The effects of tramadol on hepatic ischemia/reperfusion injury in rats

Mona F. Mahmoud, Samar Gamal, Mohamed A. Shaheen, Hassan M. El-Fayoumi

ABSTRACT

Objectives: Tramadol is a centrally acting synthetic analgesic. It has a cardioprotective effect against myocardial ischemia-reperfusion (I/R) injury in isolated rat heart. We hypothesized that tramadol may exert a similar protective effect on hepatic I/R injury. Hence, the current investigation was designed to study the possible protective effects of tramadol on experimentally-induced hepatic I/R injury in rats.

Materials and Methods: Tramadol was administered 30 min before ischemia following which the rats were subjected to 45 min of ischemia followed by 1 h of reperfusion.

Results: Tramadol attenuated hepatic injury induced by I/R as evidenced by the reduction of transaminases, structural changes, and apoptotic cell death. It decreased the level of inflammatory markers such as tumor necrosis factor-alpha (TNF-α), TNF-α/interleukin-10 (IL-10) ratio, and nuclear factor-kB gene expression. It also increased the anti-inflammatory cytokine, IL-10 levels in hepatic tissues. Furthermore, it reduced oxidative stress parameters except manganese superoxide dismutase activity.

Conclusion: The results suggest that tramadol has hepatoprotective effects against hepatic I/R injury via anti-inflammatory, antiapoptotic, and antioxidant effects.

KEY WORDS: Angioedema, anti-histamines, chronic urticaria, histaglobulin, urticaria activity score

Introduction

There are three main classes of opioid receptors, μ, δ, and κ. Previous reports showed that preventive administration of the selective μ-opioid receptor (MOR) agonist DAMGO stimulated the in vivo signaling pathways involved in hepatoprotection.[1] It was reported previously that opioid receptors have the capacity to protect different organs from hypoxia- or ischemia-induced injury.[2] Interestingly, they may be implicated in protecting the liver against ischemia-reperfusion (I/R) injury.[3] MOR plays a key role in the prevention of acute hepatic inflammation and cell death both in vivo and in vitro. It was also reported that MOR gene expression was increased in acute liver injury. Previous studies revealed that remifentanil pretreatment can reduce in vivo and in vitro hepatic injury via antioxidant and anti-inflammatory effects.[4] Another study showed that morphine administration protects against hepatic I/R injury via stimulation of opioid receptors, phosphatidylinositol-3-kinase, and Akt pathway.[5] Therefore, the administration of exogenous MOR agonist may attenuate the severity of I/R injury of the liver.

Tramadol is synthetic codeine analog. Its analgesic activity is mediated centrally. Tramadol has high oral bioavailability (70–80%). The antinociceptive effect of tramadol is due to both parent compound and its metabolite (O-desmethylated tramadol) and this metabolite is 4–6 times more potent than the tramadol itself.[6] Both tramadol and its metabolites are mainly excreted through the kidney. It is as effective as meperidine in the reduction of pain associated with labor.[7] Tramadol acts by two mechanisms. The first mechanism involved MOR as it is weak μ receptor agonist. The second...
mechanism involved the inhibition of serotonin and norepinephrine (NE) reuptake. In a previous study, tramadol protects the heart against I/R injury induced in isolated rat heart. The cardioprotective effect of tramadol was mediated through antioxidant activity and the inhibition of NE uptake. Previous studies also showed that tramadol activates nitric oxide synthase-guanulate cyclase pathway and increased NO production which mediates vasodilatation in rabbit aorta. NO reduces the interactions between neutrophils and endothelium which are essential for neutrophil accumulation at sites of inflammation in ischemic condition. Furthermore, tramadol also protects neurons against transient ischemia in rats. From the previous studies, we hypothesized that tramadol may exert a similar protective effect in hepatic I/R injury. Hence, the current investigation was designed to study the possible protective effects of tramadol on experimentally-induced hepatic I/R injury in rats and the underlying mechanism(s).

Materials and Methods

Experimental Design

Animals were randomly assigned into three experimental groups (eight animals for each group) as following: Sham + saline, I/R injury + saline, and I/R + Tramadol (50 mg/kg). Tramadol was dissolved in saline then given i.p. as a single dose, 30 min before ischemia. Blood was obtained from the retro-orbital plexus then centrifuged (3000 × g, 4°C, 20 min) for separation of serum. Serum was used to analyze liver transaminases and lactate dehydrogenase (LDH) enzyme activities. Thereafter, animals were euthanized, livers were isolated then washed twice with ice-cold saline; livers were dissected into two parts; one part was immediately immersed in liquid nitrogen and kept at −80°C for measurement of tissue parameters and the other part was kept in 10% formalin for histopathological examination.

Animals

Adult male Wistar rats, weighing 200 ± 20 g, were used in the current study. The animals were housed at constant environmental condition (25°C ± 2°C with 12-h light/dark cycle). They were fed standard chow diet and water ad libitum.

The animals were handled following the International Animal Ethics Guidelines, and the experimental procedures were approved by the Institutional Animal Ethics Committee of the Faculty of Pharmacy, Zagazig University (approval number P2-6, 2012).

Induction of Ischemia Reperfusion Injury

Rats were anesthetized by i.p. injection of ketamine (75 mg/kg). Ischemia was induced by occluding hepatic portal vein, hepatic artery, and bile duct to both the left and median lobes with a traumatic vascular clamp for 45 min then removed to start reperfusion for 1 h. I/R group subjected to partial liver ischemia (70%) followed by reperfusion. This method of partial hepatic ischemia allows for portal decompression through right and caudate lobes and so prevents mesenteric venous congestion. Sham control rats underwent the same protocol without vascular occlusion.

Drugs and Chemicals

Chemicals were purchased from El-Gomhoria Company (Cairo, Egypt) except tramadol that was obtained from October Pharm company, Egypt.

Biochemical Analysis

The serum alanine aminotransferase (ALT), aspartate aminotransferase (AST), LDH, and gamma glutamyl transferase (GGT) enzyme activities were measured using commercially available analytical kit provided by Biodiagnostic Co., Egypt.

Determination of Oxidative Stress

Oxidative stress was determined in liver tissue homogenates by measuring of malondialdehyde (MDA), glutathione peroxidase (GPx), and manganese superoxide dismutase (Mn-SOD) activity. GPx and Mn-SOD were measured photometrically (spectrophotometer, Jenway®, England, UK) using commercial kits purchased from Bio-diagnostic Co., Egypt. MDA levels were determined by the thiobarbituric acid method.

Determination of Cytokines

The level of tumor necrosis factor-alpha (TNF-α) and interleukin-10 (IL-10) in liver homogenate was detected by quantitative ELISA using ELISA kit (Quantikine, USA) and (Bio Vendor, Germany), respectively. Then, TNF-α/IL-10 ratio was calculated.

Determination of Nuclear Factor-κB Gene Expression by Quantitative Real-time Polymerase Chain Reaction

Target gene expression was assessed and related to a reference gene, β-actin. The primer sequence for nuclear factor-κB (NF-κB) was F: 5′-GTATAGGCTTAGCTTGCAGC-3′, R: 5′-TGATAAGGCTTAGCTTGCAGC-3′, and for β-actin was F: 5′-AGAACATCATCCGTGATCC-3′ R: 5′-TCACCAGCCGTGATCCGTA-3′.

Determination of Caspase-3 Activity as an Indicator of Apoptosis

Caspase-3 activity was quantified by proteolytic cleavage of the fluorogenic substrate 7-amino-4-trifluoromethylcoumarin-conjugated Asp-Glu-Val-Asp tetrapeptide (AMC-DEVDA) using a caspase-3 fluorimetric kit (Sigma Co., USA) according to the manufacturer’s instructions.

Histopathological Study

Liver tissues from rats were fixed in 10% buffered formalin. After an overnight wash, specimens were dehydrated in graded ethanol, cleared in xylene, and paraffin-embedded. Sections 5–6 µm in thickness were obtained according to routine procedures, mounted on silane-coated slides, and stored at room temperature. Slides were dewaxed in xylene, hydrated using graded ethanol, and stained for routine histological evaluation by hematoxylin and eosin. The sections were observed with a Zeiss Axiosplan light microscope (Zeiss, Berlin, Germany) and photographed with a digital camera (Canon, Hong Kong, Japan). In addition, liver sections were evaluated for apoptotic cells. Cells that showed morphological features of apoptosis (cell shrinkage, chromatin margination, and apoptotic bodies) were counted in 15 high power fields per section analyses.

Statistical Analysis

Data are expressed as a mean ± standard error of the mean. Statistical analysis was performed by using one-way analysis of variance followed by Tukey post hoc test using a computer.
Figure I/R + tramadol 2.5±0.18

Figure I/R + saline

Figure 189.7*±1.6

n 2 showed that I/R increased hepatic cell death by 125±1.6

Figure α 4. There was marked reduction in anti-inflammatory

Figure 9.6*±0.19

3b] by 16% and 38%, respectively,

140 κ 2a, cytolytic marker, by 159% when compared to the

2 illustrates that animals subjected to I/R showed a

Figure 2b. Tramadol was able to

participate in I/R injury‑derived inflammation in various tissues

in liver apoptosis score [Figure 1c]. The number of apoptotic hepatocytes was significantly decreased in tramadol treated group compared with I/R group.

Effect of Tramadol on Liver Cell Death

Figure 2 showed that I/R increased hepatic cell death by apoptosis.

It was associated with marked increase in LDH activity [Figure 2a], cytolitic marker, by 159% when compared to the sham group (P < 0.05). Furthermore, I/R caused apoptotic cell death as evidenced by the elevation of caspase-3 activity; an enzyme involved in apoptotic cell death and the increase in liver apoptosis score [Figure 2b]. Tramadol was able to decrease only apoptotic cell death without affecting the cytolitic marker [Figure 2b]. This was manifested by the reduction of the enzyme activity of caspase-3 in tramadol treated group when compared with sham group (P < 0.05).

However, tramadol did not affect LDH enzyme activity when compared to I/R group (P > 0.05).

Effect of Tramadol on Oxidative Stress

I/R induced a marked increase in oxidative stress as evidenced by marked reduction in mitochondrial Mn‑SOD [Figure 3a] and GPx [Figure 3b] by 16% and 38%, respectively, when compared with sham group. Tramadol treatment caused marked elevation in GPx content by 190% when compared with I/R group but there is no significant effect on Mn‑SOD level when compared with I/R group. I/R group showed marked increase in liver MDA level compared to sham group. Tramadol treated group showed a significant reduction in liver MDA level as compared with I/R group [Figure 3c].

Effect of Tramadol on Cytokines

Table 2 illustrates that animals subjected to I/R showed a marked increase in inflammatory cytokine (TNF-α) by 324%. The gene expression of NF‑κB was also elevated by 923% [Figure 4]. There was marked reduction in anti-inflammatory cytokine (IL‑10) by 53% in liver tissue as compared to sham group. TNF‑α/IL‑10 was significantly increased in I/R group when compared to sham group (P < 0.05). Tramadol treatment significantly reduced liver TNF‑α by 30%, gene expression of NF‑κB by 43%, and increased liver IL‑10 by 138% as compared to I/R group. It also reduced TNF‑α/IL‑10 ratio.

Discussion

Recently, studies showed that opioid receptors may participate in I/R injury‑derived inflammation in various tissues including liver. Some of these previous studies showed that

![Image](315x71 to 558x263)

Figure 1: Photomicrographs are representative of cross sections from six rats stained with hematoxylin and eosin. The sham group (a) showed normal hepatocytes arranged in branching plates radiating from the central vein. The nuclei were central and rounded and the cytoplasm was granular and acidophilic. Normal sinusoidal space and perportal area were also observed. No inflammatory activity could be seen. Ischemia/reperfusion operated rats (b) presented marked congestion (asterisk) and cellular infiltrates (arrow heads). Treatment with tramadol in group (c) showed less congestion, hepatocytes-loss, and cellular infiltrates. Bar graph showing liver apoptosis scores, (n = 10) *P < 0.05 (significantly different from Sham group); @P < 0.05 (significantly different from Ischemia/reperfusion group) (a-c) ×20

| Parameters | Sham + saline | I/R + saline | I/R + tramadol |
|------------|---------------|--------------|---------------|
| ALT (U/L)  | 60±0.88       | 189.7±1.6    | 140±3.4       |
| AST (U/L)  | 125±1.6       | 197±1.2      | 180±1.7       |
| GGT (U/L)  | 2.5±0.18      | 9.6±0.19     | 7±0.14        |

Results are presented as a means±SE (n=6-8) *P<0.05 (significantly different from Sham group), †P<0.05 (significantly different from I/R group) by one-way ANOVA and Tukey’s post hoc test. ALT=Alanine aminotransferase, AST=Aspartate aminotransferase, GGT=Gamma glutamyl transferase, I/R=Ischemia/reperfusion, ANOVA=Analysis of variance, SE=Standard error

Table 1:

Effect of ischemia/reperfusion-induced liver injury and i.p. administration of tramadol (50 mg/kg, single dose) on liver enzymes (serum alanine aminotransferase, aspartate aminotransferase, and gamma-glutamyl transferase activities)
opiod receptors have been involved in the protection against organ damage resulting from either hypoxic or ischemic events. The aim of the present study was to evaluate the effect of tramadol, as opioid receptor agonist, on hepatic I/R-induced injury and the underlying mechanism.

Our findings point to the potential protective effect of tramadol against I/R injury and the underlying mechanism. The present study showed that 45 min of ischemia followed by 1 h reperfusion was associated with a liver injury with higher concentrations of serum aminotransferases, inflammatory cytokines, and hepatic lipid peroxidation, as well as histologic changes, and apoptosis. We found that I/R was associated with increased oxidative stress. Reoxygenation of hypoxic liver tissue during reperfusion is responsible for reactive oxygen species (ROS) formation. ROS are produced from the activated Kupffer

Table 2:
Effect of ischemia/reperfusion-induced liver injury and i.p. administration of tramadol (50 mg/kg, single dose) on inflammatory cytokines (tumor necrosis factor-alpha), anti-inflammatory mediator (interleukin-10) in liver tissue

| Parameters                  | Sham + saline | I/R + saline | I/R + tramadol |
|-----------------------------|---------------|--------------|---------------|
| TNF-α (pg/mg)               | 29±1.8        | 94±1.4       | 55±2.9        |
| IL-10 (pg/mg)               | 131±3.3       | 70±1.1       | 97±2.9        |
| TNF-α/IL-10 ratio           | 0.22±0.54     | 1.3±1.2      | 0.56±1        |

Results are presented as a mean±SE (n=6-8) *P<0.05 (significantly different from Sham group); #P<0.05 (significantly different from I/R group) by one-way ANOVA and Tukey’s post hoc test. SE=Standard error; ANOVA=Analysis of variance, I/R=Ischemia/reperfusion, TNF-α=Tumor necrosis factor-alpha, IL-10=Interleukin-10.
endothelial cells, and infiltrating neutrophils. The formation of ROS may initiate oxidative stress which can lead to lipid peroxidation of hepatocytes membrane lipids. They cause the formation of lipid peroxidation product, MDA. Both GPx and Mn-SOD are consumed in quenching of these ROS. Thus, their activity was reduced following I/R as shown in the present study. Removal of oxidative stress is the primary intervention to decrease tissue injury. Superoxide dismutase, catalase, and GPx are known endogenous antioxidants, but none of these endogenous antioxidants are sufficient to compensate for oxidative stress.

Several antioxidants have been studied and reported to decrease oxidative stress in hepatic and renal tissue in experimental I/R models. The current study showed that tramadol may provide protection to the liver during I/R injury by improving activities of the endogenous antioxidant enzymes, which scavenge ROS and reduce their effects. Tramadol reduced hepatic MDA content in the liver and increased GPx activity compared to I/R group indicating a reduction of the oxidative stress in liver homogenates. This was confirmed by previous reports of Bilir et al. However, tramadol could not restore Mn-SOD activity. These protective effects could be related to the antioxidant properties of the molecule which has been shown to be able to scavenge ROS. It was shown that tramadol has antioxidant activity in vitro model system.

I/R injury has two phases: An early phase and late phase. In our study, we investigated only the early phase of liver reperfusion injury. Kupffer cells are stimulated during this phase, resulting in increased production of pro-inflammatory cytokines mainly, TNF-α. Recent studies showed that hepatocytes can also produce TNF-α following liver I/R. ROS produced during the early phase of I/R injury may up-regulate nuclear transcription factors like NF-κB and subsequently release TNF-α and IL-1. Our previous study showed that not only the expression of TNF-α was increased but also the expression of TNF-α type-1 receptors. It was suggested that TNF-α is produced just after the beginning of reperfusion. TNF-α binds to TNF-α type-1 receptors and induces inhibitory κB (IkB) serine phosphorylation and releases NF-κB. NF-κB is a nuclear transcription factor which is located in the cytoplasm. Under common conditions, NF-κB is a heterodimer, and is bound to the inhibitory unit IkB. When cells are exposed to stressful conditions such as I/R, IkB is phosphorylated by its kinase IkB kinase complex. Then, NF-κB translocates to the nucleus, where it is released from the IkB complex. NF-κB has been shown to play an important role where it increased the production of both pro-inflammatory cytokines (TNF-α, IL-1β, etc.), chemokines (IL-8, MIP-1α, and MCP-1), and adhesion molecules (ICAM, VCAM, and E-selectin) with activation of Kupffer cells and recruitment and sequestration of activated neutrophils to the liver that damages the cells. Our study showed that increased NF-κB gene expression and its subsequent translocation may aggravate hepatic damage by producing TNF-α and reducing the level of anti-inflammatory cytokines like IL-10 following reperfusion of ischemic liver. The increased production of TNF-α was also associated with increased leukocyte infiltration during I/R injury which further secretes more TNF-α and increases the extent of the damage.

Results of the present study showed that tramadol reduces NF-κB gene expression, TNF-α production, and leukocyte infiltration in I/R induced liver injury. These results are confirmed by previous studies. This anti-inflammatory effect may be responsible for the hepatoprotective effect of tramadol. Previous reports suggest that tramadol protects against myocardial and brain I/R injury via anti-inflammatory effect. Tramadol not only reduced hepatic TNF-α production but also reduced TNF-α/IL-10 ratio and elevated IL-10 level.

IL-10 suppresses the NF-κB signaling resulting in promotion of hepatocytes survival. It was reported previously that viral IL-10 gene transfer prevented hepatic I/R injury in wild-type recipients. Furthermore, it was reported that neutralization of IL-10 rendered the liver more susceptible to proinflammatory cytokines induced damage. Thus, by increasing IL-10 hepatic production, tramadol may protect the liver against I/R induced damage.

TNF-α and other inflammatory mediators formed during reperfusion are known to activate proteins implicated in apoptosis, like caspase-3. This resulted in DNA destruction and apoptotic cell death. However, some studies revealed that necrosis is the principle form of cell death. Our results showed that apoptosis is the main type of cell death that takes place during hepatic I/R injury. This was manifested in our study by the increased caspase-3 activity, the marker of apoptosis and increased number of apoptotic cells in the liver tissues. Surprisingly, our results showed that tramadol was able to suppress the increased caspase-3 activity and the subsequent apoptotic liver cell death and reduced the number of apoptotic cells.

Our results also showed marked elevation of serum ALT, AST, and GGT enzyme activities following I/R. ALT is a specific marker for hepatic parenchymal injury and AST is a nonspecific marker for hepatic injury. Tramadol reduced liver enzyme activity such as ALT, AST, and GGT when compared with I/R group. This may be attributed to the reduction of both inflammation and oxidative stress following tramadol administration.

The effect of administration of tramadol on normal rats in the absence of I/R was examined to determine whether if any
of the exhibited pharmacological effects of tramadol was due to toxic effects of the drug. The present study showed that intraperitoneal injection of tramadol (50 mg/kg) to normal rats did not affect any of the measured parameters compared to the sham group thus the pharmacological effects exhibited by tramadol are not due to the toxic effects of the selected dose of the drug.

**Conclusion**

This study is the first to evaluate the hepatoprotective effects of tramadol on hepatic I/R. The protective effects of tramadol may be related to its antioxidant and anti-inflammatory effect. These results suggest that tramadol has a beneficial role in the protection of liver from I/R injury during liver transplantation and hepatic resection.

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Nil.

**Conflicts of Interest**

There are no conflicts of interest.

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