dilatation.

Also**,** Hafez *et al*., 2015 [11] reported that in group II (treated with tramadol 12.5 mg\kg) there was mild central vein congestion and in group III (treated with tramadol 25 mg\kg) there was parenchymal changes in the form of mild few foci of hepatic necrosis and mononuclear inflammatory cell aggregation whereas in group IV (treated with 50 mg\kg tramadol) marked hepatocellular necrosis, inflammatory infiltrate, bile duct hyperplasia, hyaline degeneration and peri hepatitis were detected.

In addition, Awadalla and Salah-Eldin, 2015 [18] suggested that the tramadol-treated group showed congested central veins, hydropic degeneration, dense inflammatory portal reaction and some hepatocytes appeared with apoptotic nuclei.

In this study there were significant diffuse minimal changes in the kidney histopathlogy of the tramadol-treated group compared to the control group. The changes were mainly infiltration of the interstitial mononuclear cell in some rats with minimal vacuolization. No glomerular damage was found in all examined rats. Tubular epithelial vacuolization was found in 10%, interstitial cellular infiltration in 30%, focal necrosis in 20%, glomerular haemorrhage in 20% and degenerated tubules in 20%.

These results match with Hafez *et al*., 2015 [11] who stated that a clinical picture of drug-induced interstitial nephritis was found in renal tissues of group II (12.5 mg\kg) while in group III (25 mg\kg) there was multifocal cortical lymphocytes, macrophages, and plasma cell infiltration surrounding degenerated proximal convoluted tubules. In group IV (50 mg\kg) exaggeration of the same findings was detected.

Awadalla and Salah-Eldin, 2015 [18] emphasized that there were extensive degeneration in renal tubules accompanied with obstructed lumen, cellular infiltration, necrosis, and atrophied glomeruli with collapsed tuft and wide Bowman's space.

These findings may be explained by the toxicokinetics process of tramadol, since 30% of the tramadol is excreted through the kidney in an unchanged manner. While the remaining part of the drug is changed into active metabolites by the liver. These metabolites that are excreted through the kidneys may cause kidney dysfunction [2, 20].

In this study the tramadol-treated group showed significant diffuse changes in the brain histopathlogy compared to the control group, mainly in the form of disorganization of the cortical layers and hypercellularity as well as increased apoptotic cells. Almost all pyramidal cells appeared irregular in shape darkly stained with pyknotic nuclei and surrounded by haloes. Some pyramidal cells were shrunken and showed marked cytoplasmic vacuolization. There were areas of haemorrhage and dilated blood vessels.

Hypercellularity was found in 64%, apoptotic cells are found in 70%, inflammatory cellular infiltration in 45%, congested blood vessels in 50%, haemorrhage in 55%, degenerated neurons in 55% and cytoplasmic vacuolization in 50%.

In accordance with these data, Ghoneim *et al*., 2014 [21] reported that the frontal motor area of the cerebral cortex of a rat from the tramadol group (50 mg\kg for 30 days) showed neuronal cell disorganization, hypercellularity, increased apoptotic cells, multinuclear giant cells, extensive neurophil vacuolization and inflammatory cell infiltrations.

Also, Abou El Fatoh *et al*., 2014 [22] showed an aggregation of inflammatory cells, mainly lymphocytes and macrophage with degenerated neurons, in the cerebrum of albino rats which were administered tramadol (40 mg/kg/day BW).

In addition Khodeary *et al*., 2010 [23] mentioned that the brain regions of the group of rats treated with tramadol 30 mg\kg for 4 weeks showed disorganization, eosinophilic areas with increased cellularity, degenerated neurocytes, dilated blood vessels, shrunken neurons with pyknotic nuclei and scanty eosinophilic cytoplasm (apoptotic cells) and red neurons with shifting of nucleus towards axon (neurons with hypoxic changes). These findings may provide a possible explanation for neurological and psychological changes associated with tramadol abuse.

Repetitive or chronic exposure to uncontrollable stressor agents including drugs will gradually initiate a cascade of processes in brain which leads to profound alterations in the electrical characteristics, morphology, suppression of proliferative capacity or neurogenesis and induction of neurocytes apoptosis

of brain cells. However, animals subjected to a spontaneous recovery period of rest after chronic stress showed remarkable reversibility of these effects [24].

In this study, immunohistochemical study of the Bcl2 antigen showed decrease in its expression in the tramadol group's liver, kidney and brain specimens compared to the control and it was more obvious in the brain.

This coincides with Khodeary *et al*., 2010 [23], who reported that both low-dose (group II; 30 mg\kg) and high-dose (group III; 60 mg\kg) groups of tramadol-treated rats showed decrease in Bcl2 expression in their brains compared to the control. However, marked cytopathological injury reported in group III than group II displayed dose-dependent effect of tramadol hydrochloride (TH) on studied brain regions.

The activation of apoptotic cell death pathway namely suppression of Bcl2 proteins may play an important role in TH-induced brain damage [25]. However, we need further studies with larger number of animals and with a longer period of follow up.

CONCLUSION

Chronic usage of tramadol has toxic effects on liver as indicated by biochemical elevation of liver enzymes ALT, AST, LDH and also histopathological changes mainly in the form of hydropic degeneration and cytolysis. Moreover, it has toxic effect on kidneys; even with normal renal function there were minimal histopathological changes affecting mainly the renal tubules in the form of interstitial nephritis and normal glomeruli. Tramadol mainly affected the brain as the histopathology showed marked hypercellularity, disarrangement of nerve cells and irregular shrunken neurons. In addition, it induced apoptosis as indicated by the decrease in the protective anti apoptotic antigen Bcl2.

SOURCE OF FUNDING

The study did not receive any financial support.

CONFLICT OF INTEREST STATEMENT

The authors have no conflict of interest to declare.

ABBREVIATIONS

ALT, alanine transaminase; AST, aspartate transaminase; LDH, Lactate dehydrogenase; BUN, blood urea nitrogen; MDA, Malondialdehyde; TAC, total antioxidant capacity; GFR, glomerular filtration rate; IHC, immunohistochemistry; SGPT, Serum glutamic pyruvic transaminase; SGOT, serum glutamic-oxaloacetic transaminase; SC, sub-cutaneous; SOD, Superoxide dismutase; GSH, Glutathione; CAT; catalase.

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