

Histopathological and Molecular Studies on Tramadol Mediated Hepato-Renal Toxicity in Rats

Eatemad A. Awadalla¹, Alaa-Eldin Salah-Eldin^{1,2}

¹ Zoology Department, Faculty of Science, Aswan University, P.O. 81528, Aswan, Egypt

² Medical Laboratories Department, College of Science in AlZulfi, Majmaah University, P.O. 1712-11932, KSA

Abstract: Tramadol, a broadly used opioid in recent years, is an effective analgesic agent for the treatment of moderately severe acute or chronic pain. The liver and kidneys are responsible for the metabolism and excretion of opioids, which may cause hepatotoxicity and nephrotoxicity. The present study was designed to evaluate the toxic effects of tramadol on the liver and kidney of experimental rats. Twenty male albino rats, used in the present study, were divided into two groups; control and tramadol-treated (40 mg/kg b.w., orally) for 20 consecutive days. Livers and kidneys specimens were taken for histopathological, molecular and biochemical studies. Malondialdehyde (MDA), reduced glutathione (GSH), superoxide dismutase (SOD) and catalase (CAT) were determined in these tissues. Histopathological changes in liver and kidney were evaluated by light microscopy. Finally, RT-PCR technique was used to analysis the expression level of apoptosis-related genes, Bcl-2 and Bax, in liver and kidney tissues. Tramadol caused a significant reduction in the activities of GSH, SOD, and CAT, whilst a significant increase in the level of MDA in compared to control groups in both liver and kidney. In accordance with the biochemical findings, our data showed that tramadol treatment induced histopathological alterations in both tissues. These alterations were manifested by severe hydropic degeneration, with congested central veins of the liver and degenerated renal tubules and atrophied glomerulus. Moreover, mRNA expression level of proapoptotic marker Bax showed a significant increase, whereas the antiapoptotic Bcl-2 expression decreased significantly indicating that tramadol is harmful at the cellular level and can induce apoptotic changes in these tissues. Therefore, the toxic effects of tramadol should keep in mind, even though, the important role it plays as powerful pain management.

Keywords: Apoptosis, Bax, Bcl-2, Kidney, Liver, Oxidative Stress, Tramadol.

I. Introduction

Tramadol is an effective analgesic agent, extensively used in recent years for the treatment of moderately severe acute or chronic pain [1]. Because of its high efficacy and low addictiveness, tramadol is widely prescribed to relieve pain [2]. The mechanism of its analgesic action is complex. Most reports suggest that the analgesic activity and other clinical effects of tramadol are a result of opioid and non-opioid mechanisms. Tramadol binds to the μ -opioid receptor, although much more weakly than morphine. It also inhibits the neuronal reuptake of norepinephrine and serotonin as do the antidepressant drugs such as amitriptyline and desimpramine [3] [4] [5] [6] [7] [8].

Tramadol is metabolized in the liver by two principal pathways: O-demethylation to O-desmethyltramadol (M1) by CYP2D6 and N-demethylation to N-desmethyltramadol (M2) by CYP2B6 and CYP3A4. Only one of tramadol metabolites, M1, is pharmacologically active. Its selectivity for μ -receptors has recently been demonstrated, showing a higher affinity for opioid receptors than the parent drug [9] [10] [11]. It is transformed in the liver to O-desmethyl-tramadol, which itself is an active substance and 2-4 times more effective and potent than tramadol [12] [13] [14]. On other hand, biotransformation results in inactive metabolites, which are excreted by kidneys [15] [16].

Widespread use of tramadol makes associated hepatotoxicity a clinically and economically important problem. The liver and kidneys are responsible for the metabolism and excretion of opioids, which may cause hepatotoxicity and nephrotoxicity during its metabolism [17] [18] [19]. Tramadol and its metabolites are excreted via kidneys, consequently the kidney is considered the primary target organ for tramadol toxicity [20]. Toxic effects of opioids at the cellular level may be explained by lipid peroxidation [21]. Biological membranes contain a large amount of polyunsaturated fatty acids, which are particularly susceptible to peroxidative attacks by oxidants resulting in lipid peroxidation. Therefore, lipid peroxidation has been used as an indirect marker of oxidant-induced cell injury. A significant increase in lipid peroxidation was reported in rats receiving an acute dose of cocaine [22]. Similarly, lipid peroxides were found significantly increased among chronic heroin users [23]. In a different experimental study, isolated rat hepatocytes exhibited a marked decrease in glutathione level when incubated with various concentrations of morphine and resulted in cell death [24].

Pathological cellular insults, like oxidative stress, trigger the genetically programmed pathway of apoptosis [25]. Bcl-2 family proteins play central roles in cell death regulation and are capable of regulating

diverse cell death mechanisms that encompass apoptosis, necrosis and autophagy [26] [27] [28]. Members of the Bcl-2 family reside upstream of irreversible cellular damage and focus much of their efforts at the level of mitochondria, they play a pivotal role in deciding whether a cell will live or die. This family of proteins has expanded significantly and includes both pro- as well as anti-apoptotic molecules. Indeed, the ratio between these two subsets helps determine, in part, the susceptibility of cells to a death signal [29]. Activation of the proapoptotic molecule Bax appears to involve subcellular translocation and dimerization. In viable cells, a substantial portion of Bax is monomeric and found in the cytosol or loosely attached to membranes. Following a death stimulus, cytosolic and monomeric Bax translocated to the mitochondria where it becomes an integral membrane protein and cross-linkable as a homodimer [30] [31] [32]. The presence of an antiapoptotic Bcl-2 molecule can inhibit the activation of Bax following a death signal [31]. In contrast to Bax, Bcl-2 is an integral membrane protein heavily localized to mitochondria [33]. Moreover, Bcl-2 could be phosphorylated *in vivo* and this modification has been demonstrated to affect its antiapoptotic activity [34] [35] [36] [37] [38].

Recently, an increasingly dangerous phenomenon of tramadol abuse has been massively demonstrated in a worldwide. Hence, in the present study, histopathological, molecular, and biochemical changes induced in the liver and kidney due to the tramadol oral treatments were assessed in albino rats. The alteration in Bcl-2 and Bax expression were examined to discover their contribution in the pathogenesis and progression of liver and kidney damage due to tramadol treatment.

II. Material And Methods

2.1. Chemicals:

Tramadol tablets, each contains 225 mg tramadol hydrochloride, were obtained from October Pharma Co. (Giza, Egypt). Kits for lipid peroxidation (Malondialdehyde, MDA), reduced glutathione (GSH), superoxide dismutase (SOD), and catalase (CAT), were purchased from Bio-diagnostic Co. All other chemicals were of the highest quality available.

2.2. Animals and treatments:

Twenty, adult male albino rats (120-140g/ 4 months old) were used in the present study, obtained from the Animal-house belonged to the Egyptian Co. for Vaccines production in Helwan city. The animals were kept in a controlled light room with a photoperiod of 12 hours dark and 12 hours light at a temperature of 28 ± 2 °C. The experimental design was approved by the Animal Care and Use Committee, University of Aswan. The rats were randomly divided into two groups (control and tramadol-treated) 10 rats each. The control group was daily treated orally with saline solution; 0.9% NaCl for 20 days. Tramadol group was orally treated with tramadol hydrochloride for 20 successive days at dose 40 mg/kg body weight/daily. Twenty four hours after the last injection, rats were sacrificed by decapitation. The liver and kidneys of each rat were dissected out carefully. Each specimen was divided into two parts; one part was wrapped with aluminum foil and kept frozen at -80 °C until they were used for Reverse Transcription-Polymerized Chain Reaction (RT-PCR) analysis and measurement of MDA content and antioxidant enzymes (GSH, SOD, and CAT) activities. The other part was preserved for histopathological examination.

2.3. Determination of lipid peroxidation and antioxidant enzyme activities:

For biochemical studies, lipid peroxidation level (expressed in terms of malondialdehyde, MDA, content) was measured in the liver and kidney as described by Placer et al. [39]. CAT activity was assayed by the method reported by Aebi [40]. The activity of SOD was estimated according to Paolotti and Mocali [41], while GSH was determined by the method of Maral et al. [42]. In details, liver and kidney from both control and treated rats were minced and homogenized (10% W/V) separately in ice-cold saline, sucrose buffer (0.25 M sucrose, 1 mM EDTA and 0.05 M Tris-HCl, pH 7.4) in a Thomas Sci. Co. glass-type homogenizer (Teflon pestle). The homogenization of tissues was carried out in Teflon-glass homogenizer with a buffer containing 1.15% KCl to obtain 1:10 (W/V) whole homogenate. Homogenates were centrifuged at 3000 rpm (+4 °C) for 15 min to determine MDA, SOD, and CAT activities. While, homogenates were centrifuged at 5000 rpm for 50 min to measure the GSH activity.

2.4. Histopathological and histochemical examination:

After scarification, specimens from the liver and kidney were taken from the two studied groups. They were fixed in 10% neutral buffer formalin, embedded in paraffin, sectioned at 5 microns and prepared for the following stains:

- 1- Harris's hematoxylin and eosin stain, Gabe [43].
- 2- Mallory's triple stain for collagenous fibers, Pantin [44].
- 3- Periodic acid Schiff's (PAS) technique for general carbohydrates, Mc Manus [45].
- 4- Mercury-Bromophenol Blue method, as recommended by Mazia et al. [46] for total proteins.

2.5. Total RNA extraction from liver and kidney tissues:

Frozen liver and kidney samples of each group were thawed and used for RNA extraction. Homogenization of 200 mg of frozen tissue samples was carried after addition of 1 mL TriZol (Invitrogen, Carlsbad, CA) using a Polytron 300 D homogenizer (Brinkman Instruments, Westbury, NY). Total RNA was isolated from cells using Trizol reagent following the method of Chomczynski and Sacchi [47]. The total RNA obtained was free from protein and DNA contamination. Nucleic acid concentration was determined by optical density at 260 nm (Smart-Spec; Bio-Rad Laboratories, Hercules, CA) and RNA integrity was evaluated using an Agilent bioanalyzer (model 2100; Agilent Technologies, Foster City, CA). The purity of RNA at 260/280 OD ratio and RNA integrity was evaluated (OD 260/280 >1.8). Only a high purity samples were subjected to further manipulation.

2.6. Bcl-2 and Bax genes expression analysis using RT-PCR:

The reverse transcription step was performed by using the RT enzyme kit. Each 20 µL reaction mixture contained 5 µL Oligo(dT) (10 µM), 1 µL dNTP (10 µM), 4 µL First Strand buffer (5×), 1 µL DTT (0.1 M), 0.2 µL super Script III reverse transcriptase (200 U/µL) varied quantity of RNA template (dependent on RNA concentration) and RNase-free water to make up the volume (20 µL). Thermal cycling conditions for first strand reaction consisted of 25 °C for 5 min, 50 °C for 45 min, 70 °C for 15 min and finally maintained at 4 °C for 5 min. PCR amplification was performed using *Taq* DNA Polymerase (Perkin-Elmer, Foster City, CA, USA). Each 20 µL of the sample contained 10 µL Master mix (2 µM), 1 µL forward primer, 1 µL reverse primer for both genes of interest and internal control consecutively, 2 µL RT sample, and 4 µL RNAase-free water. The mixture was held at thermocycler and amplified for 35 cycles. Each thermocycling consisted of denaturation for 30 sec at 94 °C, varied annealing temperature for each gene of interest for 30-60 sec, and extension for 60 sec for 72 °C. β-actin gene was co-amplified with apoptotic genes Bcl-2 and Bax using the same procedures. The sense and antisense primer sequences and PCR conditions are described in Table (1) according to the gene of interest.

Table (1): Primer Sequences and PCR Conditions for Apoptotic Bcl-2 and Bax Genes.

Genes	RT-PCR Product size		Primer Sequences	Annealing Temp/Time	Cycles Number
Bax	310 bp	Sense	5'- GAC ACC TGA GCT GAC CTT GG -3'	58°C /45 sec	35 Cycles
		Antisense	5'- GAG GAA GTC CAG TGT CCA GC -3'		
Bcl-2	138 bp	Sense	5'- GGG ATG CCT TTG TGG AAC TA -3'	56°C /30 sec	35 Cycles
		Antisense	5'- CTC ACT TGT GGC CCA GGT AT -3'		
β-actin	598 bp	Sense	5'- TCA TGC CAT CCT GCG TCT GGA CCT -3'	55°C /60 sec	35 Cycles
		Antisense	5'- CGG ACT CAT CGT ACT CCT GCT TG -3'		

PCR cycle of respective genes are shown above, while temperature and time of Denaturation and Elongation steps of each PCR cycle are 94°C, 30 sec and 72°C, 60 sec, respectively.

Amplified PCR products were analyzed and electrophoresed in 2% agarose gels, then stained with ethidium bromide and visualized under UV lamp. The bands were identified based on the product size and documented using a Gel documentation system (Gel DocTMXR System, Bio-Rad, USA) and the prints were scanned. The scanned images were quantified densitometrically with the aid of NIH image program (<http://rsb.info.nih.gov/nih-image/>). The results were normalized to the levels obtained for the β-actin gene by taking a ratio of the value obtained for the gene of interest to that of β-actin and then relative to the control.

2.7. Statistical analysis:

The data were expressed as the mean ± S.E.M. and were analyzed by means of one-way analysis of variance (ANOVA). Statistical evaluation of data was done following Student's t-test. A difference was considered significant at P < 0.01.

III. Results

The role of the liver and the kidneys in tramadol metabolism and excretion predisposes them to toxic injury. Extended use of tramadol may associate with hepatotoxicity and its metabolites are excreted via kidneys make it the primary target organs for tramadol toxicity. Herein, we examined the histopathological alterations and changes in apoptotic genes expression in hepatorenal tissues.

3.1. Biochemical results:

The measured data of lipid peroxidation, superoxide dismutase (SOD), glutathione (GSH) and catalase (CAT) in the liver and kidney tissues are summarized in Table (2). Results indicated that malondialdehyde (MDA) concentration, the end product of oxidative stress, was significantly increased in the liver and kidneys ($P < 0.01$) in rats treated with tramadol. On contrary, tramadol treatment significantly ($P < 0.01$) decreased the level of SOD in both liver and kidney. Also, tramadol treatment significantly ($P < 0.01$) decreased the level of GSH in the liver and decreased ($P < 0.05$) in the kidney. In addition, tramadol treatment significantly ($P < 0.01$) decreased the level of CAT activity in the kidney and decreased ($P < 0.05$) in the liver.

Table (2): Effect of tramadol treatment on malondialdehyde (MDA), reduced glutathione (GSH), catalase (CAT) and superoxide dismutase (SOD) in hepatic and renal tissues of rats after 20 days of oral administration

Measurements Groups	Liver		Kidney	
	Control	Tramadol	Control	Tramadol
MDA Mean ± S.E.M.	3.256 ± 1.55	8.435 ⁺⁺⁺ ± 5.10	2.204 ± 1.22	6.801 ⁺⁺⁺ ± 1.23
GSH Mean ± S.E.M.	1.201 ± 0.075	0.843 ^{--a} ± 0.060	2.961 ± 0.043	0.658 ^{-a} ± 0.150
CAT Mean ± S.E.M.	1.832 ± 0.077	0.651 ^{-a} ± 0.12	2.589 ± 0.188	0.736 ^{--a} ± 0.201
SOD Mean ± S.E.M.	2.565 ± 3.31	0.572 ^{--a} ± 7.74	2.901 ± 2.155	0.485 ^{--a} ± 3.753

Results are expressed as mean ± S.E.M. of 10 animals for each group.

+++a = highly significantly increased compared with the normal at $p < 0.01$.

a = significant decreased compared with normal at $p < 0.05$.

3.2. Histopathological examination of the liver:

Examination of liver specimens taken from the control group showed normal lobular architecture with strands of normal hepatocytes running radially from central vein and sinusoids appeared containing Kupffer cells (Fig. 1a). The portal areas contained branches of the hepatic artery and bile duct, embedded in connective tissue (Fig. 1b). Also, the control group showed few collagen fibers in the central vein (Fig. 2a). The tramadol-treated group showed vacuolated hepatocytes' cytoplasm indicating hydropic degeneration, with congested central veins. Some hepatocytes appeared with apoptotic nuclei. The portal tract revealed dense inflammatory portal reaction, severe congestion of portal veins and cellular infiltration (Fig. 1c-f).

The collagen fibers were increased in the hepatic tissue of the tramadol-treated group (Fig. 2b). PAS-reaction sections of the control group showed an existence of a considerable amount of glycogen granules in the hepatocytes. The +ve PAS reaction appears in the form of dark purple granules located mainly in the ground cytoplasm, especially at one pole of the cells, reflecting the phenomenon of "glycogen flight" (Fig. 2c). However, the glycogen depletion occurred in the ground cytoplasm of tramadol-group when compared with control group (Fig. 2d). In addition, control liver revealed the general mode of protein distribution in the hepatic cells (Fig. 2e). The protein of the hepatocytes manifested a moderate reaction. The cell and nuclear membranes in addition to the chromatin bodies acquired an intense stain. The endothelial lining of the both blood sinusoids and central vein revealed a moderate reaction. Contrarily, tramadol liver sections displayed a remarkable increase in staining affinity within the majority of the hepatic cell when compared with control group (Fig. 2f).

3.3. Histopathological examination of the kidney:

Kidney of the control group showed normal features of renal tubules and Bowman's capsules (Fig. 3a). In the tramadol group, extensive changes could be seen in renal tissues comparing to control group (Fig. 3b-c). The changes include degenerated renal tubules with obstructed lumen and atrophied glomerulus with collapsed tuft and wide Bowman's space (Fig. 3b). Also, kidney in tramadol-treated animals displayed cellular infiltration with an obvious necrosis and degenerated tubules (Fig. 3c). The application of PAS reaction has presented a satisfactory profile for the localization of general carbohydrates in the normal kidney tissues as demonstrated in Fig. (3d). Nevertheless, the tramadol group revealed marked depletion in the PAS-reactivity in the kidney tissue components when compared with control groups (Fig. 3e).

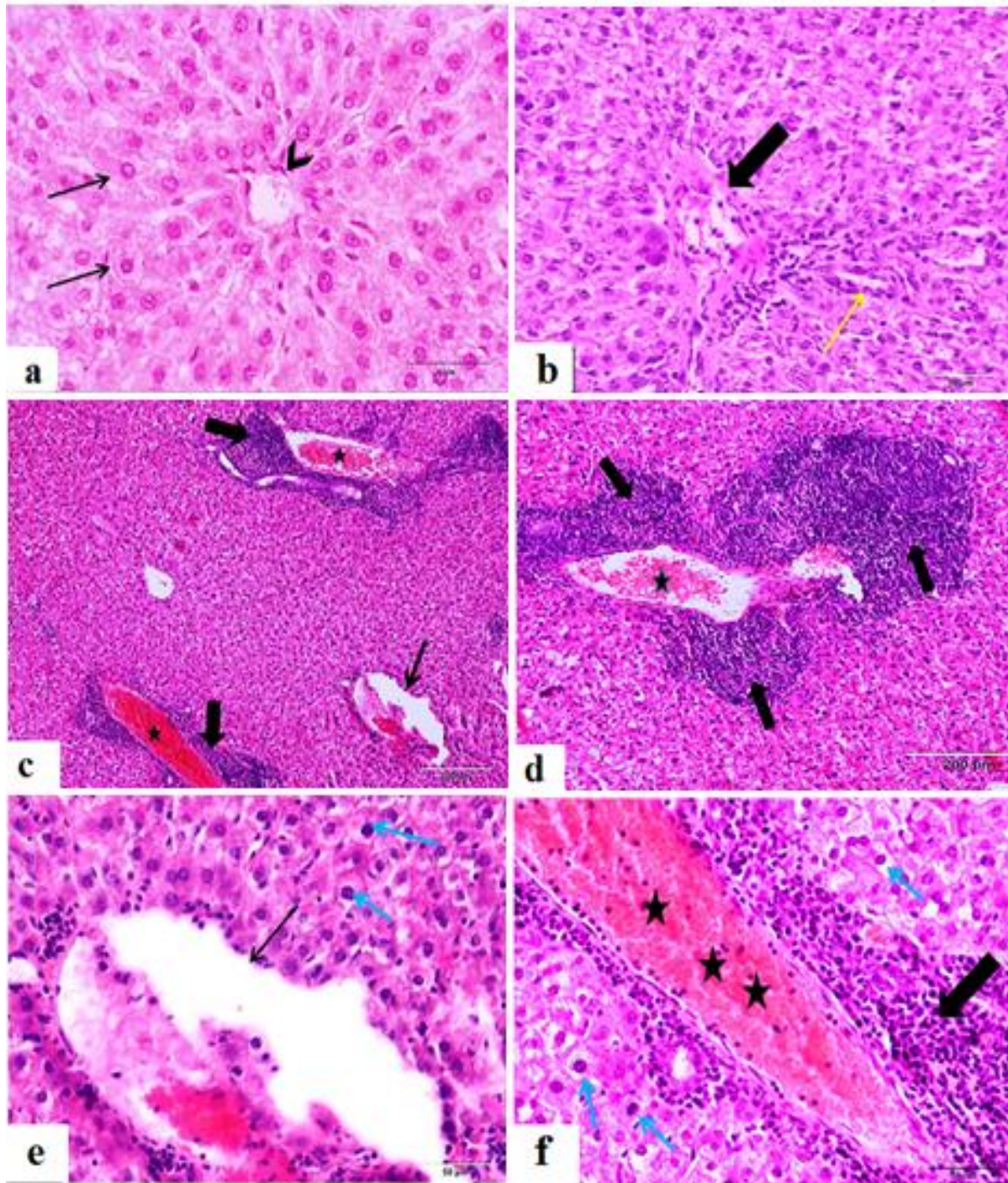


Figure (1): Sections of control liver (a-b) showing normal appearance of the hepatocytes (arrows) and a prominent central vein (arrowhead) with portal area containing hepatic artery (thick arrow) and bile ductule (yellow arrow) (H&E, original magnification: "a & b": 400X = 50 μ m). Liver sections of tramadol group (c-f) showing congested blood vessels (thin arrow & *) with huge inflammatory of lymphocytes (thick arrows) and degenerated hepatocytes with spotty necrosis (blue arrow) (H&E, original magnification, "c & d": 100X = 200 μ m; "e & f": 400X = 50 μ m).

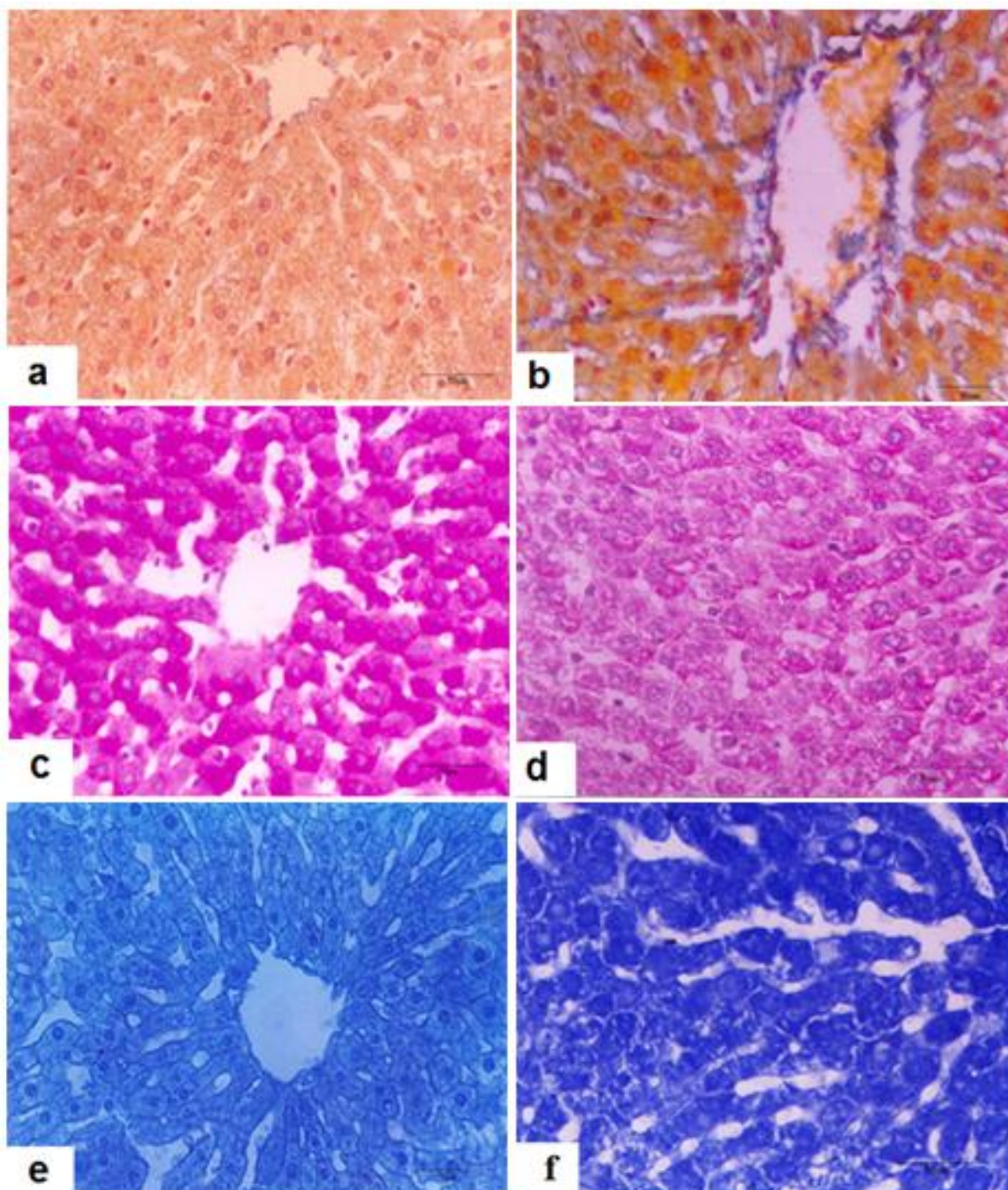


Figure (2): Liver section of the control group (a) shows normal distribution of the collagen fibers around the central vein as well as blood sinusoids. Liver section of the tramadol group (b) exhibits a pronounced increase in thickness of the collagenous fibers surrounding dilated central vein and blood sinusoids (Mallory triple stain, original magnification: 400X = 50 μ m). Liver section of control liver (c) shows strong PAS-reaction in the centro-lobular hepatocytes, being stained with intense magenta color, indicating their richness of polysaccharides (specifically glycogen granules). Liver section of the tramadol group (d) shows a weak PAS-reactivity of the most of hepatic cells. Liver section of control liver (e) shows moderate reaction of the protein in the hepatocytes. Tramadol liver section (f) shows remarkable increase in staining affinity within the most of hepatic cells (Bromophenol blue, original magnification: 400X = 50 μ m).

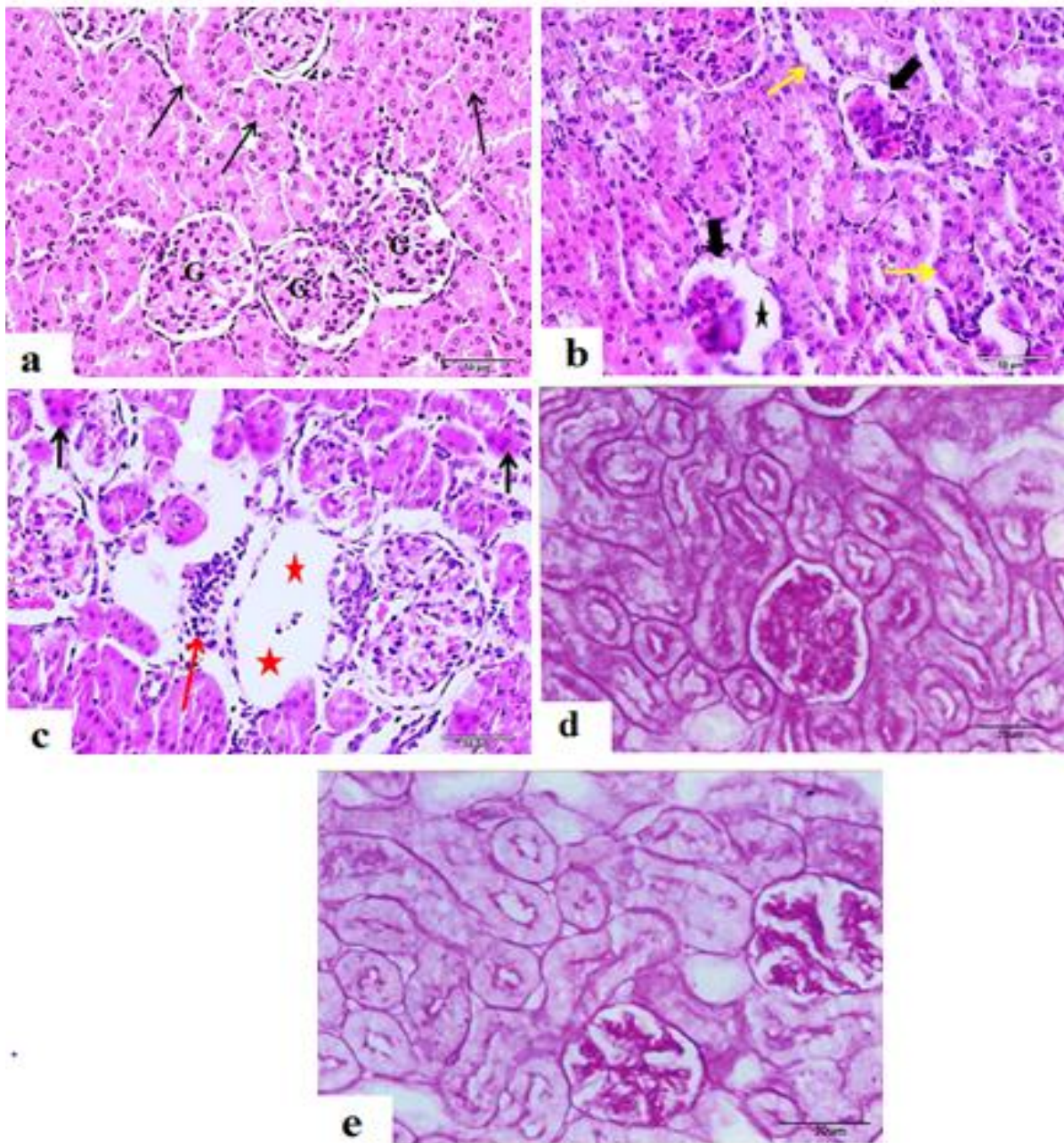


Figure (3): Section of control group (a) showing a normal structure of glomeruli (G) and normal architecture of renal tubules (thin arrows). Sections of tramadol-treated group (b and c) showing atrophied glomeruli with collapsed tuft (thick arrows), wide Bowman's space (black star) and degenerated tubules in (b) (donated by yellow arrow). In (c); treated kidney displays cellular infiltration (red arrow), wide spacing (red star) and degenerated tubules (arrow) are also seen (H&E, original magnification: 400X and bar = 50 μ m). Kidney section of control group (d) is showing a strong reactivity of PAS in all cortical renal tissues. Kidney section of the tramadol group (e) shows remarkable depletion of PAS-reactivity in the most cortical renal tissues (PAS, original magnification: 400X = 50 μ m).

3.4. Expressions of apoptotic factors in the liver tissue:

In liver tissues, Bcl-2 and Bax expression level in the control group was considered as a reference to evaluate their expressions level in the tramadol-treated rats. Housekeeping β -actin gene was used to normalize and calculate the expression level of Bcl-2 and Bax genes in treated group. The results are given in Fig. 4(a-c) showing the effect of tramadol treatment on Bcl-2 and Bax mRNA expression in liver of animals after 20 days of experimental. Normalization using β -actin as an internal control, the Bcl-2 gene expression is studied in the PCR bands at 138 base pair (Fig. 4a). Tramadol treated animals showed a significant ($p < 0.05$) decrease in Bcl-2 gene expression in the liver tissues compare to control one. While, Bax gene expression results are shown in

Fig. (4b) and the PCR bands size located at 310 base pair. Bax gene expression in the tramadol-treated animals significantly ($p < 0.05$) increases compare to control group after normalization with β -actin as an internal control. Illustration in Fig. (4c) showing a densitometric analysis data of the expressed bands of Bcl-2 and Bax after normalized with that of β -actin, and then calculated as relative to the control group in 10 rats. Values donated by (*) are significantly different ($P < 0.05$) compared to control.

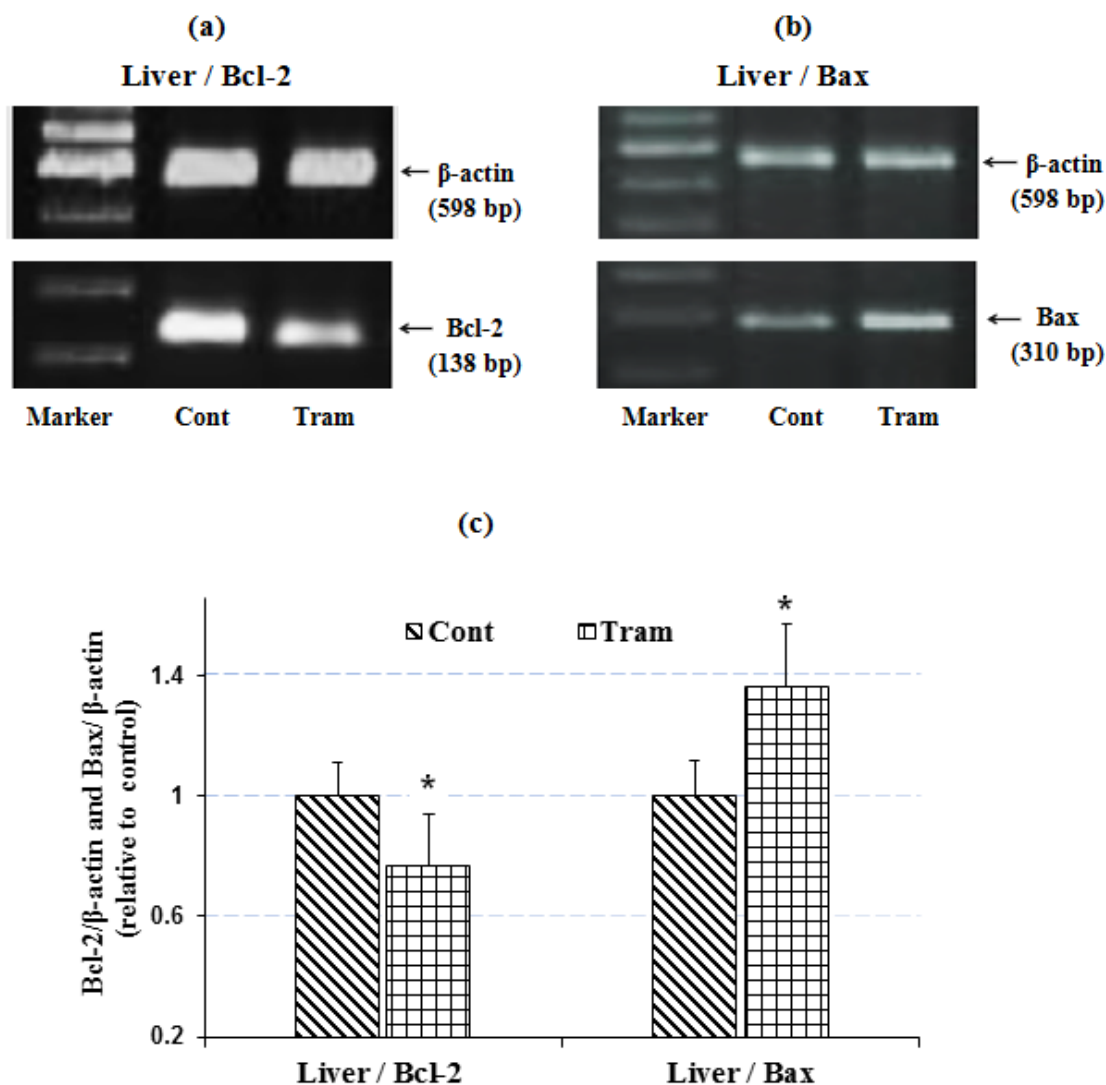


Figure (4): RT-PCR analysis of Bcl-2 and Bax expressions in liver tissues. Data of control (Cont) and tramadol-treated rats (Tram) are shown after 20 days of experiment. RNA was extracted and reverse transcribed (1 μ g) and then RT-PCR analysis was carried out for Bcl-2 (a) and Bax (b) genes. Data of densitometric analysis of the expresses bands of Bcl-2 and Bax were normalized with that of β -actin and then calculated as relative to the control group (c). (*) Values are significant different ($P < 0.05$) compared to control.

3.5. Expressions of apoptotic factors in the kidney tissue:

In kidneys, the expression level of the Bcl-2 and Bax genes in the control group was considered as a reference to calculate and normalize the expressions level in the tramadol-treated group. The RT-PCR data were normalized to the levels obtained for the β -actin gene; by taking a ratio of the value obtained for the gene of interest to that of β -actin and then relative to the control. The results are given in Fig. (5a-c), showing the effect of tramadol treatment on Bcl-2 and Bax mRNA expression in kidneys of albino rats. The results of Bcl-2 gene expression are given in Fig. (5a) and Bcl-2 amplified bands found at 138 base pair. Using β -actin as an internal control, Bcl-2 expression showed a significant ($p < 0.05$) decrease in the kidney tissues of tramadol-treated rats compare to control group. In the meantime, Bax gene expression was studied and the expression results are shown in Fig. (5b) at a PCR-product size equal to 310 base pair. Calibration using β -actin as an internal control, the expression of Bax gene in the tramadol-treated animals significantly ($p < 0.05$) increase compare to the

control group. Fig. (5c) showing a densitometric analysis data of the Bcl-2 and Bax bands after normalized with that of β -actin in 10 rats, and then calculated as relative to the control group. Labeled value by (*) are significant different ($P < 0.05$) in comparison with control.

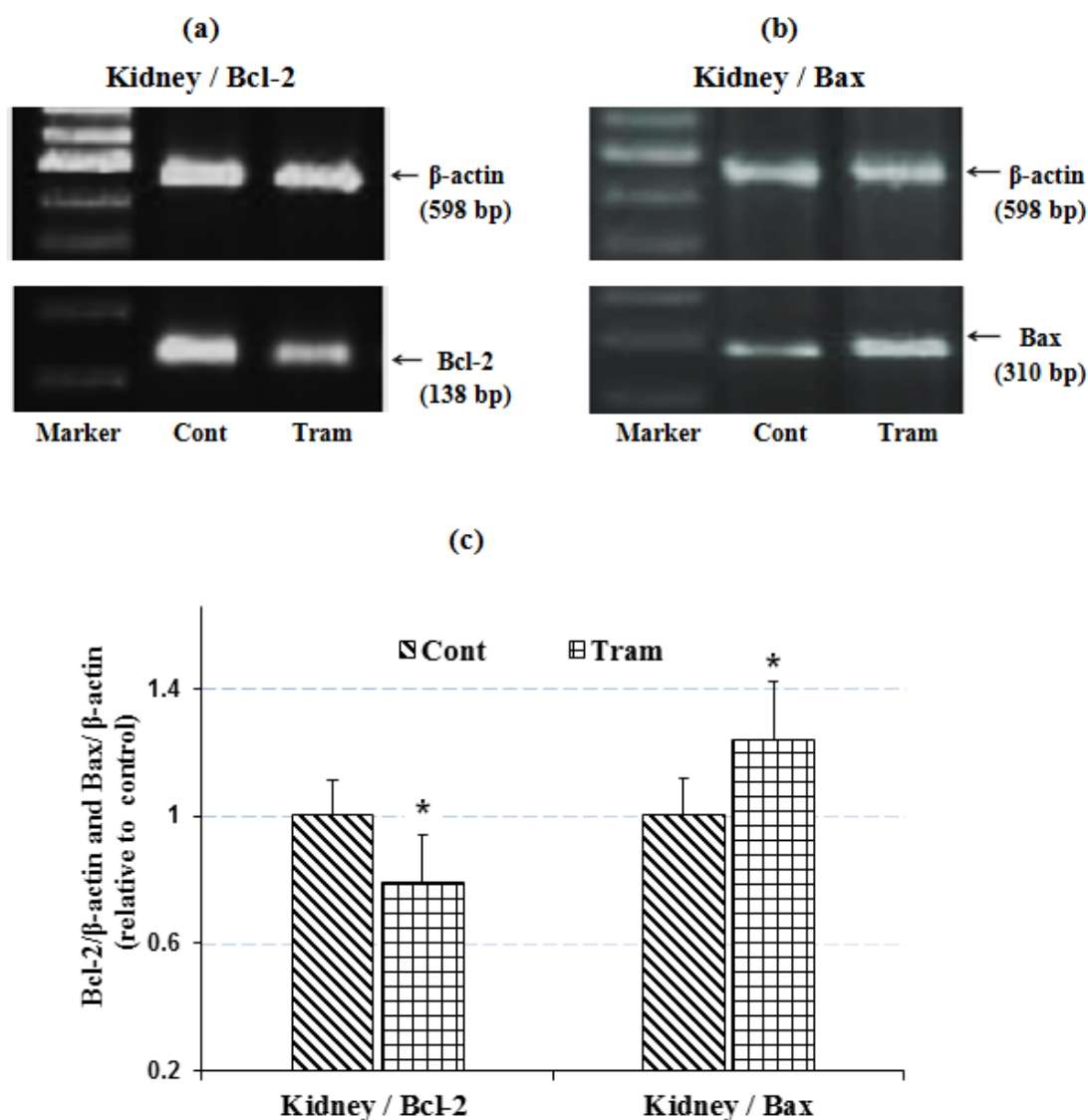


Figure (5): RT-PCR analysis of Bcl-2 and Bax expressions in kidney tissues. Data of control (Cont) and tramadol-treated rats (Tram) are shown after 20 days of experiment. RNA was extracted and reverse transcribed (1 μ g) and then RT-PCR analysis was carried out for Bcl-2 (a) and Bax (b) genes. Data of densitometric analysis of the expresses bands of Bcl-2 and Bax were normalized with that of β -actin and then calculated as relative to the control group (c). (*) Values are significant different ($P < 0.05$) compared to control.

IV. Discussion

Every drug has been associated with hepatotoxicity due to the essential role of the liver in drug metabolism [48] [49]. Hepatic metabolism is, first and principal, mechanism that converts drugs and other compounds into products that are more easily excreted and that usually have a lower pharmacologic activity than the potent compound [16] [49]. The hepatic function in drug metabolism involves converting drugs and other compounds into products that are more easily excreted [17] [19] [49]. Metabolites may have a higher activity and/or a greater toxicity than the original drug. These metabolites, excreted via kidneys, may also cause a cellular damage and, thus, a kidney dysfunction [50]. Therefore, the central role of liver and kidney in drug metabolism predisposes them to toxic injury.

Opioids are the most potent and effective analgesics available and have become accepted as appropriate treatment for acute, cancer and non-cancer chronic pain [51] [52] [53]. Tramadol is rapidly absorbed

orally; a peak concentration is detected 2-3 hours post oral dose. It has extensive tissue distribution. Thirty percent of the drug is excreted through the kidneys in an unchanged manner. Elimination half-life is 5-6 hours, while the remaining is metabolized in the liver by N- and O-demethylation, followed by conjugation with glucuronic acid and sulphate. The active metabolite, O-desmethyl tramadol shows higher affinity for the μ -opioid receptors and has twice the analgesic potency of the parent drug [54].

It was reported that MDA is widely accepted as a sensitive biomarker of lipid peroxidation. It is considered a useful measure of oxidative stress status [55]. In addition, oxidative stress was not only by augmenting lipid peroxidation but also by inhibiting the antioxidant enzymes activities [56]. The current study recorded that tramadol treatment induced oxidative stress. Our data proved that tramadol treatment leads to an increase in MDA level, while there is a decrease in the activities of GSH, SOD and CAT enzymes in both liver and kidney tissues. Our data coincided with Elkhateeb et al. [57] who reported that a significant increase in the MDA level with a highly significant decrease in the glutathione peroxidase (GPx) level were observed in tramadol-treated animals. Furthermore, the present results in parallel with Nehru and Anand [58], who reported that reactive oxygen species (ROS) generation and lipid peroxidation are responsible for tramadol-induced nephrotoxicity. The previous theory coincides with our results, which revealed an increase in MDA level and a decrease GPx level in kidneys of rats receiving tramadol. Abdel-Zaher et al. [59] reported that administration of tramadol to mice resulted in depletion of GPx and subsequent potentiation of lipid peroxidation in kidney cortical slices resulting in inhibition of protein synthesis, and mitochondrial damage. The present biochemical data could be attributed to the suggestion of Ismail et al. [60], who suggested that the antioxidant enzymes were found to contain a transition metal as a cofactor. The interaction of tramadol with metals of these enzymes may explain the observed inhibition in the activities of these enzymes.

In the present study, the histopathological results supported the toxic effect of tramadol on the liver. We found hydropic degeneration, with congested central veins and necrotic signs in some hepatocytes. These degenerative changes are in agreement with previous histopathological studies indicated the necrosis, vacuolization, central vein dilation, hemorrhage, cytolysis and complete cell membrane degeneration in hepatocytes in tramadol long-term treated mice [61] [63]. Our data supported by Loughrey et al. [63], who reported that tramadol administration in adult male rats for one month was accompanied by hepatic congestion, hemorrhage, and necrosis. In addition, El-Wessemy [64] reported a loss of architecture, congested central veins, and expanded portal area with edema and inflammatory reaction in rats treated with tramadol. Also, the present data coincide with Samaka et al. [65], who found liver parenchymal changes in form of sinusoidal dilation, focal necrosis, marked congestion and hemorrhage were seen in group treated with acute dose of tramadol. Portal changes; marked inflammatory cellular infiltrate, mild fibrosis and bile duct proliferation were noticed in-group treated with a chronic dose of tramadol.

In addition, our results are coincided by results of Elmanama et al. [66], who emphasized that treatment of tramadol is more harmful to the liver and causes a serious cellular toxicity and a liver failure. Moreover, our results in parallel with findings of Elkhateeb et al. [57], who found that tramadol treatment induced severe pathological changes in the liver. These changes included hydropic degeneration of hepatocytes, apoptotic nuclei and congestion of central veins in the tramadol group. There were also, proliferated bile ducts, cellular infiltration, hemorrhage and fibrosis of blood vessels. Regarding the present data concerned with collagen fibers in the hepatic tissues. We found that the collagen fibers were increased in the hepatic tissue of the tramadol-treated group comparing with control group. This result in accordance with Altindag et al. [67], who suggested that the increased collagen fibers occur due to decreased collagen metabolism that may be related with oxidative stress.

Also, our histopathological results were confirmed the toxic effects of tramadol on the kidney after administration of tramadol for successive twenty days, in the form of degenerated renal tubules to obstructed lumen and atrophied glomerulus with collapsed tuft and wide Bowman's space. Our results are in agreement with the findings of Atici et al. [68], who observed renal tubular vacuolization, mononuclear cell infiltration, focal necrosis and hemorrhage as well as an increase in creatinine levels in rats receiving opioids. These observations can be considered as evidence of renal damage. Similar results were reported by Elkhateeb et al. [57], who found atrophied glomerulus with collapsed tuft, wide Bowman's space, degenerated tubules, cellular infiltration and hemorrhage in tramadol treated group. Elmanama et al. [66] suggests that the long-term use of tramadol has negative impacts on kidney functionality. Others studies reached similar results [62] [69] [70]. Regarding the present histochemical results, we found considerable depletion in the amount of glycogen granules in both hepatic and renal tissues. In addition, we found remarkable increase in the amount of the total protein in the hepatic tissues. Our results may be a secondary event following tramadol-induced lipid peroxidation of hepatic and renal tissues. Lipid peroxidation of cell membranes leads to loss of membrane fluidity, changes in membrane potential and an increase in membrane permeability, all of which lead to alteration of the chemical compound of the cells [22].

The expression oxidative stress is used to describe various deleterious processes resulting from an imbalance between the excessive formation of ROS and limited antioxidant defenses [71]. The effects of ROS are wide-ranging, but three reactions are particularly relevant to cell injuries which are lipid peroxidation of membranes, oxidative modification of proteins and lesions in DNA [72]. Recent studies have demonstrated that ROS and the resulting oxidative stress play a pivotal role in apoptosis [73]. However, there are conflicting results in the literature concerning the effects of opioids on apoptosis. *In vitro* studies using specific cell lines showed that opioids might induce or enhance apoptosis [74]. In addition, tramadol hydrochloride and/or its active metabolite may produce an excessive release of ROS leading to single- or double-strand DNA breaks [75]. Apoptosis provides a mechanism for the disposal of cells damaged by toxicants without perturbing the homeostatic balance of their environment. However, the importance of apoptosis in toxicology has been underestimated given the difficulty of identifying apoptotic cells either *in vivo* or *in vitro* models [76].

Many families of proteins are involved in the regulation of programmed cell death [77]. Bcl-2 is the most important family among them. Under normal conditions, Bax is present in a monomeric form in the cytosol or loosely attached to membranes [30]. Unlike Bax, Bcl-2 is mainly localized as an integral mitochondrial membrane protein, and forms heterodimers with Bax to prevent mitochondrial changes in apoptosis [78]. Hence, dysregulation between pro- and anti-apoptotic proteins may initiate or inhibit the development of cellular apoptosis. Prolonged morphine administration resulted in an up-regulation of the proapoptotic Bax protein besides a downregulation of the antiapoptotic Bcl-2 protein [79]. This intracellular imbalance in Bax/Bcl-2 ratio accelerated apoptosis of lymphocytes by morphine with subsequent activation of caspase-3 [80]. Run in agreement with previous studies, our data recorded a significantly increased in the expressions level of proapoptotic Bax, while, a conversely to the antiapoptotic Bcl-2 protein expression which was significantly decrease in the liver and kidney tissues of tramadol-treated group as compared to control. Khodeary et al. [81] reported the upregulation of Bax besides down-regulation of Bcl-2 in rat neurocytes were a significantly pronounced in tramadol hydrochloride treated group with high dose than low dose indicating dose-dependent effects of tramadol. Run in agreement with previous studies, the upregulation of Bax besides down-regulation of Bcl-2 in rats liver and kidney suggested that tramadol-induced damage in a manner of activation of apoptotic cell death pathway in these tissues.

V. Conclusion

In conclusion, tramadol treatment in rats induced histopathological alterations and changes in apoptotic genes expression in a manner reveal tramadol toxicity to hepatorenal tissues. Therefore, the toxic effects of opioids should be kept in mind, even though, the important role tramadol plays as a powerful pain management.

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