



Pulmonary, Gastrointestinal and Urogenital Pharmacology

Therapeutic potential of cannabidiol against ischemia/reperfusion liver injury in rats

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ABSTRACT

The therapeutic potential of cannabidiol, the major non-psychoactive *Cannabis* constituent, was investigated in rats exposed to ischemia/reperfusion liver injury. Ischemia was induced by clamping the pedicle of the left hepatic lobe for 30 min, and cannabidiol (5 mg/kg, i.v.) was given 1 h following the procedure and every 24 h thereafter for 2 days. Ischemia/reperfusion caused significant elevations of serum alanine aminotransferase and hepatic malondialdehyde, tumor necrosis factor- α and nitric oxide levels, associated with significant decrease in hepatic reduced glutathione. Cannabidiol significantly attenuated the deterioration in the measured biochemical parameters mediated by ischemia/reperfusion. Histopathological examination showed that cannabidiol ameliorated ischemia/reperfusion-induced liver damage. Immunohistochemical analysis revealed that cannabidiol significantly reduced the expression of inducible nitric oxide synthase, cyclooxygenase-2, nuclear factor- κ B, Fas ligand and caspase-3, and increased the expression of survivin protein in ischemic/reperfused liver tissue. These results emphasize that cannabidiol represents a potential therapeutic option to protect the liver against hypoxia–reoxygenation injury.

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1. Introduction

Ischemia/reperfusion injury is a major determinant in many clinical conditions such as liver resection and liver transplantation. Hepatic pedicle clamping (Pringle's maneuver) is often used during liver surgery to reduce intraoperative bleeding. However, the resulting ischemia/reperfusion causes liver failure and contributes to high postoperative morbidity and mortality (Belghiti et al., 1999; Serracino-Inglott et al., 2001). Tissue injury occurs during the ischemic phase and much injury arises upon restoring the blood supply (reperfusion phase). Reperfusion of ischemic hepatic tissue initiates complex cellular events leading eventually to necrosis and apoptosis of liver cells (Kim et al., 2004; Nagai et al., 2007). Several lines of evidence indicate that oxidative stress with increased free radical generation and intense inflammatory reaction with increased production of proinflammatory cytokines play a crucial role in the pathogenesis of ischemia/reperfusion liver injury. Also, previous studies demonstrated that antioxidants and anti-inflammatory agents effectively protected against liver damage induced by ischemia/reperfusion (Kim et al., 2010; Subhas et al., 2010; Zhang et al., 2006).

Cannabidiol is the major non-psychoactive cannabinoid component derived from the plant *Cannabis sativa*. It possesses powerful antioxidant and anti-inflammatory activities. However, the exact mechanisms of action of cannabidiol remain obscure. In contrast to the other cannabinoids, cannabidiol is known to have a very low affinity for the cannabinoid CB₁ and CB₂ receptors. The antioxidant and anti-inflammatory effects of cannabidiol may be due to its direct action or mediated through a new abnormal cannabinoid, non-CB₁ and non-CB₂, receptor (Begg et al., 2005; De Petrocellis and Di Marzo, 2010). Cannabidiol may also exert its beneficial effects by inhibiting adenosine uptake and activating transient receptor potential vanilloid-1 (Bisogno et al., 2001; Carrier et al., 2006). Previous reports proved that cannabidiol may have therapeutic utility in a number of conditions involving inflammation and oxidative stress, including diabetes mellitus, rheumatoid arthritis and neurodegenerative disorders (Blake et al., 2006; Iuvone et al., 2009; Rajesh et al., 2010). The protective effect of cannabidiol was also demonstrated in animal models with cerebral and myocardial ischemia/reperfusion by attenuating the oxidative stress and inflammatory response (Alvarez et al., 2008; Durst et al., 2007; Hayakawa et al., 2009; Walsh et al., 2010). A recent study revealed that cannabidiol pretreatment significantly protected against liver ischemia for 60 min followed by reperfusion for 24 h (Mukhopadhyay et al., 2011).

Therefore, the present study was conducted to evaluate the therapeutic effect of cannabidiol given to rats 1 h after being exposed to liver ischemia, and every 24 h thereafter for 2 days. Also, the possible mechanisms underlying this therapeutic effect were investigated.

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2. Materials and methods

2.1. Animals

Male Sprague–Dawley rats, weighing 200 ± 10 g were obtained from the Animal House, College of Medicine, King Faisal University. The animals were kept at standard housing facilities (24 ± 1 °C, $45 \pm 5\%$ humidity and 12 h light/dark cycle). They were supplied with standard laboratory chow and water ad libitum, and left to acclimatize for 1 week before the experiments. The experimental protocol was approved by the Local Animal Care Committee and the experimental procedures were carried out in accordance with international guidelines for care and use of laboratory animals.

2.2. Drugs

Cannabidiol powder (Cayman Chemical Company, USA) was dissolved in a vehicle solution of 1% Tween 80 and 2% DMSO in saline. The dose of cannabidiol used in this study was selected based on our preliminary experiments and in accordance with previous reports (Durst et al., 2007).

2.3. Experimental design

The rats were randomly divided into four equal groups ($n = 10$, each). The first group was sham-operated and served as control. Liver ischemia/reperfusion was induced in animals of the second and third groups. The second and third group animals received the vehicle of cannabidiol or cannabidiol at a dose of 5 mg/kg, i.v. (through the tail vein), respectively, 1 h following the procedure and every 24 h thereafter for two consecutive days. The rats of the fourth group were sham-operated and then received cannabidiol as mentioned before.

2.4. Surgical procedure

The surgical technique was followed as described previously by Kooij et al. (1994). Following anesthesia with thiopental (40 mg/kg, i.p.), a mid-line abdominal incision was performed and the liver hilum was gently exposed. A fine nontraumatic vascular clip was applied at the pedicle supplying the left lobe of the liver. This allowed for selective interruption of the blood supply of the left hepatic lobe, while preserving that of the right lobe. Thus, gastrointestinal congestion and hemodynamic instability accompanying complete occlusion of the hepatic pedicle were avoided. Warm saline was injected in the abdomen which was temporarily closed. The ischemic phase persisted for 30 min, then the clip was removed and the abdominal wall was sutured to start a 72 h-reperfusion phase. For the sham-operated groups, the same procedures were done except for clamping the vascular pedicle of the left hepatic lobe.

2.5. Sample preparation and biochemical studies

The animals were euthanized 24 h following the last dose of cannabidiol. Blood samples were collected, left for 60 min to clot, and then centrifuged for 10 min at 2430 g to obtain clear sera which were stored at -20 °C. Subsequently, serum level of alanine aminotransferase was measured using colorimetric assay kit according to the recommendations of the manufacturer (Randox Laboratories Ltd., UK).

The left hepatic lobe was removed, washed with ice-cold saline and kept at -80 °C and subsequently homogenized in cold potassium phosphate buffer (0.05 M, pH 7.4). The homogenates were centrifuged at 2430 g for 10 min at 4 °C. The resulting supernatant was used for determination of malondialdehyde and reduced glutathione levels using colorimetric assay kits according to the manufacturer's instructions (Biodiagnostic, Egypt). The level of nitric oxide was assayed using

colorimetric assay kit as indicated by the manufacturer (Cayman Chemical Company, USA). Also, the level of tumor necrosis factor- α in liver homogenates was determined by enzyme-linked immunosorbent assay (ELISA) using rat tumor necrosis factor- α immunoassay kit according to the recommendations of the manufacturer (R&D Systems, USA).

2.6. Histopathological examination

Parts of left hepatic lobe obtained from each animal were fixed in 10% formalin solution, dehydrated in ascending grades of alcohol and embedded in paraffin. Sections at 4 μ m-thickness were taken, stained with hematoxylin and eosin (H&E) and examined under light microscope by a pathologist unaware of the treatment protocol.

2.7. Immunohistochemical examinations

Four μ m thick sections were deparaffinized, rehydrated, and endogenous peroxidase activity was blocked with H_2O_2 in methanol. Sections were pre-treated in citrate buffer (pH 6.0) in a microwave. Sections were incubated at room temperature with rabbit monoclonal anti-inducible nitric oxide synthase, anticyclooxygenase-2, anti-nuclear factor- κ B, anti-Fas ligand, anti-survivin and anticaspase-3 antibodies (Thermo Scientific, USA, dilution 1:1000). UltraVision detection System (Thermo Scientific) was used as follows; sections were incubated with biotinylated goat anti-polyvalent, then with streptavidin peroxidase and finally with diaminobenzidine plus chromogen. Slides were counterstained with hematoxylin, visualized under light microscope and the extent of cell immunopositivity was assessed. The number of immunopositive cells was counted in 30 separate microscopic fields and the mean was calculated for each group. The same procedures were repeated using normal rabbit serum instead of the primary antibody to obtain negative control and indicate the specificity of the antibody.

2.8. Statistical analysis

All values are expressed as mean \pm S.E.M. The results were analyzed by one-way analysis of variance (ANOVA) followed by Tukey test for multiple comparisons using SPSS for Windows (version 11). Differences were considered significant at $P < 0.05$.

3. Results

3.1. Effects of cannabidiol on serum alanine aminotransferase

Significant elevation of serum alanine aminotransferase level was observed in rats exposed to ischemia/reperfusion liver injury as compared to the sham-operated animals. Cannabidiol treatment resulted in significant reduction in the serum level of alanine aminotransferase (Table 1).

3.2. Effects of cannabidiol on liver biochemical analysis

Cannabidiol significantly suppressed hepatic lipid peroxidation and prevented the depletion of reduced glutathione level resulted from ischemia/reperfusion. This was accompanied by significant reductions of ischemia/reperfusion-induced elevations of tumor necrosis factor- α and nitric oxide in liver homogenates (Table 1).

3.3. Effects of cannabidiol on liver histopathology

Ischemia/reperfusion caused marked liver damage in the form of ballooning degeneration and cytoplasmic vacuolation of hepatocytes with sinusoidal dilatation and congestion. Cannabidiol treatment markedly attenuated the ischemia/reperfusion-induced liver tissue

Table 1
Effects of cannabidiol treatment on serum alanine aminotransferase, and hepatic levels of malondialdehyde, reduced glutathione, tumor necrosis factor- α and nitric oxide in rats exposed to ischemia/reperfusion (IR) liver injury.

	Sham	Vehicle + IR	Cannabidiol + IR	Cannabidiol + sham
Alanine aminotransferase (U/L)	64.12 \pm 5.61	483.28 \pm 35.74 ^a	174.25 \pm 11.93 ^{a,b}	77.87 \pm 4.85
Malondialdehyde (nmol/g tissue)	81.62 \pm 5.74	209.55 \pm 11.48 ^a	111.06 \pm 8.41 ^b	73.15 \pm 6.28
Reduced glutathione (mmol/g tissue)	5.91 \pm 0.41	1.18 \pm 0.09 ^a	4.79 \pm 0.37 ^b	4.88 \pm 0.27
Tumor necrosis factor- α (pg/100 mg tissue)	17.49 \pm 1.12	92.25 \pm 6.83 ^a	32.17 \pm 2.91 ^{a,b}	13.85 \pm 1.28
Nitric oxide (nmol/100 mg tissue)	99.48 \pm 7.62	187.33 \pm 13.94 ^a	131.28 \pm 9.83 ^b	86.81 \pm 6.26

All the values are expressed as mean \pm S.E.M., n = 10 in each group.

^a $P < 0.05$ versus sham-operated (sham) group.

^b $P < 0.05$ versus vehicle + IR group.

injury and restored the same histopathological picture observed with the sham-operated group (Fig. 1).

3.4. Effects of cannabidiol on liver immunohistochemistry

Ischemia/reperfusion resulted in significant increases in the immunoreactivity of inducible nitric oxide synthase, cyclooxygenase-2, nuclear factor- κ B, Fas ligand, survivin and caspase-3 in the cytoplasm of the hepatocytes as compared to the sham-operated group. However, cannabidiol-treated rats showed significant reductions in the expression of inducible nitric oxide synthase, cyclooxygenase-2, nuclear factor- κ B, Fas ligand and caspase-3, associated with more significant increase in the expression of survivin protein in the liver tissue as compared to the ischemia/reperfusion, cannabidiol non-treated group (Figs. 2–7, and Table 2).

4. Discussion

The present study showed that post-ischemic cannabidiol treatment effectively protected against liver tissue damage mediated by ischemia/reperfusion in rats. Also, the present work, in agreement with previous studies, confirmed the important role of increased lipid peroxidation, depletion of antioxidant defenses and increased production of proinflammatory cytokines in the pathogenesis of ischemia/reperfusion-induced liver injury (Kim et al., 2010; Subhas et al., 2010; Zhang et al., 2006). In addition, increased nitric oxide production in the liver tissue was reported to be involved in the pathogenesis of ischemia/reperfusion injury (Wang et al., 2009; Yao et al., 2009). This can be explained by the ability of tumor necrosis factor- α to up-regulate the inducible nitric oxide synthase enzyme (Morris and Billiar, 1994). Excess nitric oxide reacts with superoxide anion to generate peroxynitrite radical that causes cell

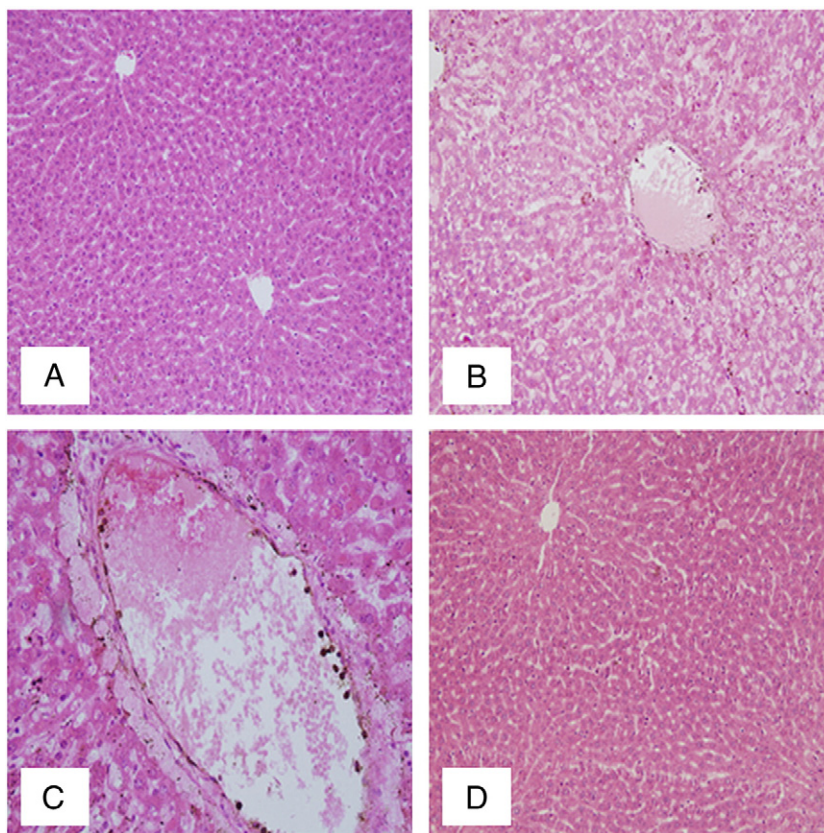


Fig. 1. Photomicrographs of rat liver (H&E) from: (A, 200 \times) sham-operated control group showing normal hepatic architecture; (B, 200 \times and C, 400 \times) ischemia/reperfusion group without cannabidiol treatment showing widespread necrosis, cytoplasmic vacuolization and ballooning degeneration of hepatocytes with dilated congested sinusoids; (D, 200 \times) ischemia/reperfusion cannabidiol-treated group showing a histological picture comparable to that of the control group.

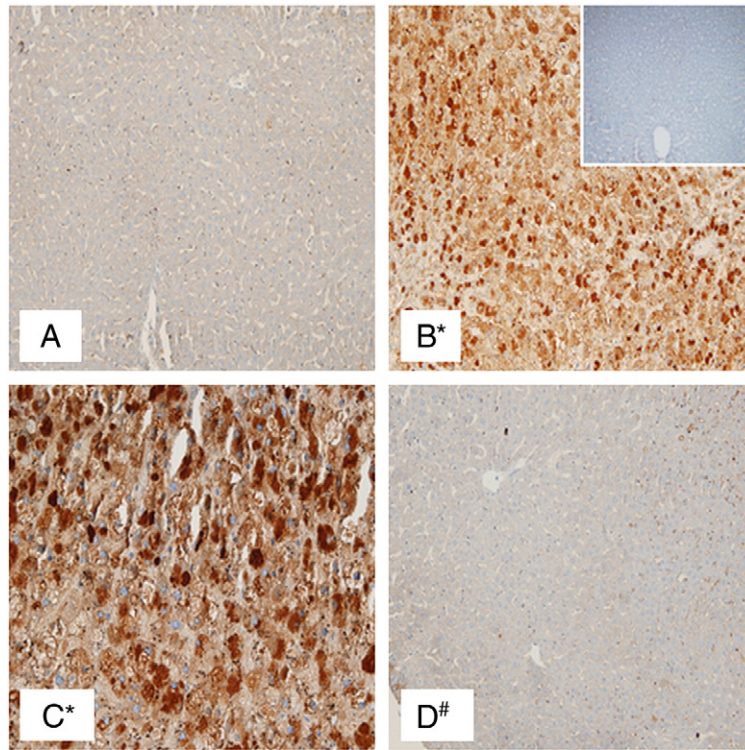


Fig. 2. Immunohistochemical staining of inducible nitric oxide synthase in rat liver from: (A, 200 \times) sham-operated control group showing no expression of inducible nitric oxide synthase; (B, 200 \times and C, 400 \times) ischemia/reperfusion group without cannabidiol treatment showing a significant increase in inducible nitric oxide synthase immunoreactivity in the cytoplasm of hepatocytes, (B, inserted figure, 200 \times) a negative control using normal rabbit serum instead of the primary antibody was included to indicate the specificity of the antibody; (D, 200 \times) ischemia/reperfusion cannabidiol-treated group showing a significant reduction in inducible nitric oxide synthase immunostaining. Brown color indicates inducible nitric oxide synthase positivity. Numerical data and statistical significance are shown in [Table 2](#).

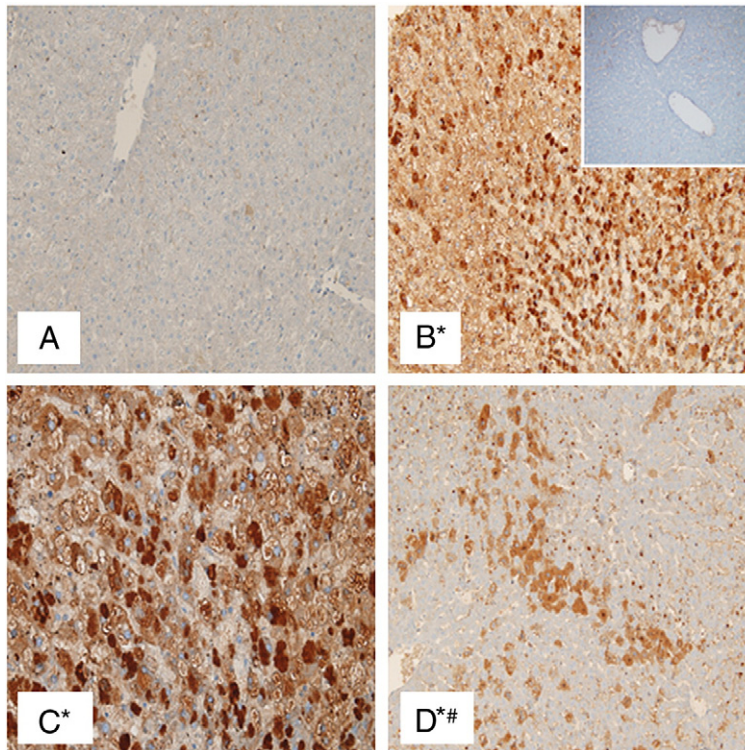


Fig. 3. Immunohistochemical staining of cyclooxygenase-2 in rat liver from: (A, 200 \times) sham-operated control group showing no expression of cyclooxygenase-2; (B, 200 \times and C, 400 \times) ischemia/reperfusion group without cannabidiol treatment showing a significant increase in cyclooxygenase-2 immunoreactivity in the cytoplasm of hepatocytes, (B, inserted figure, 200 \times) a negative control using normal rabbit serum instead of the primary antibody was included to indicate the specificity of the antibody; (D, 200 \times) ischemia/reperfusion cannabidiol-treated group showing a significant reduction in cyclooxygenase-2 immunostaining. Brown color indicates cyclooxygenase-2 positivity. Numerical data and statistical significance are shown in [Table 2](#).

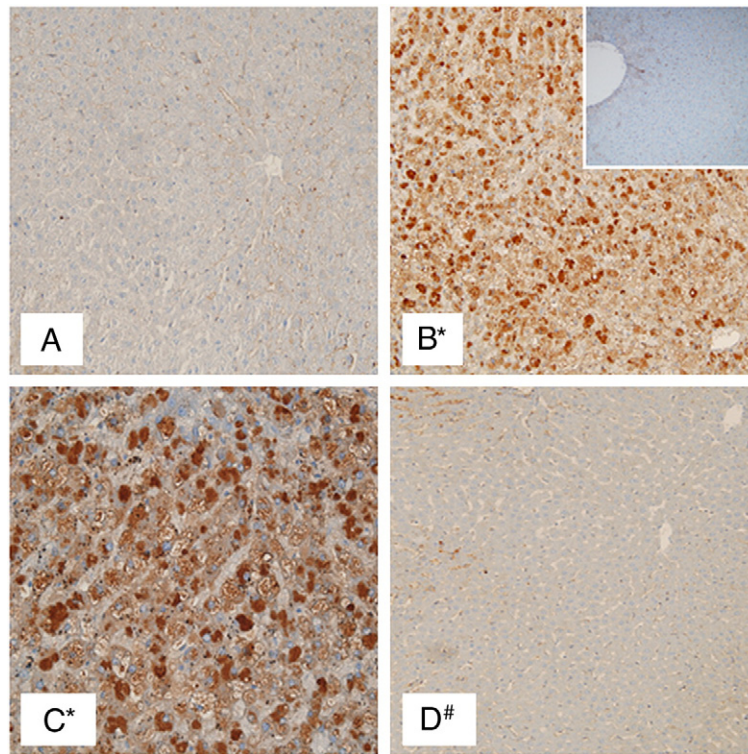


Fig. 4. Immunohistochemical staining of nuclear factor- κ B in rat liver from: (A, 200 \times) sham-operated control group showing no expression of nuclear factor- κ B; (B, 200 \times and C, 400 \times) ischemia/reperfusion group without cannabidiol treatment showing a significant increase in nuclear factor- κ B immunoreactivity in the cytoplasm of hepatocytes, (B, inserted figure, 200 \times) a negative control using normal rabbit serum instead of the primary antibody was included to indicate the specificity of the antibody; (D, 200 \times) ischemia/reperfusion cannabidiol-treated group demonstrating a significant reduction in nuclear factor- κ B immunostaining. Brown color indicates nuclear factor- κ B positivity. Numerical data and statistical significance are shown in Table 2.

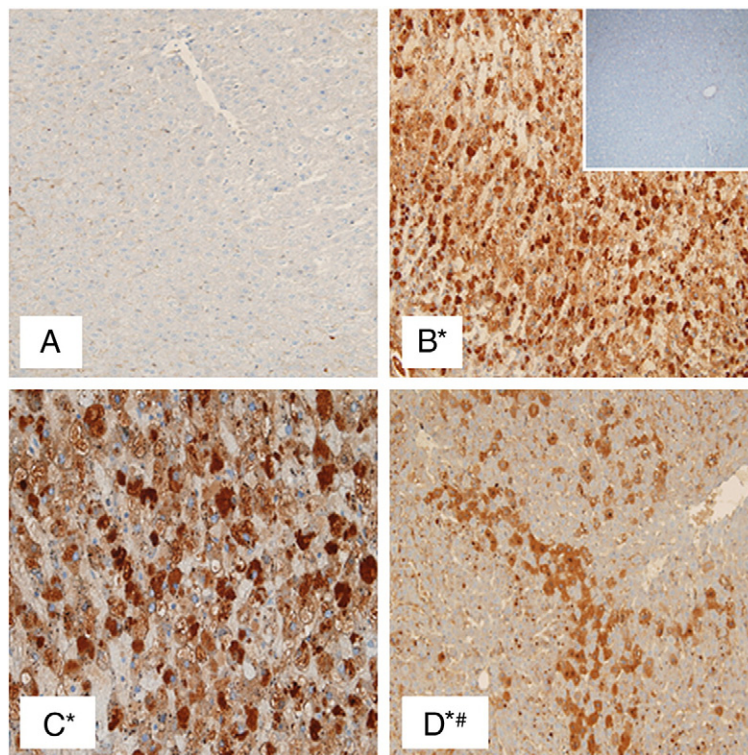


Fig. 5. Immunohistochemical staining of Fas ligand in rat liver from: (A, 200 \times) sham-operated control group showing no expression of Fas ligand; (B, 200 \times and C, 400 \times) ischemia/reperfusion group without cannabidiol treatment showing a significant increase in Fas ligand immunoreactivity in the cytoplasm of hepatocytes, (B, inserted figure, 200 \times) a negative control using normal rabbit serum instead of the primary antibody was included to indicate the specificity of the antibody; (D, 200 \times) ischemia/reperfusion cannabidiol-treated group demonstrating a significant reduction in Fas ligand immunostaining. Brown color indicates Fas ligand positivity. Numerical data and statistical significance are shown in Table 2.

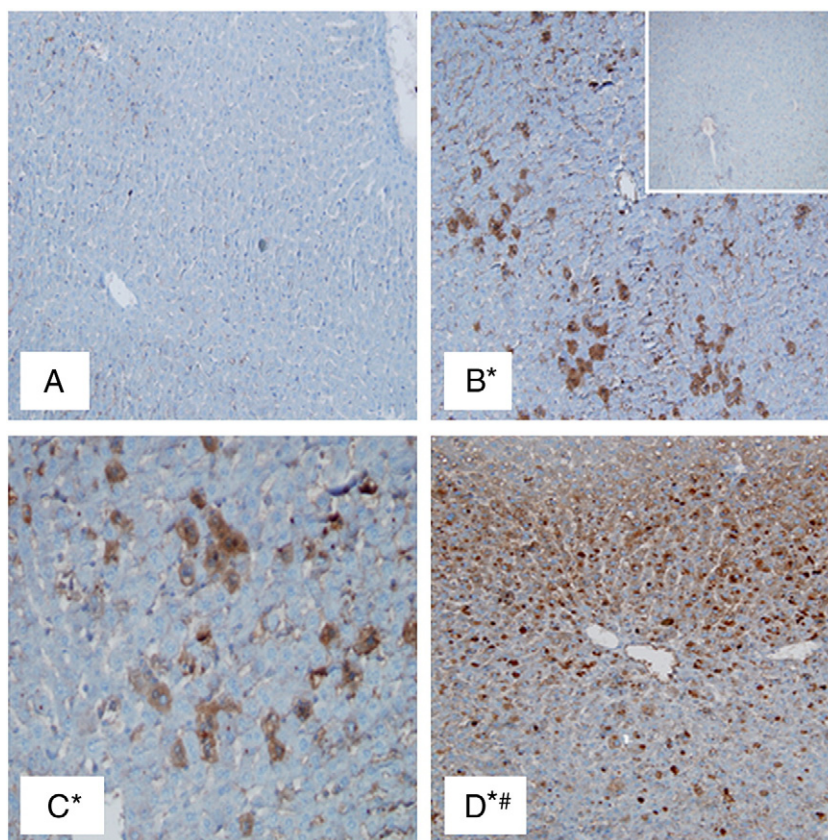


Fig. 6. Immunohistochemical staining of survivin in rat liver from: (A, 200 \times) sham-operated control group showing no expression of survivin; (B, 200 \times and C, 400 \times) ischemia/reperfusion group without cannabidiol treatment showing a significant increase in survivin immunoreactivity in the cytoplasm of hepatocytes, (B, inset figure, 200 \times) a negative control using normal rabbit serum instead of the primary antibody was included to indicate the specificity of the antibody; (D, 200 \times) ischemia/reperfusion cannabidiol-treated group showing a more significant increase in surviving immunostaining. Brown color indicates survivin positivity. Numerical data and statistical significance are shown in Table 2.

damage by oxidizing and nitrating cellular macromolecules. Also, excess nitric oxide depletes intracellular reduced glutathione increasing the susceptibility to oxidative stress (Clancy and Abramson, 1995). Moreover, ischemia/reperfusion is known to induce cyclooxygenase-2 with subsequent increased production of inflammatory prostaglandins responsible for further hepatic injury (Eum and Lee, 2004; Yun et al., 2010).

Cannabidiol has been shown to have prominent antioxidant and anti-nitrative properties in several disease models. It inhibits NADPH oxidases (Pan et al., 2009) implicated in the generation of reactive oxygen species during liver ischemia/reperfusion (Liu et al., 2008; Shaik and Mehvar, 2010). It also scavenges lipid peroxidation products during free radical reactions (Borrelli et al., 2009), and suppresses excess nitric oxide production preventing nitrosative stress (Pan et al., 2009; Ruiz-Valdepeñas et al., 2011). In addition, cannabidiol exhibits anti-inflammatory activity by reducing the release of proinflammatory cytokines and inflammatory prostaglandins (Costa et al., 2004; Rajesh et al., 2010).

Ischemia/reperfusion is also known to induce nuclear factor- κ B with subsequent cascade of events responsible for liver tissue injury (Yamaguchi et al., 2010). Cannabidiol has ability to inhibit the activation of nuclear factor- κ B signaling pathway which promotes the transcription of tumor necrosis factor- α , inducible nitric oxide synthase, cyclooxygenase-2 and NADPH oxidase genes (Rajesh et al., 2010; Wu et al., 2009). This is in accordance with the present results which revealed that cannabidiol treatment significantly suppressed lipid peroxidation, restored the depleted reduced glutathione, attenuated the overproduction of tumor necrosis factor- α and nitric oxide, and reduced the expression of nuclear factor- κ B, inducible nitric oxide synthase and cyclooxygenase-2 in the liver of rats exposed to ischemia/reperfusion.

Also, it was demonstrated that ischemia/reperfusion can induce liver cell apoptosis by activating the Fas/Fas ligand pathway (Kang et al., 2009; Zhang et al., 2010). Cross-linking of Fas ligand to its Fas cell surface receptor triggers cell apoptosis by inducing recruitment of the Fas-associated protein with death domain, which eventually leads to activation of the caspase family of proteases (Yin and Ding, 2003). Reactive oxygen species, tumor necrosis factor- α and activated nuclear factor- κ B are believed to be the main factors responsible for increased expression of Fas ligand and induction of apoptotic cell death (Shetty et al., 2002).

The survivin gene, a new member of the anti-apoptotic protein family, is generally accepted as the most powerful antiapoptotic gene in vivo. Its mechanism of regulating apoptosis involves various complex factors. One relatively clear mechanism is that upon activation of pro-apoptotic cell signaling, survivin is released from the mitochondria and forms a survivin-hepatitis B X-interacting protein complex which inhibits the activation of caspase-3 and caspase-9 (Altieri, 2006; Kelly et al., 2011; Ouhtit et al., 2007). Survivin also suppresses apoptotic cell death by inhibiting Fas stimulation (Ryan et al., 2009). It seems that nuclear factor- κ B plays an important role in the up-regulation of survivin expression at a transcriptional level (Kelly et al., 2011). Similar to the present results, a recent study showed that ischemia/reperfusion increased the expression of survivin protein in the liver tissue, suggesting a compensatory mechanism for protection of liver cells, and ischemic preconditioning caused more significant increase in survivin expression and protected against liver cell apoptosis (Li et al., 2008).

The present study revealed that cannabidiol treatment significantly inhibited the expression of caspase-3 activity and liver cell apoptosis

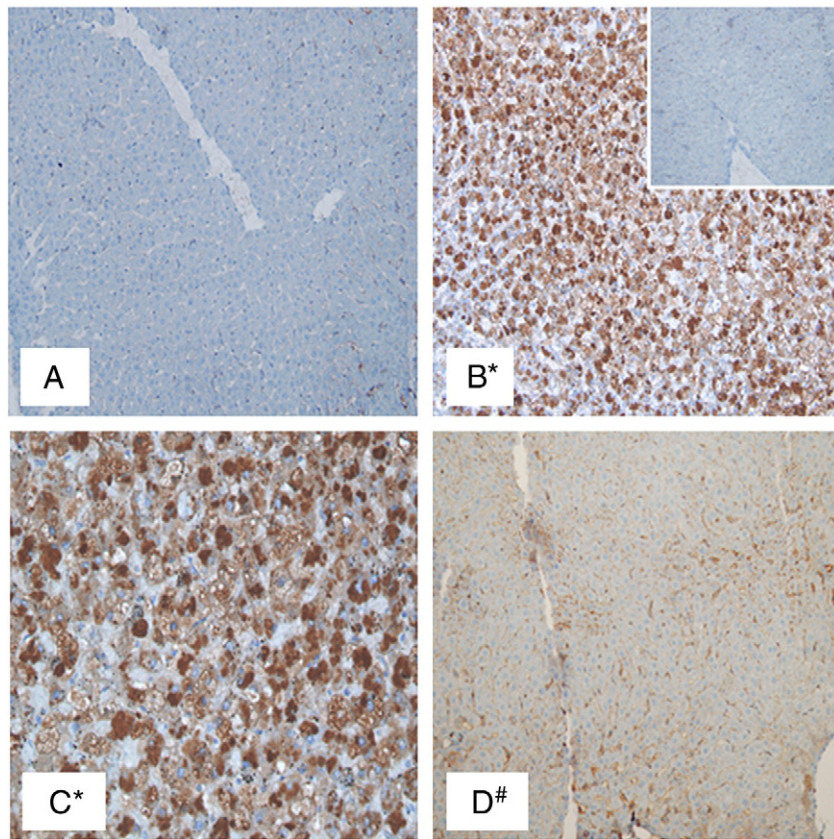


Fig. 7. Immunohistochemical staining of caspase-3 in rat liver from: (A, 200 \times) sham-operated control group showing no expression of caspase-3; (B, 200 \times and C, 400 \times) ischemia/reperfusion group without cannabidiol treatment showing a significant increase in caspase-3 immunoreactivity in the cytoplasm of hepatocytes, (B, inset figure, 200 \times) a negative control using normal rabbit serum instead of the primary antibody was included to indicate the specificity of the antibody; (D, 200 \times) ischemia/reperfusion cannabidiol-treated group showing a significant reduction in caspase-3 immunostaining. Brown color indicates caspase-3 positivity. Numerical data and statistical significance are shown in Table 2.

resulted from ischemia/reperfusion. The antiapoptotic activity observed with cannabidiol treatment can be attributed to the inhibition of Fas ligand expression and induction of survivin protein, and to the best of our knowledge, this is the first study showing the effect of cannabidiol on Fas/Fas ligand system and survivin gene. Also, free radical scavenging, anti-inflammatory action with reduced tumor necrosis factor- α production and inhibition of nuclear factor- κ B may contribute to the antiapoptotic effect of cannabidiol. However, further study on Fas/Fas ligand pathway and survivin may provide an effective path to intervene in ischemia/reperfusion-induced apoptosis.

A recent study demonstrated that cannabidiol pretreatment significantly attenuated the elevations of serum aminotransferases, decreased

oxidative and nitrative stress, and reduced the inflammatory response in liver tissue in a mouse model of hepatic ischemia/reperfusion injury (Mukhopadhyay et al., 2011). This study indicated that the protective effect afforded by cannabidiol was not mediated through the classical CB_{1/2} receptors, and cannabidiol most probably exerts direct antioxidant and anti-inflammatory actions.

The results of the present study indicate that post-ischemic cannabidiol treatment significantly protected against ischemia/reperfusion-induced liver injury in rats. The antioxidant, anti-inflammatory and antiapoptotic activities can be considered the main factors responsible for the hepatoprotective effect of cannabidiol. Therefore, cannabidiol may be a feasible therapeutic candidate to prevent liver tissue injury

Table 2
Effects of cannabidiol treatment on the percentage expression of inducible nitric oxide synthase, cyclooxygenase-2, nuclear factor- κ B, Fas ligand, survivin and caspase-3 in the liver of rats exposed to ischemia/reperfusion (IR) liver injury.

	Sham	Vehicle + IR	Cannabidiol + IR	Cannabidiol + sham
Inducible nitric oxide synthase	ND	18.54 \pm 1.46 ^a	1.59 \pm 0.14 ^b	ND
Cyclooxygenase-2	ND	19.93 \pm 1.49 ^a	5.86 \pm 0.47 ^{a,b}	ND
Nuclear factor- κ B	ND	16.14 \pm 1.19 ^a	2.16 \pm 0.18 ^b	ND
Fas ligand	ND	22.64 \pm 1.97 ^a	6.92 \pm 0.51 ^{a,b}	ND
Survivin	ND	4.21 \pm 0.35 ^a	14.28 \pm 1.19 ^{a,b}	ND
Caspase-3	ND	17.88 \pm 1.59 ^a	2.01 \pm 0.13 ^b	ND

All the values are expressed as mean \pm S.E.M., n = 10 in each group.

ND = non-detectable.

^a $P < 0.05$ versus sham-operated (sham) group.

^b $P < 0.05$ versus vehicle + IR group.

resulting from ischemia/reperfusion which is a major clinical challenge particularly during liver transplantation.

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