



Protective effects of St. John's wort in the hepatic ischemia/reperfusion injury in rats

Süleyman Atalay¹ , Belkis Soylu² , Aslı Aykaç³ , Ayliz Velioglu Ögünç⁴ , Şule Çetinel⁵ , Naziye Özkan⁵ , Can Erzik⁶ , Ahmet Özer Şehirli⁷

ABSTRACT

ORCID IDs of the authors:

S.A. 0000-0001-9033-2719;
B.S. 0000-0003-3833-8050;
A.A. 0000-0002-4885-5070;
A.V.Ö. 0000-0001-8453-6491;
Ş.Ç. 0000-0003-1912-3679;
N.Ö. 0000-0003-3524-0672;
C.E. 0000-0002-7914-1169;
A.Ö.Ş. 0000-0002-5175-9290.

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Objectives: The purpose of this study was to investigate possible protective effects of St. John's wort in the hepatic ischemia/reperfusion injury.

Material and Methods: The hepatic artery, portal vein, and bile duct were all clamped for 45 minutes to induce ischemia in rats, and after that reperfusion for 1 hour. SJW was administered orally, once a day for 3 days before ischemia/reperfusion. The aspartate aminotransferase, alanine aminotransferase, tumor necrosis factor, and interleukin levels were measured in the serum samples. Luminol chemiluminescence, lucigenin luminol chemiluminescence levels; myeloperoxidase. The sodium-potassium ATPase (Na⁺/K⁺ ATPase) activity was determined in the liver tissue, and caspase-3 and caspase-9 activity with the bcl-2/bax ratio were measured by the western blot analysis.

Results: The St. John's wort administration recovered the aspartate aminotransferase, alanine aminotransferase, tumor necrosis factor, and IL-1β levels serum parameters meaningfully, while ischemia/reperfusion caused an increase in luminol chemiluminescence, lucigenin luminol chemiluminescence, myeloperoxidase, caspase-3, and caspase-9 activity and led to a decrease in the B-cell lymphoma-2/bcl-2-associated X protein (bcl-2/bax) ratio and the Na⁺/K⁺ ATPase activity.

Conclusion: The obtained results indicate protective effects of St. John's wort on the ischemia/reperfusion injury through various mechanisms, and we are able to suggest that St. John's wort can clinically create a new therapeutic principle.

Keywords: Apoptosis, inflammatory, ischemia/reperfusion, St. John's wort

INTRODUCTION

The ischemia/reperfusion (I/R) injury occurs in various clinical situations, such as hepatic trauma, hemorrhagic shock, resection of a large intrahepatic tumor, and liver transplantation (1, 2).

The ischemia/reperfusion causes the hepatic neutrophil accumulation. In addition, the Kupffer cells become activated and release proinflammatory cytokines such as reactive oxygen species and the tumor necrosis factor (TNF-α) and IL-1 (3, 4). These cytokines activate most of the proteins involved in apoptosis, such as caspase-3 and caspase-9, bcl-2/bax ratio, as well as mitochondria cytochrome-c released to cytoplasm. Increased cytokine levels as a result of inflammation are associated with increased aldosterone levels, leading to the intensification of inflammation by inhibiting the Na⁺/K⁺-ATPase pump (5). The sequential events initialized by these substances results in the DNA damage and cell death (6). During I/R, with the myeloperoxidase-mediated neutrophil activation, the assay of excess production of free radicals was represented by using luminol chemiluminescence (CL) and lucigenin CL as indicators. By luminol-mediated measurements, the levels of hydroxyl (OH[•]), hydrogen peroxide (H₂O₂), hypochlorite, and hydroperoxyl radicals were assayed, whereas by lucigenin-mediated measurements, the levels of superoxide (O₂⁻) free radicals were assayed. During I/R, as a result of neutrophil activation, the levels of the luminol and lucigenin were found to be elevated (7).

Hypericum perforatum (St. John's wort; SJW) is commonly used in the treatment of mild to moderate depression. Apart from depression, SJW is used traditionally for in-wound healing, first-degree burns, and myalgia externally (8, 9). There are also arguments claiming that *Hypericum perforatum* or its' bioactive component, hypericin, may be useful in the treatment of various types of cancer (10).

In the light of such information, our study aims to investigate protective effects of SJW in the case of liver damage arising from the I/R implementation on the left and middle hepatic artery, portal vein, and bile duct.

MATERIAL AND METHODS

Animals and Conditions

The experiment was carried out with the permission of the Ethics Committee (date of permission: 11/18/2013 and protocol code: 95.2013.mar) of the Marmara University Experimental Animal Research Centre. The rats were procured from and the experiments were held in the same laboratory.

¹Department of General Surgery, Haydarpaşa Numune Training and Research Hospital, İstanbul, Turkey

²Department of Pharmacology, Marmara University School of Pharmacy, İstanbul, Turkey

³Department of Biophysics, Near East University School of Medicine, Nicosia, Turkish Republic of Northern Cyprus

⁴Department of Biochemistry, Marmara University Vocational School of Health Related Professions, İstanbul, Turkey

⁵Department of Histology-embryology, Marmara University School of Medicine, İstanbul, Turkey

⁶Department of Medical Biology, Marmara University School of Medicine, İstanbul, Turkey

⁷Department of Pharmacology, Near East University School of Dentistry, Nicosia, Turkish Republic of Northern Cyprus

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Corresponding Author Ahmet Özer Şehirli

e-mail: ahmetozersehirli@neu.edu.tr

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In this research, a total of 32 ten-weeks-old male albino Wistar rats were used, and they were weighing between 200 and 250 grams. The rats were randomly divided into four groups of eight. The rats were kept in wire cages under standard laboratory conditions, with each of the eight cages containing four rats. Tap water and standard rat food were released throughout the experiment.

Each rat was kept in a jar containing ether for 40 to 60 seconds for the purpose of anesthesia induction. After the anesthesia induction, 100 mg/kg ketamine (Ketalar, Parke-Davis, Eczacıbaşı-Istanbul), 1 mg/kg intraperitoneally (ip), chlorpromazine (Largactil, Eczacıbaşı-Istanbul) 1 mg/kg ip were applied as maintenance anesthesia.

In our research, rats were placed in supine position during anesthesia. The abdominal front walls were shaved by bistoury and sterilized using a povidone iodine solution. Afterwards, the abdomen was opened by midline incision. The hepatic artery, portal vein, and bile ducts of the rats were explored, and ischemia was induced by clamping for a 45 minutes. At the end of these 45 minutes, the rats were decapitated after undergoing a 60 min reperfusion (11). SJW (SOLGAR) (300 mg/kg, p.o.) (12) or a physiological saline solution (PSS) were administered for 3 days before ischemia and reperfusion.

Control (Sham opere-C) Group: Two cc. blood and liver tissue samples were collected 60 min after the standard operation without clamping the hepatic artery, portal vein, and bile ducts and without any further operation.

Saint John's Wort (SJW) Group: Three days before the sham operation of the rats and for 3 days before the sham operation, a 300 mg/kg SJW dose was administered orally.

Hepatic Ischemia/Reperfusion (HI) Group: The PSS was administered orally for 3 days before the day of hepatic I/R. Following the standard operation, ischemia was induced by the vascular clamping of the hepatic artery, portal vein, and bile duct. The rats were only subjected to a 45 min ischemia followed by a 60 min reperfusion.

Hepatic Ischemia/Reperfusion Group which was administered SJW (HI-SJW): For 3 days before the day of hepatic ischemia/reperfusion, a 300 mg/kg SJW dose was administered orally. The rats were only subjected to a 45 min ischemia followed by a 60 min reperfusion.

At the end of the reperfusion period, 2 cc. blood and liver tissue samples were taken from the decapitated rats. The resected liver tissues were washed with cold 0.9% NaCl and wrapped in aluminum foil to be stored at -80°C for biochemical research. Some samples were also stored in formaldehyde for histological research. The luminol and lucigenin levels, bcl-2/bax ratio, $\text{Na}^{+}/\text{K}^{+}$ -ATPase, caspase-3, and caspase-9 activities of the frozen samples were examined once thawed at the room temperature. Structural damage of the samples stored in formaldehyde was histologically evaluated. The plasma that was separated by centrifuging the blood sample (at 3000 rpm/15 min) was stored at -70°C . The aminotransferase (AST), alanine aminotransferase (ALT), tumor necrosis factor (TNF- α), and interleukin (IL-1 β) levels were measured after the frozen samples were thawed at the room temperature.

Examinations of Serum

Determination of AST (Biolabo Europe S.A. Catalog No: REF 92025, 02160 Maizy, France) and ALT (Biolabo Europe SA Catalog Number: REF 80027, 02160 Maizy, France) in the serum is a kinetic spectrophotometric method and is measured using the Opera Technican Bayer Autoanalyzer device. TNF- α (Bio Source Europe S.A. Catalog No.KRC 3014, Nivelles, Belgium) and IL-1 β (ELISA, Bio Source Catalog No.KRC0011, Nivelles, Belgium) were determined using appropriate rat kits and measured by the enzyme-linked immunosorbent assay.

Examinations of Tissues

Determination of free oxygen radicals in tissues was conducted by the chemiluminescence method (13, 14). Tissues were placed into a 2 mL phosphate buffer solutions-4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (PBS-Hepes), and luminol (0.2 mM) and lucigenin (0.2 mM) were added to the two tubes (4 mL for each tube), which included the samples of the same tissue. The photonic activity resulting from the reactions between the reactive oxygen species, luminol, and lucigenin was recorded by using an illuminometer for 10 min, and the area-under-curve was calculated.

Determination of the *myeloperoxidase* (MPO) level in the tissues was performed by the Hillegeas method (15). The tissues that were removed immediately following decapitation were washed by PSS to remove any blood and environmental contamination. This step was followed by drying the tissues with filter paper and weighing them. By homogenizing the liver tissue by 50 mM K_2HPO_4 (pH: 6), a 10% homogenate was prepared, and the tissue was centrifuged at $41.400\times g$ at a temperature of 4°C for 10 min. The supernatant was discarded, and the samples, which were homogenized again by adding 0.5% hexadecyl-trimethyl ammonium bromide to the solution, were re-frozen, thawed, and sonicated 3 times. Next, the samples were centrifuged at $41.400\times g$ at a temperature of 4°C for 10 min. 50 mM K_2HPO_4 (pH: 6), 20 mM H_2O_2 , and o-Dianisidine-2 HCl was added to the supernatant obtained, and it was incubated in a 37°C water bath for 3 min. Then the color reaction was stopped by adding 2% sodium azide. Later, the samples were centrifuged at $41.400\times g$ at a temperature of 4°C for 10 min. The supernatant was obtained, and thus the absorbance of the color formed was measured using a spectrophotometer at 460 nm.

The $\text{Na}^{+}/\text{K}^{+}$ -ATPase activity of tissues was determined using the method of Reading and Isbir (16). The liver tissue was homogenized with a 10% sucrose solution, and then was centrifuged at 3000 rpm for 10 min. 0.1 ml supernatant was obtained, and this was followed by the incubation of homogenates with an appropriate medium containing 3 mM ATP. The Mg^{2+} -ATPase activity was detected in the presence of 1 mM ouabain, while the total ATPase activity was determined in the presence of 100 mM NaCl, 5 mM KCl, 6 mM MgCl_2 , 0.1 mM EDTA, 30 mM Tris HCl (pH: 7.4). The difference between the total ATPase activity and Mg^{2+} -ATPase was evaluated as the $\text{Na}^{+}/\text{K}^{+}$ -ATPase activity (16), and the specific activity of the enzyme is expressed as $\text{nmol Pi mg}^{-1} \text{ protein h}^{-1}$. The protein concentration of the supernatant was measured by the Lowry method (17).

Determination of the bcl-2/bax ration, caspase-3, and caspase-9 activities tissue homogenization

Protease inhibitors (0.2 mM PMSF, 1 µM pepstatin, 1 µg/mL leupeptin, and 10 µg/mL soybean trypsin), 10% glycerol, 50 mM NaCl, 2 mM DTT, 20mM Tris buffer with pH=7.4, which contains 1 mM EGTA and 1 mM EDTA, were used to prepare homogenates for molecular analysis of tissues that were stored at -80°C.

The tissues were transferred into 50 ml falcons and were homogenized on ice using the ultra-turrax homogenizer (90s) in the homogenization buffer, which was added according to their weight (1:2, w/v). After the homogenates were centrifuged at 2.000×g for 10 minutes, the supernatant portion was centrifuged again at 12.000× g for 60 min. The mitochondrial pellet formed as a result of the second centrifuge was centrifuged at 12.000× g for 1 h after being washed with a 500 µl homogenization buffer. Pellets containing 50 mM Tris-HCl (pH: 7.4), 5% glycerol, 1 mM EDTA, 5 mM DTT, protease inhibitors, and the fractions, which were incubated by vortexing for 1.5 h using 0.05% Triton X-100 buffer, were used as mitochondrial extracts. All processes were performed at +4 °C (16). The protein amounts of mitochondrial extracts were determined using the Lowry method (17).

SDS-PAGE and western blot

The tissues were analyzed by the Lowry method to determine their protein amounts and were then mixed with a 4X buffer (to include 100 µg proteins) and denatured for 6 minutes.

The samples were loaded to electrophoresis setup with gel containing 12% polyacrylamide for 120 min. The gel was transferred to the nitrocellulose membrane at 125mA for 90 min. Membranes were incubated by a buffer containing TBS-T (TBS containing 0.05% Tween 20) and 3% BSA for 14 h at +4°C. The day after bracketing, the membranes were incubated throughout the night using different concentrations of polyclonal antibodies of apoptosis [bcl 2 (1: 200), bax (1: 200), caspase-3 (1: 100), and caspase-9 (1: 100)] proteins.

Apart from the antibodies that were treated, all the membranes were incubated throughout the next night with the β-actin antibody (1: 200), at 4°C to be able to perform the normalization analysis. After each incubation, membranes were washed using a buffer containing TBS-T (TBS containing 0.05% Tween 20) for 3×15 min. In the next stage, they were incubated for another hour with a secondary antibody containing alkaline phosphatase conjugated to rabbit monoclonal anti-goat

immunoglobulin G. After the secondary antibody incubation, membranes were washed once more using TBS-T for 3×15 min. Following the secondary antibody incubation, distinctive bands were identified on the membranes after being washed using TBS-T for 3×15 min one more time and using blue tetrazolium chloride/5-Bromo-4chloro-3indolyl phosphate color improvement solution (18, 19).

Histopathologic grading method (light microscope)

Having placed the tissues in 10% formaldehyde, they were washed by tap water for at least 3 h or 1 night. The remaining alcohol concentration was used for dehydration (15 min with 70% alcohol, 15 min with 90% alcohol, 30 min with 96% alcohol, 2x30 min 100% alcohol, 100% toluene for 2x30 min), and after that, they were steeped in paraffin for 1 night at 60°C. The next day the tissues were embedded in paraffin blocks. Following the blocking process, the tissues were sectioned to 5 to 6 mm thick pieces and placed onto the lames where they were left in toluene for 2 h to eliminate the paraffin. The aim of the process was to reduce to water as the alcohol concentration was gradually decreased (2 min with 100% alcohol, 2 min with 90% alcohol, 2 min with 70% alcohol, and finally placed into distilled water.). After a 15 min treatment with hematoxylin, they were steeped in tap water for empurpling. In addition to 5 minutes of staining with Eosin, distilled water was applied and as alcohol concentrations were increased again, the process was conducted (2 min with 70% alcohol, 2 min with 90% alcohol, 2 min with 96% alcohol, and 10 min with 100% alcohol). Then it was washed with toluene twice (1st bath for 5 min and 2nd one for 10 min), the tissue was covered with Entellan. Finally, it was examined with a light microscope.

Statistical Analysis

In our study, we compared the groups of the AST, ALT, TNF-α, IL-1β levels in serum and GSH, MDA, luminol, lucigenin levels, bcl-2/bax ratio and MPO, Na⁺/K⁺-ATPase, caspase-3 and caspase-9 activities, (which can be determined from the liver tissue) were investigated using the one-way analysis of variance. A Tukey test was used for pair-wise comparisons. P-values less than 0.05 were considered significant.

RESULTS

In the Hepatic Ischemia (HI) group, it could be observed that the AST, ALT, TNF- α, and IL-1β levels in the serum were significantly higher than in the control group. On the other hand, in the group to which SJW was applied, the increase was significantly slower in the levels of the HI group, approaching the values of the control group (Table 1).

Table 1. In the rat hepatic ischemia/reperfusion model (HI), The aminotransferase (AST), alanine aminotransferase (ALT), tumor necrosis factor (TNF-α), and interleukin (IL-1β) values of all groups. SJW: Saint John's wort.

| | Control | SJW | HI | HI-SJW |
|---------------|----------|----------|---------------|-------------|
| AST (U/L) | 66.2±7.5 | 68.2±5.4 | 120.5±9.3*** | 71.5±10.1** |
| ALT (U/L) | 61.3±5.6 | 68.7±6.2 | 133.8±14.6*** | 83.5±7.8** |
| TNF-α (pg/mL) | 55.4±3.2 | 48.2±3.1 | 82.2±3.9*** | 56.6±3.9*** |
| IL-1β (pg/mL) | 365±22 | 326±16 | 475±29* | 380±18* |

Average value±standard error

*p<0.01, ***p<0.001 Comparisons with respect to control group

p<0.05, **p<0.01, *p<0.001 Comparisons with respect to HI group

The luminol and lucigenin levels in the liver tissues of the HI group were found to be higher than in the control group. It was observed that this increase was prevented considerably in the HI group that was administered the treatment with SJW (Figure 1).

On one hand, HI increased the neutrophil infiltration in the liver in parallel with a significant increase of the MPO values with respect to the control group; on the other hand, the MPO activity decreased significantly in the SJW administered group, and the results of the MPO values were observed to be close to the control group MPO values (Figure 2a).

The Na⁺/K⁺-ATPase activities in the liver tissues were found to be low in the HI group with respect to the control

group. It was observed that this decrease was prevented considerably in the HI group, which was treated with SJW (Figure 2b).

When the bcl-2/bax ratio was evaluated with respect to the control, the SJW and HI-SJW group's ratios were observed to be high, and the ratio was observed to be low in the HI group. The rate of decrease was considerably slower in the HI group treated with SJW (Figure 3a, Table 2).

The caspase-3 and caspase-9 activities with respect to control group were found to be low in the SJW group, while they were found to be high in the HI group. This increase was significantly recovered in the HI-SJW group (Figure 3b, c, Table 2).

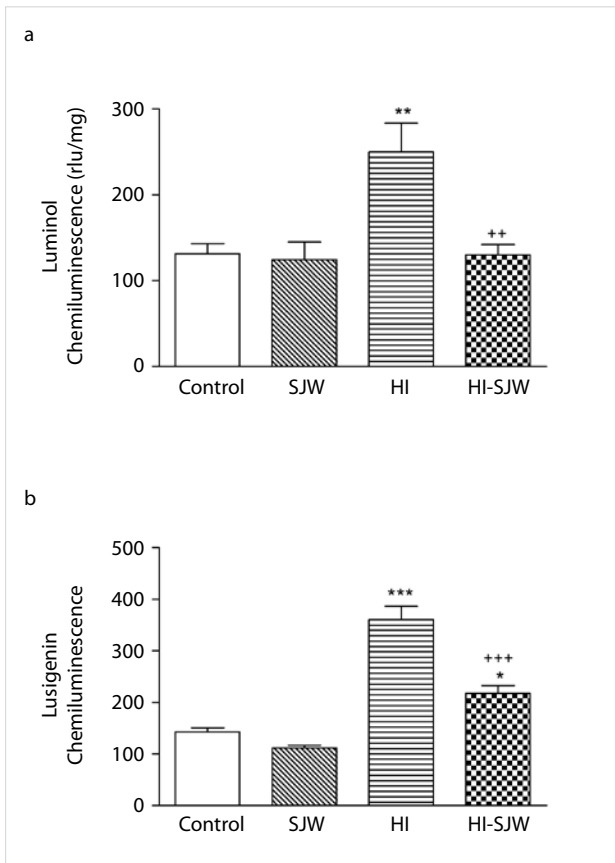


Figure 1. a, b. In the rat hepatic ischemia/reperfusion model (HI), tissues from all groups luminol (a) and lucigenin values (b) SJW: Saint John's wort
*p<0.01, **p<0.01, ***p<0.001 Comparisons with respect to control groups, **p<0.01, ***p<0.001 Comparisons according to the I/R group

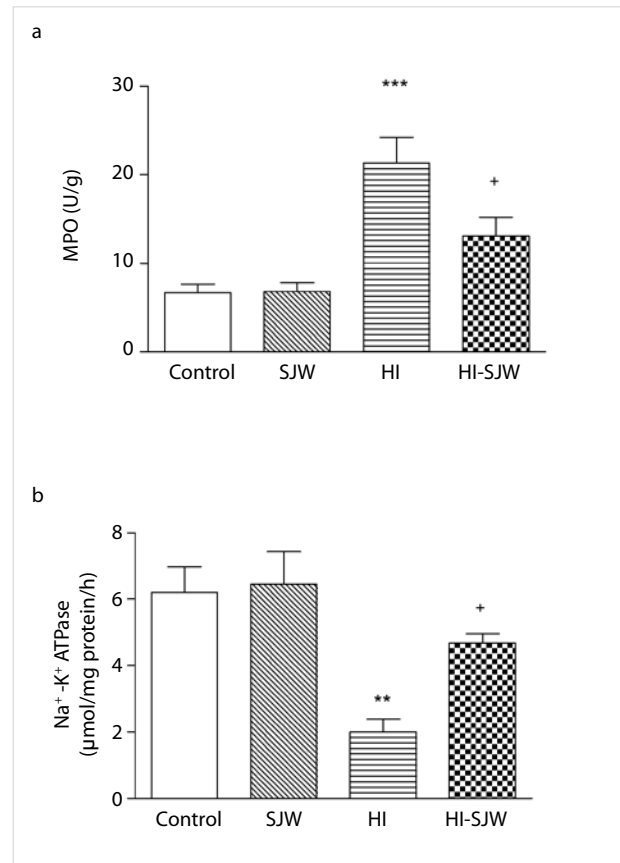


Figure 2. a, b. In the rat hepatic ischemia/reperfusion model (HI), tissues from all groups myeloperoxidase (MPO) (a), Na⁺K⁺ATPase values (b) SJW: Saint John's wort
p<0.01, *p<0.001 Comparisons with respect to control groups, +p<0.05 Comparisons according to the HI group

Table 2. In the rat hepatic ischemia/reperfusion model (HI), the (b-cell lymphoma 2/ B-cell lymphoma 2 associated Xprotein (bcl-2/bax) ratio, and caspase-3 and caspase-9 activities in all groups. SJW: Saint John's wort

| | Control | SJW | HI | HI-SJW |
|-------------------|---------|--------------|--------------|-------------------|
| bcl-2/bax ratio | 1 | 1.35±0.01*** | 0.76±0.02*** | 1.18±0.02***, +++ |
| caspase-3/β-actin | 1 | 0.75±0.02*** | 1.16±0.03*** | 0.87±0.02*** |
| caspase-9/β-actin | 1 | 0.86±0.02** | 1.19±0.03*** | 0.87±0.03**, +++ |

Average value ± standard error

** p<0.01, ***p<0.001 Comparisons with respect to control group

+++p<0.001 Comparisons with respect to HI group

In the histopathological examination of the control group, it is observed that hepatocytes and sinusoids were intact, and liver parenchyma was found to be maintaining a normal morphology (Figure 4a). In the SJW administered group, it was observed that hepatocytes and sinusoids were intact, and liver

parenchyma was found to be maintaining a normal morphology (Figure 4b). In the HI group, a widespread congestion and hepatocyte degeneration, caused by HI, have been observed (Figure 4c). In the HI group that was administered the treatment with SJW, low sinusoidal and hepatocellular degeneration was observed (Figure 4d).

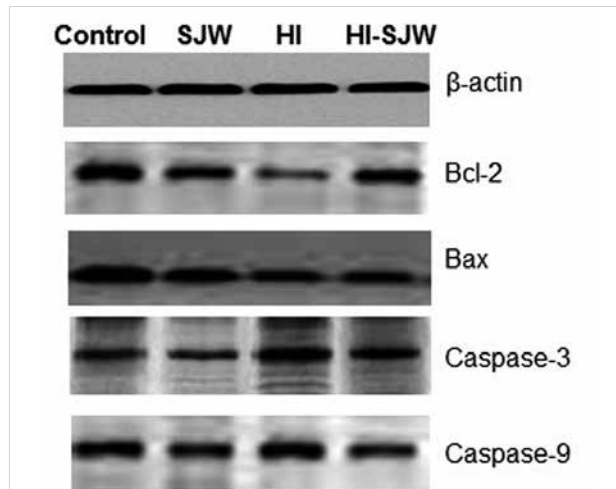


Figure 3. In the rat hepatic ischemia/reperfusion model, Bcl-2/Bax ratio and caspase-3 and caspase-9 activities of all groups.
SJW: Saint John's wort

DISCUSSION

A hepatic I/R injury may occur in most of clinical practices including transplantation of organs, hepatectomy, trauma, and vascular diseases (20, 21). Various factors contribute to the hepatic I/R injury, namely the Kupffer cell activation, oxidative stress, and upregulation of proinflammatory cytokine signaling.

During an ischemia period, several functional changes occur at cellular levels that promote cell injury. More specifically, the oxidative phosphorylation levels go down. This situation results in the ATP depletion. In addition, during the ischemia period, the lack of oxygen to hepatocytes causes mitochondrial deenergization, and finally the swelling of the sinusoidal endothelial cells and the Kupffer cells. The production of reactive oxygen species, activation of Kupffer cells, upregulation of proinflammatory cytokines, resulting in neutrophil-mediated injury are the major factors contributing to inflammation-associated damages (22).

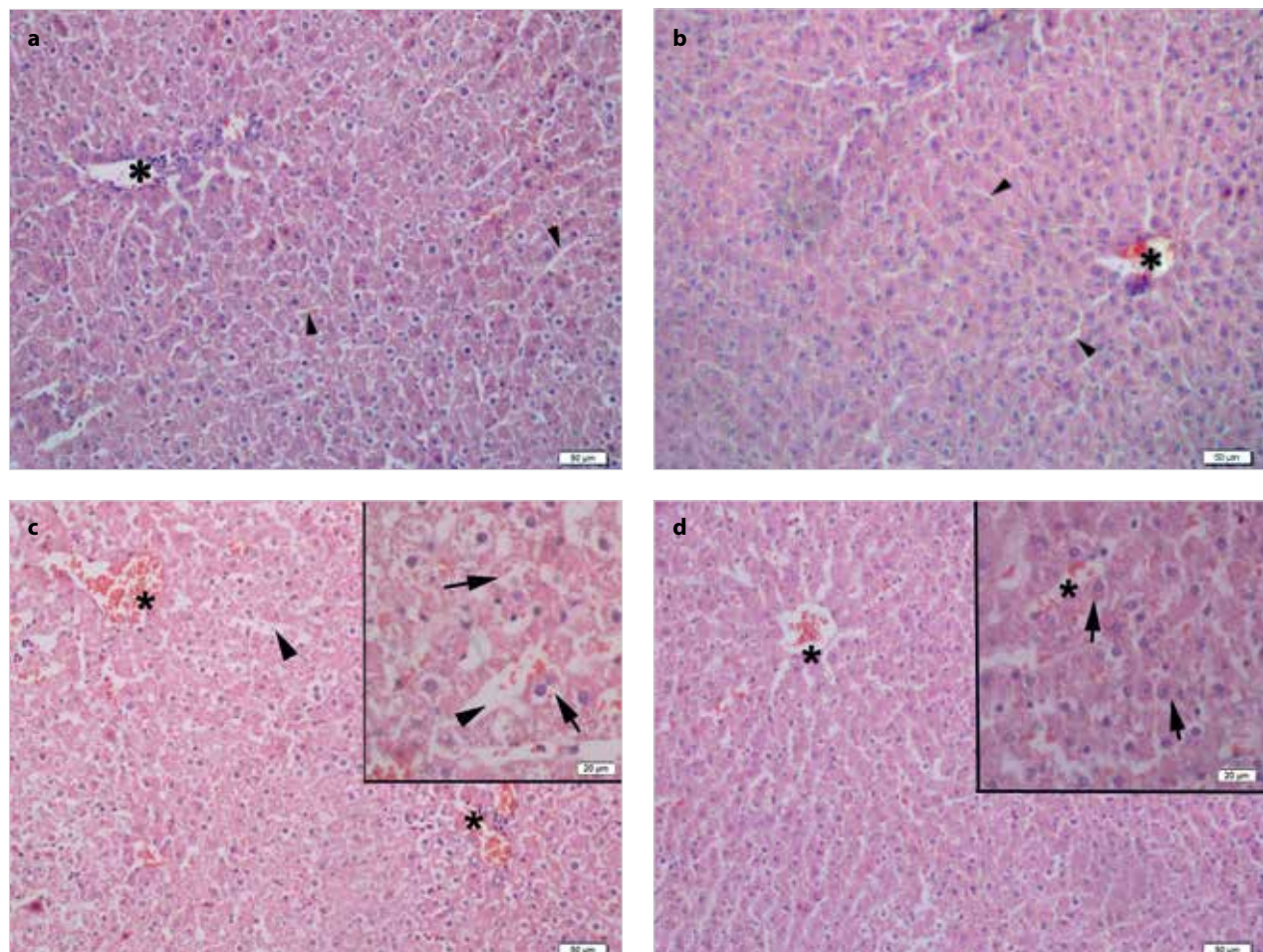


Figure 4. a-d. (a) Control group; central vein with smooth contours (*) and sinusoids (►) (a), SJW group, central vein with smooth contours (*) and sinusoids (►) (b), HI group, dilatation of sinusoids, hepatocellular degeneration (arrows) and widespread congestion in the central vein (*) (c), HI-SJW group, reduced dilatation in sinusoids (arrows) and reduced congestion in the central vein (*) (d)

Many agents have been investigated for prevention of the I/R injury (1, 22, 23). However, a study that would test SJW alone in the hepatic I/R injury and stated quantitative histopathological findings, has not yet been documented in the literature.

In a study conducted on rats, comparing the effects of the ischemia period, the rats were subjected to bilateral ischemia for 45 minutes, and this was followed by reperfusion for 1 hour. The values of AST and ALT were measured. This ischemia and reperfusion period were found to be associated with the extent of muscle edema as well as increased values of AST and ALT (23). In Kupffer cells isolated from the rat liver, an increase in the TNF- α levels after I/R were discovered to be 5 times greater than the increase of the TNF- α levels in the control group. Besides, it has been shown in studies conducted on rats that the use of anti-TNF- α antibodies in the hepatic I/R cases reduces liver damage. The effects of the oral use of *H. perforatum* in an animal model of acute inflammation, carrageenan-induced pleurisy, were evaluated. It was also determined that an acute inflammatory response characterized by fluid accumulation in the pleural cavity in animals contains an increased production of TNF- α and IL-1 β . All parameters of inflammation were reduced by the administration of the *H. perforatum* extract (24). In our study, the AST, ALT, TNF- α , and IL-1 β levels of the hepatic I/R group were found to be significantly higher than in the control group. On the other hand, in the SJW administered group was significantly reduced versus HI group and approached the levels in the control group.

Luminol is an indicator of the hydrogen peroxide and hypochlorous acid, hydroxyl radical formation, and lucigenin is an indicator of the superoxide anion radical formation (25). In this study, luminol levels in the liver tissues of the HI group were found to be higher than in the control group. It was observed that this increase was considerably prevented in the HI group that was administered the SJW treatment. Lucigenin levels in the HI group were found to be higher than the control group, and this increase was considerably reduced in the I/R group, which was administered with SJW.

Myeloperoxidase is an important enzyme that is found in neutrophils, and it is involved in the ROS production (26, 27). In recent studies, in the hepatic I/R injury, it is often stated that as an indicator of neutrophil infiltration, an increase in the MPO activity causes the endothelial dysfunction and inflammation (28, 29). Similarly, our study showed that HI increased the MPO values compared with the control group in the liver tissue, while in the SJW group in our study, the MPO activity was substantially reduced, and the results were close to the MPO values of the control group.

The Na⁺/K⁺-ATPase is an important membrane enzyme that plays a key role in the hepatocyte structure and physiology by ensuring that sodium and potassium are found in all cell membranes (30, 31). During liver transplantation, this is an indicator of the tissue viability and hepatic function. It is reported in a study that the Na⁺/K⁺-ATPase level was degraded after the hepatic I/R (32). Parallel to these studies, our study results also show that the Na⁺/K⁺-ATPase activity of the HI group was lower than that of the control group. In the HI group, which was treated with SJW, this decline was significantly prevented.

When the bcl-2/bax ratio was evaluated with respect to the control group, the ratios of the SJW group and the HI-SJW group were observed to be high, and the ratio was observed to be low in the HI group. This decrease was considerably prevented in the HI group treated with SJW.

The caspase-3 and caspase-9 activity with respect to the control group was found to be low in the SJW group, while it was found to be high in the HI group. This increase was significantly recovered in the HI-SJW group.

It is known that SJW have antidepressant, anti-inflammatory, analgesic, antibacterial, and wound-healing effects (33). In recent years, inflammatory models have shown a protective effect of SJW with antioxidant properties by inhibiting free radicals and lipid peroxidation (34). In addition to this, SJW has been shown to reduce the levels of proinflammatory cytokines and prevent PMN leukocytes from accumulating in the inflamed area (35). In our study, it has also been shown that either directly or indirectly, SJW prevents tissue damage by inhibiting both cytokine activation as a result of free radicals and the activation of apoptotic agents during inflammatory events.

CONCLUSION

We have, as a result, shown in our experimental study that the injury caused by hepatic I/R was significantly reduced by administering SJW. There is a need for further comparative experimental and clinical studies in the clinical use of SJW for this purpose. Particularly, the recovery effect of SJW in the I/R injury may lead to clinical and experimental research that will offer new treatment methods.

Ethics Committee Approval: Ethics committee approval was received for this study from the ethics committee of Marmara University Experimental Animal Research Centre (18.11.2013/95.2013)

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