European Review for Medical and Pharmacological Sciences 2023; 27: 10322-10333

Pre-treatment with pregabalin reduces liver ischemia-reperfusion injury in rats: tissue protection with an analgesic

V. UMMAN[1], N. KEPIL[2], H. UZUN[3], E. GOKSOY[1]

[1]Department of General Surgery, Istanbul University Cerrahpasa School of Medicine, Istanbul, Turkey
[2]Department of Pathology, Istanbul University Cerrahpasa School of Medicine, Istanbul, Turkey
[3]Department of Biochemistry, Istanbul University Cerrahpasa School of Medicine, Istanbul, Turkey

Abstract. – OBJECTIVE: Ischemia-reperfusion injury is thought to be the most important factor affecting the success of liver surgery. Pregabalin has been studied to prevent ischemic reperfusion injury in many organs. The aim of this study was to investigate the role of pregabalin in preventing liver ischemic injury.

MATERIALS AND METHODS: 40 male Wistar-Albino rats, 6-8 weeks old, were divided into 5 groups. Four groups other than the sham group were subjected to hepatic ischemia for 1 hour, followed by 2 hours of reperfusion. Effects of 30 mg/and 60 mg/kg pregabalin were evaluated by aspartate aminotransferase (AST), alanine aminotransferase (ALT), tumor necrosis factor α (TNF-α), nuclear factor-kappa B (NF-κB), interleukin (IL)-6 levels, measured in blood samples collected before and after ischemia. Apoptosis was measured by caspase-3, and tissue samples were evaluated for ischemia by histopathologic examination.

RESULTS: The 60 mg pregabalin group was significantly superior (p=0.024) to the N-acetylcysteine group and the 30 mg pregabalin group for AST levels (p=0.612 and p=0.807, respectively). The difference between before and after ischemia-reperfusion blood TNF-α levels was higher in the 60 mg pregabalin group, but not significantly different from the 30 mg pregabalin and N-acetylcysteine groups (p>0.05). Tissue TNF-α levels showed that 60 mg and 30 mg pregabalin treatment was more effective than no-treatment (p=0.011, p=0.033, respectively), but not superior to N-acetylcysteine (p>0.05).

CONCLUSIONS: It has been found that ischemia-reperfusion causes damage to the liver, and this damage may be irreversible if no treatment is given. Our study group, pregabalin molecule was found to be significantly effective in preventing ischemia-reperfusion injury and may have a therapeutic advantage over N-acetylcysteine.

Key Words:
Liver, Liver ischemia-reperfusion, Liver transplantation, Pregabalin, Liver cancer, Analgesic.

Introduction

Liver transplantation and liver resection surgery have dramatically improved the quality of life and life expectancy of patients with chronic liver disease or liver cancer. In both surgical procedures, the liver is subjected to ischemia-reperfusion injury (IRI) for a period of time, with temporary clamping of the hepatic pedicle containing the hepatic artery and portal vein (Pringle maneuver) to ensure the control of bleeding. During this period, an inflammatory cascade due to ischemia and reperfusion begins, causing cellular damage in the liver. If the duration of ischemia is long, the changes caused by ischemic damage can also be life-threatening.

Ischemia-Reperfusion Injury

Neutrophil infiltration is one of the key causes of the inflammatory cascade that leads to liver ischemia-reperfusion damage. The role of Kupffer cells, mitochondrial reactive oxygen products, toll-like receptors, high-mobility group box 1 (HMGB1) pathway and autophagy in liver IRI has been well established[1]. Studies[2] in this field have demonstrated that the IRI cascade starts when immune system cells and signaling pathways identify pathogen-derived molecular patterns (PAMPs), or damage-associated molecular patterns (DAMPs) produced from damaged or dying cells.

All cells in the immune system express pattern recognition receptors, which allow them to recognize endogenous molecules called DAMPs. Toll-like receptors (TLRs) are one class of DAMP receptors. All types of liver cells include TLRs, which sense DAMPs and induce an inflammatory response after reperfusion. TLR stimulation causes the nuclear factor-kappa B (NF-κB) pathway to be activated, which attracts additional leuko-

Corresponding Author: Veysel Umman, MD; e-mail: veysel.umman@aya.yale.edu

cytes and causes the production of cytokines and chemokines. One of these endogenous ligands, DNA, is released from damaged hepatocytes and binds to TLR9, promoting additional neutrophil activation.

Nitric oxide (NO), a vital element in vasodilation, undergoes a reaction with reactive oxygen species such as superoxide. This reaction decreases the readily available amount of NO, which then results in an augmented generation of reactive hydroxyl radicals. The liver microcirculation and its hepatocytes and sinusoidal endothelial cells sustain damage through these pathways. This damage advances through neutrophil migration, platelet aggregation and subsequently altered capillary permeability[2-5]. Ischemia and reperfusion have also been demonstrated to enhance the transcription of multiple genes in hepatocytes *via* transcription factors, including heat shock proteins and NF-κB[6]. Ischemia-reperfusion injury accounts for approximately 10% of transplant failures and can lead to acute and chronic rejection[7].

Pregabalin and Its Effect on Ischemia-Reperfusion Injury

Pregabalin is a drug in the class of anticonvulsants with a chemical structure similar to that of gamma-aminobutyric acid and is used for analgesia, antiepilepsy, diabetic neuropathy, postherpetic neuralgia, anxiety, fibromyalgia, complex regional pain syndrome and various clinical conditions, particularly to manage neurogenic pain[8-16].

Pregabalin does not interact with liver enzymes, does not bind to plasma proteins and 95% is excreted through the kidneys. Recent studies[13] have investigated the efficacy of pregabalin in preventing ischemia reperfusion-induced damage in multiple organs. Pregabalin exhibits neuroprotective properties on ischemia/reperfusion and reduces caspase-dependent apoptosis, as well as markers of inflammatory and oxidative stress.

Pregabalin is thought to have a role in protecting against ischemic injury through the inhibition of the HMGB1 pathway and caspase enzyme activation. Additionally, pregabalin is advantageous in clinical situations such as liver failure, resection, or transplant due to its lack of metabolism by the liver and absence of enzymes like the hepatic cytochrome P450 system[17]. Therefore, in our study, we aimed to investigate the role of pregabalin in the prevention of ischemic liver injury.

Materials and Methods

The project was initiated with the approval of Istanbul University Animal Experiments Ethics Committee (İstanbul Üniversitesi Hayvan Deneyleri Etik Kurulu - HADYEK) with acceptance number 2018/25. It was funded by Istanbul University Scientific Research Projects (İstanbul Üniversitesi Bilimsel Araştırma Projeleri - BAP) with the project code TTU-2018-31521. Forty Wistar-Albino 6-8-week-old male rats were obtained from the Istanbul Aziz Sancar Experimental Research Institute, where the experiments were performed.

Experimental

Forty animals were divided into 5 groups, and all 4 groups, except for the sham group, were subjected to liver ischemia for 1 hour, followed by 2 hours of reperfusion. In the first group, only laparotomy (sham) was performed, and the abdomen was closed without ischemia. The second group underwent 1 hour of ischemia, followed by 2 hours of reperfusion without any medication. Groups 3, 4, and 5 received therapeutic agents 30 minutes before surgery. The third group received 150 mg/kg intraperitoneal N-acetylcysteine (NAC) 30 minutes before ischemia, the fourth group received 30 mg/kg pregabalin, and the fifth group received 60 mg/kg pregabalin (Table I). The first group is referred to as sham, the second as no-treatment, the third as NAC, the fourth as PGB30, and the fifth as PGB60.

Preparation of Pregabalin Suspension

To determine the pregabalin administration method, we considered prior research on pharmacological agent bioavailability in rats. It was determined that an intraperitoneal solution was the appropriate route[18]. Since there is no intravenous form of pregabalin, a suspension was prepared.

The average weight of the experimental animals was approximately 250 grams. Pregabalin doses of 30 mg/kg and 60 mg/kg were chosen for the two study groups in accordance with the existing literature review[13]. Higher doses of pregabalin have been reported to cause peripheral edema and decreased efficacy; therefore, these two most commonly used doses were chosen.

When determining the weight-based dosage, a quantity of 7.5 mg of pregabalin per animal was calculated. The commercially available pregabalin product contains 75 mg of pregabalin with additional excipients. Since the tablet is soluble

Table I. Study protocol and groups.

Groups (n: 8, for each group)	Sampling	IP medication used 30 minutes before ischemia	Operation	Ischemia	Reperfusion	Sampling
Group 1 (Sham/control)	Blood sampling	Non	Sham (Laparotomy only)	No	No	Blood and tissue sampling
Group 2 (No treatment: ischemia - reperfusion only)		Non	Laparotomy + portal vessels ligation	1 hour	2 hours	
Group 3 (NAS: N-acetyl-cysteine)		150 mg NAS				
Group 4 (PGB30: Pregabalin 30 mg)		30 mg PGB				
Group 5 (PGB60: Pregabalin 60 mg)		60 mg PGB				

in water, it was dissolved in 10 ml of a 0.9% phosphate saline vehicle solution. The suspension of 7.5 mg of pregabalin was then injected intraperitoneally into each rat in a volume of 1 ml. (75 mg/10=7.5 mg).

Intraperitoneal administration was preferred due to its superior bioavailability in contrast to oral administration. It facilitates swifter and immediate plasma concentrations, while also avoiding the necessity for hepatic metabolism for drug activation.

Liver Ischemia Reperfusion Model in Rats

The primary technique for investigating the IRI model in rats involves inducing a partial ischemic injury through clamping of the hepatic artery and vein using an atraumatic bulldog clip[19]. The ischemia-reperfusion model, as previously described in many studies[20-23] conducted at our clinic, was executed identically.

In our experimental setup, anesthetized rats were secured to the operating table with tape. A heating pad was attached to the table to maintain their body temperature during the two-hour reperfusion period following laparotomy. A heating lamp was also utilized for this purpose.

During the surgical procedure, a stopwatch was used to measure ischemia and reperfusion durations. Additionally, any changes in color in the median lobe and left lateral lobe of the rats were observed following hemi-hepatic clamping for ischemia (Figure 1).

Blood samples were collected from the tail vein before the start of ischemia. Intracardiac blood samples were collected from each animal after 1 hour of ischemia, followed by 2 hours of reperfusion. The animals were then sacrificed by means of exsanguination.

Tissue samples were collected from the brain, liver, lung, and kidney for subsequent biochemical and pathological analysis. These tissues were snap-frozen in liquid nitrogen and then stored at -80 degrees Celsius. Furthermore, all blood samples underwent centrifugation at 3,000 rpm and the resulting serum samples were stored in appropriately labeled Eppendorf tubes and frozen at -80 degrees Celsius to facilitate joint analysis.

Biochemical Analysis

Commercially available rat aspartate aminotransferase (AST), and rat alanine aminotransferase (ALT) ELISA kits were used to determine serum AST and ALT levels before and after ischemia in all rats. Pre-ischemic blood samples were collected from the tail vein, whereas post-ischemic samples were acquired intracardially. The apoptosis markers caspase-3, B cell lymphoma-2 (BCL-2) and BAX were analyzed with commercially available ELISA kits. DAKO brand IS61430-2 BCL2 Oncoprotein [124] FLEX RTU 6 mL for BCL-2, and CellSignaling brand CST 9664T caspase-3 [5A1E] Conc. 0.02 mL (1:400) for caspase-3 were used.

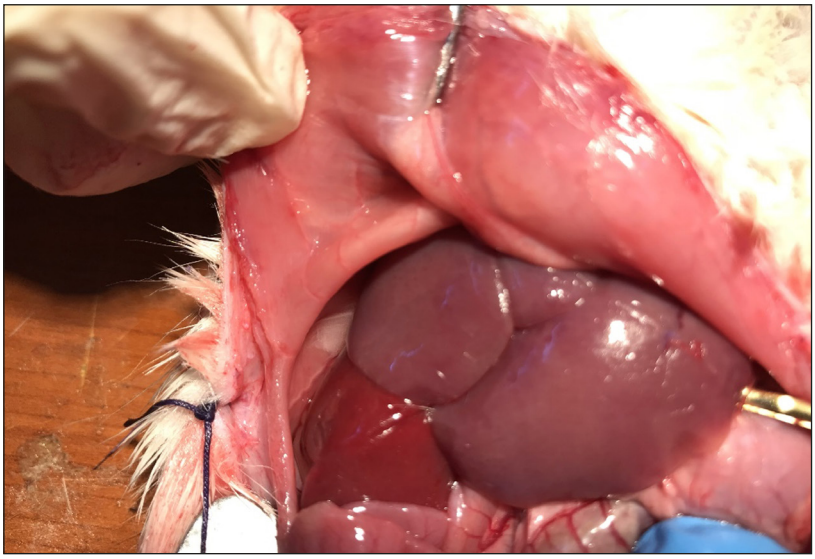

Figure 1. Liver color change after hepatic artery and vein clamping.

Tissue Analysis

Livers and other tissues from all groups of rats were harvested after 1 hour of ischemia, followed by 2 hours of reperfusion. These livers were removed at -80°C, weighed using a precision balance (Dikomsan, FGH), and placed in 2 ml Eppendorf tubes. They were homogenized in a homogenizer (Next Advance Bullet Blender Storm 24) for 3 minutes by adding beads up to their weight (mg) and freshly prepared PBS 4 times their weight. 20% tissue homogenates were collected in 1.5 ml Eppendorf tubes. The tubes were centrifuged at 3,000 rpm for 10 minutes and the biochemical parameters of the supernatants were analyzed.

Histological Examination

The liver tissue collected after the experiment was fixed in formalin. It was then examined histopathologically after hematoxylin-eosin staining (Tissue-Tek Prisma Plus machine). A Ventana benchmark XT IHC instrument was also used for caspase-3 staining. Liver specimens were blindly grouped according to modified Suzuki criteria and evaluated for ischemic injury by light microscopy. Both Suzuki and caspase staining were scored.

Interpretation of Results

All blood and tissue samples obtained from the animals were numbered by the surgical team. Automated biochemical analysis and histopathologic examination were subsequently conducted, with our team's biochemist and pathologist performing these tasks in a blinded manner.

Statistical Analysis

SPSS version 17.0 (SPSS Inc., Chicago, IL, USA) was used for statistical analysis. Categorical variables were presented as frequency and percentage, while continuous variables were displayed as mean±standard deviation. The Fisher's Exact test was used to evaluate categorical data. Continuous variables were assessed by using the One-way ANOVA test. Post-hoc Tukey test was used for pairwise comparisons. Paired *t*-test was used for the before-after comparisons. A p-value<0.05 was considered significant.

Results

Blood Biochemical Tests

Blood samples obtained from the tail vein of the rats before ischemia and intracardiac blood samples after 1 hour of ischemia, followed by 2 hours of reperfusion, were analyzed in all groups. AST, ALT, tumor necrosis factor α (TNF-α), NF-κB and interleukin-6 (IL-6) values were evaluated (Table II).

AST Levels

When AST values were compared between the 5 groups before and after ischemia-reperfusion, it was found that the mean AST value was 41.38 (±5.04) before ischemia in the no-treatment group and increased to 49.53 (±6.83) after ischemia. In the NAC treatment group, the mean AST was 37.55 (±1.76) before ischemia and 36.97 (±1.54) after reperfusion. In the PGB30 treatment group,

Table II. Comparison of the before and after blood AST, ALT, TNF-α, NF-κB and IL-6 levels of the groups.

Parameters		Group 1 (Sham)	Group 2 (No treatment)	Group 3 (NAS)	Group 4 (PGB30)	Group 5 (PGB60)	*p*-value*
AST	Before	37.4±2.2	41.4±5.0	37.6±1.8	38.9±2.1	38.4±3.1	
	After	35.7±4.5	49.5±6.8	37±1.5	38.6±2.1	34.8±2.6	
	Difference	1.7±5.4[a]	-8.2±7.8[b]	0.6±3.1[a]	0.3±3.0[a]	3.5±3.5[a]	**<0.001**
	p-value**	0.394	**0.021**	0.612	0.807	**0.024**	
ALT	Before	11.3±0.7	21.8±5.9	15.4±1.7	16.7±7.9	11.5±1.1	
	After	11.5±3.4	17.4±3.0	11.6±2.0	12.7±1.9	10.6±1.0	
	Difference	-0.2±3.4	4.4±8.4	3.8±1.7	3.9±8.4	0.9±0.6	0.371
	p-value**	0.879	0.182	**<0.001**	0.224	**0.004**	
TNF-α	Before	44.3±6.3	55±7.6	52.6±2.4	51.1±2.6	50.7±3.5	
	After	45.5±8.6	67.3±3.5	49.2±2.1	46.2±2.6	46.1±3.3	
	Difference	-1.1±13.4[a,b]	-12.4±9.0[b]	3.4±3.1[a]	4.9±2.2[a]	4.6±5.6[a]	**<0.001**
	p-value**	0.818	**0.006**	**0.017**	**<0.001**	0.053	
NF-κB	Before	1.0±0.1	1.2±0.4	1.1±0.1	1.0±0.2	1.0±0.2	
	After	0.9±0.2	1.6±0.3	0.9±0.1	0.9±0.2	0.9±0.3	
	Difference	0.1±0.2[a]	-0.3±0.4[b]	0.2±0.1[a]	0.1±0.3[a]	0.1±0.2[a]	**0.001**
	p-value**	0.115	**0.044**	**<0.001**	0.207	0.160	
IL-6	Before	1.3±0.4	2.1±0.4	1.7±0.2	1.7±0.2	1.7±0.2	
	After	1.4±0.4	2.3±0.5	1.7±0.2	1.5±0.2	1.5±0.2	
	Difference	-0.1±0.2	-0.2±0.8	0±0.2	0.2±0.2	0.2±0.3	0.196
	p-value**	0.289	0.408	0.651	0.055	0.155	

Results were presented as mean±standard deviation. *: One-way ANOVA, **: Paired *t*-test. There is no significant difference between groups that have the same superscript letter. Post-hoc comparisons: for AST: No treatment *vs*. Sham $p=0.003$, No treatment *vs*. NAS $p=0.009$, No treatment *vs*. PGB30 $p=0.013$, No treatment *vs*. PGB60 $p<0.001$. For TNF-α: No treatment *vs*. Sham $p=0.051$, No treatment *vs*. NAS $p=0.003$, No treatment *vs*. PGB30; $p=0.001$, No treatment *vs*. PGB60 $p=0.001$. For NF-κB: No treatment *vs*. Sham $p=0.012$, No treatment *vs*. NAS $p=0.001$, No treatment *vs*. PGB30; $p=0.006$, No treatment *vs*. PGB60 $p=0.007$.

the mean AST value decreased from 38.91 (±2.12) before ischemia to 38.64 (±2.14) after reperfusion. In the PGB60 treatment group, the mean AST value was 38.36 (±3.06) before ischemia and 34.84 (±2.60) after reperfusion.

AST values before ischemia and after reperfusion in each group were statistically analyzed by paired *t*-test. In the no-treatment group, the *p*-value was 0.021 when before and after ischemia and reperfusion values were compared. In the PGB60 group, the *p*-value was found to be significant at 0.024.

The ANOVA test was performed to determine whether the difference in AST values between the groups was significant or not. There was a significant difference between the groups in terms of AST change ($p=0.0003$). A post-hoc test was performed to find the groups that caused the difference in AST values. According to Tukey's test, when the no-treatment group was compared to NAC and PGB30, PGB60 groups, the *p*-value was 0.009 in the NAC group, 0.013 in the PGB30, and 0.0003 in the PGB60 group. There was a significant difference in AST values between the PGB60 and no-treatment group ($p=0.0003$).

ALT Levels

When ALT values were compared between the 5 groups, the mean ALT was 15.37 (±1.65) in the NAC group before ischemia and decreased to 11.57 (±1.96) afterward. In the PGB30 group the mean ALT was 16.68 (±7.92) before ischemia and decreased to 12.73 (±1.83) afterward. In the PGB60 group, ALT value, which was 11.50 (±1.14) before ischemia, decreased to 10.59 (±0.95) in the next measurement.

When ALT values before ischemia and after reperfusion were compared within the groups by paired *t*-test, a significant difference was observed

in group 3 and group 5. The *p*-value was 0.0003 in the NAC group and 0.004 in the PGB60 group.

A one-way ANOVA test was used to compare the measurement changes of 5 groups. There was no significant difference between the groups in terms of ALT change.

TNF-α, NF-κB, and IL-6 Levels

TNF-α, NF-κB and IL-6 values obtained from the tail vein before ischemia and intracardiac blood samples after reperfusion were analyzed in all groups. After reperfusion, the mean TNF-α value in the no-treatment group was 67.32, while the mean values in the NAC, PBG30 and PGB60 treatment groups were 49.16, 46.19, and 46.08, respectively. Similarly, while the mean NF-κB value after reperfusion was 1.55 in the no-treatment, the mean values in the NAC, PBG30 and PGB60 treatment groups were 0.88, 0.88 and 0.87, respectively. Similarly, the mean value of IL-6 in the no-treatment group was 2.34, while the mean values in the NAC, PBG30 and PGB60 treatment groups were 1.68, 1.49 and 1.54, respectively.

A paired *t*-test was used to determine the statistical significance of TNF- α, NF-κB, and IL-6 values before and after ischemia-reperfusion. After comparing TNF-α blood values before ischemia and after reperfusion, significant *p*-values were found for the no-treatment group (0.006), NAC group (0.017), and PGB30 group (0.0004). When NF-κB values before and after ischemia-reperfusion were compared, *p*-values were found to be significant as 0.044 in the no-treatment group and 0.0004 in the NAC group. No statistically significant difference was found when IL-6 before and after ischemia-reperfusion values were compared.

TNF-α, NF-κB and IL-6 values before and after ischemia-reperfusion were compared between the groups by ANOVA test, and statistical significance was investigated. TNF-α and NF-κB values were statistically different between the groups (p=0.0003, p=0.001, respectively).

Tukey's test was performed as a post hoc test find the origin of the statistical difference between the groups. When the TNF-α value was compared between the no-treatment and the NAC, PGB30, and PGB60 treatment groups, a statistically significant difference was found (*p*-values 0.003, 0.01, 0.001, respectively). NF-κB value showed a significant difference between no-treatment and all treatment groups (*p*-values=0.012, 0.001, 0.006, 0.007 compared to groups no-treatment, NAC, PGB30 and PGB60, respectively).

Tissue Biochemical Tests

Tissue TNF-α, NF-κB, and IL-6 levels

After all tissues were collected at the end of the experiment, TNF-α, NF-κB, and IL-6 levels were analyzed, and biochemical and histological examination results of the tissues were evaluated.

Upon analyzing the tissue TNF-α, NF-κB, and IL-6 values were analyzed, it was observed that the no-treatment group exhibited the highest mean values for TNF-α, NF-κB and IL-6 when compared to the other groups. The mean TNF-α values in no-treatment, NAC, PBG30, and PGB60 were 87.13, 66.23, 73.53, and 71.59, respectively. Similarly, the mean values of NF-κB and IL-6 were lower in the NAC, PGB30 and PGB60 groups compared to the no-treatment group. IL-6 averages for the no-treatment, NAC, PGB30, and PGB60 groups were 2.14, 1.72, 1.72, 1.72 and 1.67, respectively. The PGB60 group had the lowest IL-6 average with pregabalin treatment at 60 mg/kg.

The ANOVA test was performed to compare the 5 groups among themselves, and a significant difference was found between the groups in terms of TNF-α and IL-6 values. The ANOVA test *p*-value for TNF-α was 0.0003, and the ANOVA test *p*-value for IL-6 was 0.046. A post hoc test was performed to find the origin of this difference between the groups. All groups were compared by the Tukey test for TNF-α and IL-6. When TNF-α values were compared between all groups, the no-treatment group showed a significant difference compared to all other groups. In terms of TNF-α values, the no-treatment group showed very high significance with the NAC group (p=0.0003), with PGB30 group (p=0.033) and the PGB60 group (p=0.011). When IL-6 values were compared between all groups, no significant difference was found between the groups in the Tukey test (Table III).

Histopathological Results

After histologic examination of the tissues, Suzuki scores within the groups showed that 62.5% in the no-treatment group had a Suzuki 2 score, while 37.5% had a Suzuki 1 score (Figures 2 and 3). In the NAC group, 25% had Suzuki 1 score, 62.5% had Suzuki 2 score, and 12.5% had Suzuki 3 score. In the PGB30 group, 50% had Suzuki 1, and 25% each Suzuki 2 and 3 scores. In the PGB60 group, 12.5% had Suzuki 1, 50% had Suzuki 2 and 37.5% had Suzuki 3 scores. When the distribution of Suzuki scores between the groups was analyzed statistically with Fisher's test, the

Table III. Comparison of the tissue TNF-α, NF-κB and IL-6 levels of the experimental groups.

	Group 1 (Sham)	Group 2 (No treatment)	Group 3 (NAS)	Group 4 (PGB30)	Group 5 (PGB60)	*p*-value*
TNF-α	67.9±13.9[a]	87.1±5.0[b]	66.2±6.3[a]	73.5±8.8[a]	71.6±7.9[a]	<0.001
NF-κB	1.2±0.4	1.8±0.4	1.4±0.4	1.3±0.5	1.4±0.3	0.120
IL-6	1.7±0.2[a]	2.1±0.4[a]	1.7±0.4[a]	1.7±0.2[a]	1.7±0.4[a]	0.046

Results were presented as mean±standard deviation. *: One-way ANOVA. There is no significant difference between groups that have the same superscript letter. Post-hoc comparisons for TNF-α: No treatment *vs.* Sham p=0.001, No treatment *vs.* AS p<0.001, No treatment *vs.* PGB30 p=0.033, No treatment *vs.* PGB60 p=0.011.

p-value was found to be 0.134 and not significant (Table IV).

In the studies performed for BCL-2 in tissues, no significant examination was performed, and staining could not be evaluated, possibly due to the error in staining caused by the kit, so the results were not interpreted in the study.

Caspase levels were examined in the tissues and scored as 0, 1, and 2. In the sham group, 62.5% had a score of 0, 12.5% had a score of 1 and 25% had a score of 2. In the no-treatment group, 87.5% had a score of 0, 12.5% had a score of 1. In the NAC group, 62.5% had a score of 0, and 37.5% had a score of 1. In the PGB30 group, 75% had a score of 0 and 25% had a score of 1. In the PGB60 group, 50% had a score of 0 and 50% had a score of 1.

When the statistical significance of the caspase score distribution between the groups was analyzed with Fisher's test, the p-value was not found to be significant at 0.362.

Discussion

Liver ischemic reperfusion injury is a prevalent issue encountered in various clinical contexts, and it can set off a cascading chain of events that could affect not only the liver but also the whole body, including the lungs, kidneys, intestines, adrenal glands, and pancreas[24]. In this study, our objective was to examine the potential of the pregabalin molecule, which has previously been studied[8-15] for its effects on skeletal muscle, spinal cord, and cerebral damage, to prevent liver ischemia-reperfusion injury. Similar to previous studies found in the literature, our study shows that the use of the pregabalin molecule was effective in preventing ischemic reperfusion injury. However, to the best of our knowledge, this is the only study to investigate the efficacy of pregabalin in liver ischemia-reperfusion.

Liver ischemia-reperfusion injury has been studied in many experimental animal models,

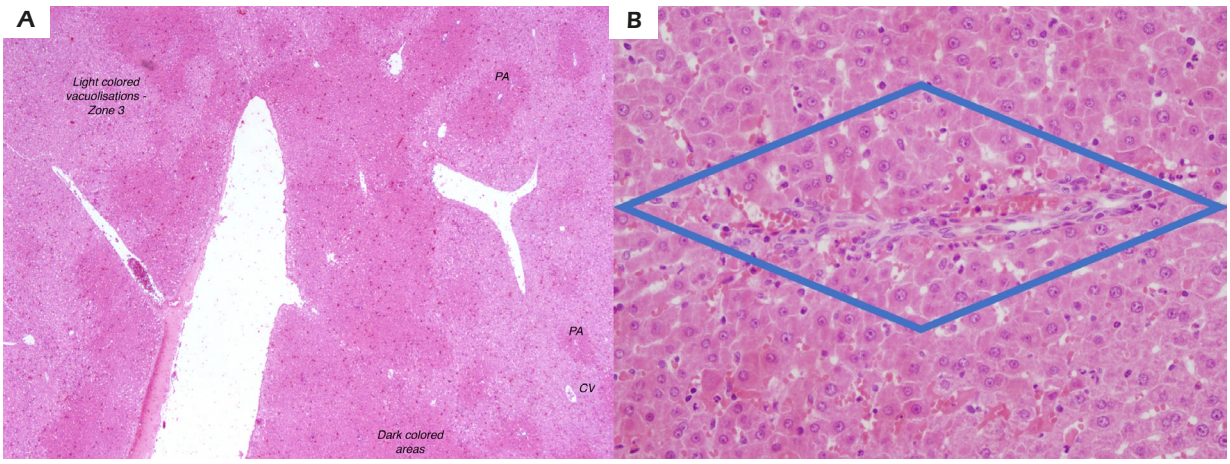

Figure 2. **A**, Light-colored areas (areas matching zone 3) swollen-damaged areas. **B**, Portal area containing artery-venous and biliary ducts. Ductal hepatocytes in the plate show focal necrosis and apoptosis. (Light microscopy with hematoxylin eosin staining - 40x enlargement).

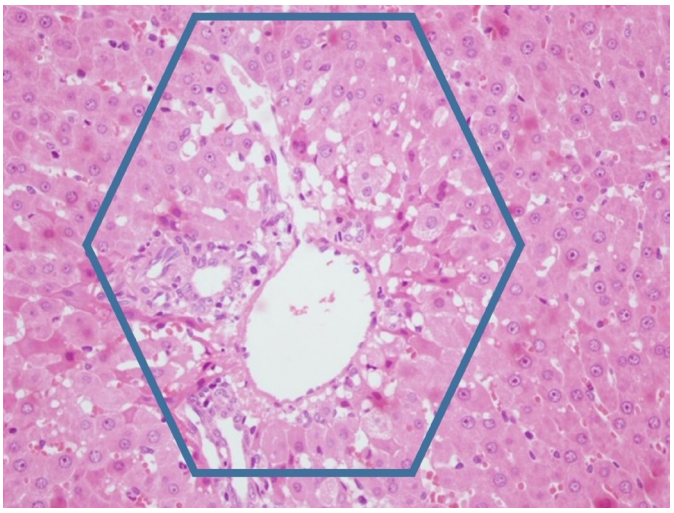

Figure 3. Extensive necrosis around the portal area, roof collapse. (Light microscopy with hematoxylin eosin staining - 40x enlargement).

including rats and mice, rabbits, dogs, and pigs. In this study, rats were preferred to facilitate manipulation during surgery. Prevention of ischemia-reperfusion injury has been extensively researched[25-27], with multiple molecules tested to determine their efficacy in reducing or exacerbating the damage. One of these methods is ischemic preconditioning to reduce the effects of warm ischemia[18,28]. In addition, preservation fluids have been employed to mitigate cold ischemia injury following organ removal. Furthermore, ongoing research[29-31] is exploring the therapeutic potential of novel molecules added to these fluids. A study[32] conducted on pigs used a hypothermic, oxygenated, portable perfusion machine to prevent ischemia and reperfusion injury to minimize damages after organ removal. Randomized controlled trials[33] are being carried out to investigate the application of machine perfusion in humans, resulting in favorable outcomes.

This injury has been linked to the complex signaling mechanisms of the liver's innate immune system, the molecular mechanism of ischemia and subsequent reperfusion injury, and the following cascade started by the release of DAMP and PAMP molecules[34]. The HMGB1 molecule and the TLR-4 receptor are among the most studied and identified DAMP molecules and pattern recognition receptors. In a study[35] conducted in mice, it was shown that a 60-70% decrease in nuclear HMGB1 expression in mice receiving HMGB1 siRNA treatment reduced hepatic ischemia-reperfusion injury and decreased hepatic TLR4, TLR2, TNF-α, IL-1β, IL-6, monocyte chemoattractant protein-1 (MCP-1), and receptor for advanced glycation end-products (RAGE) expression. Additionally, agents that prevent the binding of this molecule to the RAGE receptor by reducing the extracellular HMGB1 ratio have demonstrated efficacy in the prevention of isch-

Table IV. Comparison of histological examination of the experimental groups.

Scoring		Group 1 (Sham)	Group 2 (No treatment)	Group 3 (NAS)	Group 4 (PGB30)	Group 5 (PGB60)	*p*-value*
Suzuki	1	6 (75)	3 (37.5)	2 (25)	4 (50)	1 (12.5)	0.134
	2	2 (25)	5 (62.5)	5 (62.5)	2 (25)	4 (50)	
	3	0 (0)	0 (0)	1 (12.5)	2 (25)	3 (37.5)	
Caspase	0	5 (62.5)	7 (87.5)	5 (62.5)	6 (75)	4 (50)	0.362
	1	1 (12.5)	1 (12.5)	3 (37.5)	2 (25)	4 (50)	
	2	2 (25)	0 (0)	0 (0)	0 (0)	0 (0)	

Results were presented as frequency (%). *Fisher's Exact test.

emia-reperfusion[36,37]. Several studies[38-40] have shown that HMGB1 is effective in ischemia-reperfusion injury through TLR-4 receptor and NF-κB pathway together with TLR-4. It was observed[41] that HMGB1 release was decreased by TLR-4 receptor antagonism, and ischemia-reperfusion injury was reduced by blocking this interaction. In another study[14] investigating its role in preventing hyperglycemic brain damage and neurotoxicity in rats, low levels of HMGB1, TLR4, NF-κB, IL-1β, and TNF-α expression and improved neurological outcomes were observed in rats treated with pregabalin. Since the HMGB1-initiated cascade is an effective pathway in liver ischemia-reperfusion and the pregabalin molecule is active in this pathway, we aimed to investigate its role in preventing liver damage in our study. This effect of pregabalin has never been studied before.

In our study, in the no-treatment group, which underwent ischemia and reperfusion without any therapeutic agent, the AST levels in the blood collected at the end of reperfusion showed an increase compared to the samples collected before ischemia. The increased AST values observed after 1 hour of ischemia, followed by 2 hours of reperfusion, indicate the expected liver damage and efficacy of our experimental model. These findings align with previous research performed in our clinic and in accordance with existing literature[20-23].

The NAC treatment served as the medical control group and the established gold standard antioxidant for addressing ischemia-reperfusion injury. NAC treatment has demonstrated[42,43] the ability to reduce and inhibit glutathione, malondialdehyde, serum alanine aminotransferase levels, histopathological changes, caspase-3 activity, post-reperfusion apoptosis, and endoplasmic reticulum stress levels caused by reactive oxygen species. We aimed to investigate the effectiveness of pregabalin treatment to prevent oxidative damage compared to NAC, which is known to be effective in many animal studies in the literature, particularly in liver ischemia-reperfusion. NAC treatment showed a statistically significant decrease in AST, ALT, and TNF-α levels. In addition, there was a statistically significant decrease in tissue TNF-α and NF-κB levels in the NAC-treated group.

When pregabalin treatment was evaluated, specifically on AST results, it was observed that the highest significant difference in *p*-values was observed in the 60 mg/kg pregabalin group, then in the NAC group, and finally in the 30 mg/kg pregabalin treatment group. ALT levels also showed a decrease in the treatment groups similar to AST levels. The change in ALT levels was highly significant in the NAC group and highly significant in the 60 mg/kg pregabalin group. These results suggest that the effectiveness of pregabalin may surpass that of NAC at a dosage of 60 mg/kg, possibly through divergent mechanisms of action.

When examining the TNF-α, NF-κB, and IL-6 levels obtained from the blood samples before and after ischemia-reperfusion, the mean difference was higher in the no-treatment group compared to the NAC, PGB30, and PGB60 treatment groups. When these results were analyzed, it was found that TNF-α results showed the most significant difference among all groups in the PGB60 group. 60 mg/kg pregabalin was superior to 30 mg/kg pregabalin and the NAC control treatment. The results of NF-κB levels did not show a parallel effect with TNF-α results. NAC treatment was found to be more significant in comparison to 30 mg/kg and 60 mg/kg pregabalin treatments. However, the regression effect of pregabalin treatment on NF-κB results was yet again confirmed. The IL-6 results were not statistically significant.

Tissues collected at the conclusion of the experiment underwent histological examination in a blinded manner. Ischemia-reperfusion injury was microscopically scored to assess the extent of congestion, vacuolization, and necrosis. The total Suzuki score was then calculated. When the Suzuki scores between groups were analyzed by the Fisher's test, the distribution was not statistically significant, which might be attributed to the small sample size of our study group. When the caspase levels were examined, and the statistical significance of the score distribution was analyzed with the Fisher's test in the specimens scored as 0, 1, and 2 in terms of caspase, the *p*-value was not significant at 0.362. In view of the underlying cause of these results, the hematoxylin and eosin staining and the immunohistochemical staining were performed by automated machines so that human error is not taken into account. Since all tissues were examined by the same pathologist and blinded, there was no observer bias in the evaluation of the tissues. While there was an increase in markers in the biochemical assays, the lack of histologically similar damage may be due to the animals not being alive after reperfusion, so that the damage was not yet localized at the tissue level. Moreover, the overall duration of the experiment may not have been long enough to demonstrate this damage.

In this experiment, we demonstrated that the comparable efficacy of pregabalin and NAC in preventing ischemia-reperfusion injury. Addition-

ally, pregabalin showed superiority over NAC in specific parameters, potentially attributed to distinct mechanisms of action. The molecule pregabalin, which has also been shown to have beneficial effects on wound healing[44], is metabolized without imposing an additional burden on the liver. It has also been shown[45] to prevent cognitive decline after abdominal surgery in rats. After liver resection surgery and liver transplantation, patients often complain of excruciating pain that is poorly controlled with the use of analgesics. Finding the optimal analgesic becomes a challenge when considering the limited number of analgesics usable in such patients without causing additional liver stress. Given its protective properties against ischemia-reperfusion injury, cognitive benefits, wound healing effects, and non-metabolism in the liver, it would be interesting to undertake clinical studies to verify the efficacy and safety of this drug in the particular field of liver surgery.

Conclusions

In the rat ischemia-reperfusion model, we found that in the absence of any therapeutic agent, ischemia-reperfusion injury to the liver is severe and can be detected through biochemical markers in both blood and tissue. The ischemia-reperfusion cascade may be amenable to intervention with NAC. We also found that pregabalin molecule was effective in interrupting ischemia-reperfusion injury and 60 mg/kg pregabalin treatment was significantly superior to N-acetylcysteine treatment and 30 mg/kg pregabalin treatment in preventing ischemia-reperfusion injury according to AST levels among biochemical markers and according to blood TNF-α levels. According to these results, pregabalin treatment was effective in preventing liver ischemia-reperfusion injury, it was superior to N-acetylcysteine when evaluated by certain parameters, and 60 mg/kg pregabalin treatment was superior to 30 mg/kg pregabalin treatment. In line with these findings, it was concluded that further studies should be conducted to determine the most effective dose of the pregabalin molecule, the pathways of its action, and its potential clinical use in liver surgeries.

Ethics Approval
The Ethical Consent of the study was approved by Istanbul University Animal Experiments Ethics Committee (İstanbul Üniversitesi Hayvan Deneyleri Etik Kurulu - HADYEK) with acceptance number 2018/25.

Informed Consent
Not applicable.

Availability of Data and Materials
All data necessary to support the protocol is available upon reasonable request.

Conflicts of Interest
All the authors declare that they have no conflict of interest.

Funding
This research was funded by Istanbul University Scientific Research Projects (İstanbul Üniversitesi Bilimsel Araştırma Projeleri - BAP) with the project code TTU-2018-31521.

Authors' Contributions
VU designed the experiment, conducted the experiment, wrote the main manuscript text, tables, and figures, and revised the text. NK provided the pathological resources and performed the histopathological examination and analysis, wrote the pathological findings and reviewed the paper. HU provided the resources and performed the biochemical analysis, and interpreted the pathological findings and reviewed the paper. EG worked on the conceptualization of the study, designed the experiment, wrote and revised the text. All authors approved of the publication.

ORCID ID
Veysel Umman: 0000-0003-1760-7346
Nuray Kepil: 0000-0001-5494-6422
Hafize Uzun: 0000-0002-1347-8498
Ertugrul Goksoy: 0000-0003-2306-718X

References

1) Zhao Y, Cai H, Zhou P, Lin S, Pan Y, Liang X. Protective effect of ulinastatin on hepatic ischemia reperfusion injury through autophagy activation in Chang liver cells. J Cell Biochem 2019; 120: 14960-14970.
2) Ramalho FS, Fernandez-Monteiro I, Rosello-Catafau J, Peralta C. Hepatic microcirculatory failure. Acta Cir Bras 2006; 21: 48-53.
3) Elias-Miro M, Massip-Salcedo M, Jimenez-Castro M, Peralta C. Does adiponectin benefit steatotic liver transplantation? Liver Transpl 2011; 17: 993-1004.
4) Huang H, Tohme S, Al-Khafaji AB, Tai S, Loughran P, Chen L, Wang S, Kim J, Billiar T,

Wang Y, Tsung A. Damage-associated molecular pattern-activated neutrophil extracellular trap exacerbates sterile inflammatory liver injury. Hepatology 2015; 62: 600-614.

5) Papadopoulos D, Siempis T, Theodorakou E, Tsoulfas G. Hepatic ischemia and reperfusion injury and trauma: current concepts. Arch Trauma Res 2013; 2: 63-70.
6) Tacchini L, Radice L, Pogliaghi G, Bernelli-Zazzera A. Differential activation of heat shock and nuclear factor kappaB transcription factors in postischemic reperfused rat liver. Hepatology 1997; 26: 186-191.
7) Mendes-Braz M, Elias-Miró M, Jiménez-Castro MB, Casillas-Ramírez A, Ramalho FS, Peralta C. The current state of knowledge of hepatic ischemia-reperfusion injury based on its study in experimental models. J Biomed Biotechnol 2012; 2012: 298657.
8) Aşcı S, Demirci S, Aşcı H, Doğuç DK, Onaran İ. Neuroprotective Effects of Pregabalin on Cerebral Ischemia and Reperfusion. Balkan Med J 2016; 33: 221-227.
9) Kazanci B, Ozdogan S, Kahveci R, Gokce EC, Yigitkanli K, Gokce A, Erdogan B. Neuroprotective Effects of Pregabalin Against Spinal Cord Ischemia-Reperfusion Injury in Rats. Turk Neurosurg 2017; 27: 952-961.
10) Wang RR, Lou GD, Yu J, Hu TT, Hou WW, Chen Z, Zhang SH, Seltzer Z. Oral Administration of Pregabalin in Rats before or after Nerve Injury Partially Prevents Spontaneous Neuropathic Pain and Long Outlasts the Treatment Period. Pharmacology 2016; 97: 251-258.
11) Ha KY, Carragee E, Cheng I, Kwon SE, Kim YH. Pregabalin as a neuroprotector after spinal cord injury in rats: biochemical analysis and effect on glial cells. J Korean Med Sci 2011; 26: 404-411.
12) Yoon JS, Lee JH, Son TG, Mughal MR, Greig NH, Mattson MP. Pregabalin suppresses calcium-mediated proteolysis and improves stroke outcome. Neurobiol Dis 2011; 41: 624-629.
13) Celik M, Kose A, Kose D, Karakus E, Akpinar E, Calik M, Dostbil A, Calikoglu C, Aksoy M, Ozel L. The double-edged sword: effects of pregabalin on experimentally induced sciatic nerve transection and crush injury in rats. Int J Neurosci 2015; 125: 845-854.
14) Song Y, Jun JH, Shin EJ, Kwak YL, Shin JS, Shim JK. Effect of pregabalin administration upon reperfusion in a rat model of hyperglycemic stroke: Mechanistic insights associated with high-mobility group box 1. PLoS One 2017; 12: e0171147.
15) Ozturk L, Dogan HT, Kilicarslan A, Aydin ME, Ozer A, Demirtas H, Kilic Y, Iriz E, Kucuk A, Bayraktar AC, Kavutcu M, Arslan M. Effect of different doses of pregabalin on skeletal muscle ischaemia-reperfusion injury in rats. Bratisl Lek Listy 2017; 118: 417-422.
16) Murawiec S, Chudek J, Nieves W, Almgren-Rachtan A, Jedrzejczak J. Increasing the dosage of pregabalin in patients with focal epilepsy decreases the frequency of seizures and ameliorates symptoms of anxiety, depression and insomnia. Eur Rev Med Pharmacol Sci 2020; 24: 13015-13024.
17) Ben-Menachem E. Pregabalin pharmacology and its relevance to clinical practice. Epilepsia 2004; 45: 13-18.
18) Turner PV, Brabb T, Pekow C, Vasbinder MA. Administration of substances to laboratory animals: routes of administration and factors to consider. J Am Assoc Lab Anim Sci 2011; 50: 600-613.
19) Yuan GJ, Ma JC, Gong ZJ, Sun XM, Zheng SH, Li X. Modulation of liver oxidant-antioxidant system by ischemic preconditioning during ischemia/reperfusion injury in rats. World J Gastroenterol 2005; 11: 1825-1828.
20) Ertürk S. Splenektomi işleminin karaciğer regenerasyonu üzerine etkileri. Ulusal Tez Merkezi. Gov.tr. Accessed September 21, 2023. Available at: https://tez.yok.gov.tr/UlusalTezMerkezi/tezDetay.jsp?id=OgeiUkz5.
21) Kaptanoglu L, Kapan M, Kapan S, Goksoy E, Oktar H. Effects of nimodipine and pentoxyfylline in prevention of hepatic ischemic damage in rats at normal and hypothermic conditions. Eur J Pharmacol 2008; 587: 253-256.
22) Aytac E. Karaciğer iskemi-reperfüzyon hasarında bakteriyel translokasyon ve oksidatif hasar. Ulusal Tez Merkezi. Gov.tr. Accessed September 21, 2023. Available at: https://tez.yok.gov.tr/UlusalTezMerkezi/tezDetay.jsp?id=OgeiUkz5-cOF1cNAOy_CnQ&no=OgeiUkz5-cOF1cNAOy_CnQ.
23) Tasci Y. Total hepatik vasküler oklüzyon ve pringle manevrası ile oluşturulan İskemi-Reperfüzyon modelinde oksidatif hasar ve karaciğer histopatolojisinin değerlendirilmesi. Ulusal Tez Merkezi. Gov.tr. Accessed September 21, 2023. Available at: https://tez.yok.gov.tr/UlusalTezMerkezi/tezDetay.jsp?id=R5wyRbqZ-62vYI61mwg0Pg&no=O-ZlO2wsyorm1fLwf5SyYfA.
24) Nastos C, Kalimeris K, Papoutsidakis N, Tasoulis MK, Lykoudis PM, Theodoraki K, Nastou D, Smyrniotis V, Arkadopoulos N. Global consequences of liver ischemia/reperfusion injury. Oxid Med Cell Longev 2014; 2014: 906965.
25) Aydin I, Sehitoglu I, Ozer E, Kalkan Y, Tumkaya L, Cure MC, Cure E. High dose zoledronic acid increases ischemia-reperfusion damage of the liver. Eur Rev Med Pharmacol Sci 2021; 25: 3567-3575.
26) Wang M, Zhang J, Zhang J, Sun K, Li Q, Kuang B, Wang MMZ, Hou S, Gong N. Methyl eugenol attenuates liver ischemia reperfusion injury via activating PI3K/Akt signaling. Int Immunopharmacol 2021; 99: 108023.
27) Zito G, Miceli V, Carcione C, Busà R, Bulati M, Gallo A, Iannolo G, Pagano D, Conaldi PG. Human Amnion-Derived Mesenchymal Stromal/Stem Cells Pre-Conditioning Inhibits Inflammation and Apoptosis of Immune and Parenchymal Cells in an In Vitro Model of Liver Ischemia/Reperfusion. Cells 2022; 11: 709.
28) Radojkovic M, Stojanovic M, Stanojevic G, Radojkovic D, Gligorijevic J, Ilic I, Stojanovic N. Isch-

emic preconditioning vs adenosine vs prostaglandin E1 for protection against liver ischemia/reperfusion injury. Braz J Med Biol Res 2017; 50: e6185.

29) Martins RM, Pinto Rolo A, Soeiro Teodoro J, Furtado E, Caetano Oliveira R, Tralhão JG, Marques Palmeira C. Addition of berberine to preservation solution in an animal model of ex vivo liver transplant preserves mitochondrial function and bioenergetics from the damage induced by ischemia/reperfusion. Int J Mol Sci 2018; 19: 284.

30) Pantazi E, Zaouali MA, Bejaoui M, Folch-Puy E, Ben Abdennebi H, Varela AT, Rolo AP, Palmeira CM, Roselló-Catafau J. Sirtuin 1 in rat orthotopic liver transplantation: an IGL-1 preservation solution approach. World J Gastroenterol 2015; 21: 1765-1774.

31) Franco-Gou R, Mosbah IB, Serafin A, Abdennebi HB, Roselló-Catafau J, Peralta C. New preservation strategies for preventing liver grafts against cold ischemia reperfusion injury. J Gastroenterol Hepatol 2007; 22: 1120-1126.

32) Compagnon P, Levesque E, Hentati H, Disabato M, Calderaro J, Feray C, Corlu A, Cohen JL, Ben Mosbah I, Azoulay D. An oxygenated and transportable machine perfusion system fully rescues liver grafts exposed to lethal ischemic damage in a pig model of DCD liver transplantation. Transplantation 2017; 101: e205-e213.

33) Parente A, Tirotta F, Pini A, Eden J, Dondossola D, Manzia TM, Dutkowski P, Schlegel A. Machine perfusion techniques for liver transplantation - A meta-analysis of the first seven randomized-controlled trials. J Hepatol 2023; S0168-8278: 00399.

34) Lu L, Zhou H, Ni M. Innate immune regulations and liver ischemia reperfusion injury. Transplantation 2016; 100: 2601-2610.

35) Zhao G, Fu C, Wang L, Zhu L, Yan Y, Xiang Y, Zheng F, Gong F, Chen S, Chen G. Down-regulation of nuclear HMGB1 reduces ischemia-induced HMGB1 translocation and release and protects against liver ischemia-reperfusion injury. Sci Rep 2017; 7: 46272.

36) Zhang W, Li F, Ye Y, Liu Y, Yu S, Cen C, Chen X, Zhou L, Tang X, Yu J, Zheng S. Isoglycyrrhizinate magnesium enhances hepatoprotective effect of FK506 on ischemia-reperfusion injury through HMGB1 inhibition in a rat model of liver transplantation. Transplantation 2017; 101: 2862-2872.

37) Tsaroucha AK, Valsami G, Kostomitsopoulos N, Lambropoulou M, Anagnostopoulos C, Christodoulou E, Falidas E, Betsou A, Pitiakoudis M, Simopoulos CE. Silibinin effect on Fas/FasL, HMGB1, and CD45 expressions in a rat model subjected to liver ischemia-reperfusion injury. J Invest Surg 2018; 31: 491-502.

38) Wang Y, Wu S, Yu X, Zhou S, Ge M, Chi X, Cai J. Dexmedetomidine protects rat liver against ischemia-reperfusion injury partly by the α2A-adrenoceptor subtype and the mechanism is associated with the TLR4/NF-κB pathway. Int J Mol Sci 2016; 17: 995.

39) Yang H, Zhou H, Zhuang L, Auwerx J, Schoonjans K, Wang X, Feng C, Lu L. Plasma membrane-bound G protein-coupled bile acid receptor attenuates liver ischemia/reperfusion injury via the inhibition of toll-like receptor 4 signaling in mice. Liver Transpl 2017; 23: 63-74.

40) Kamo N, Ke B, Ghaffari AA, Shen X, Busuttil RW, Cheng G, Kupiec-Weglinski JW. ASC/caspase-1/IL-1β signaling triggers inflammatory responses by promoting HMGB1 induction in liver ischemia/reperfusion injury. Hepatology 2013; 58: 351-362.

41) Mcdonald K-A, Huang H, Tohme S, Loughran P, Ferrero K, Billiar T, Tsung A. Toll-like receptor 4 (TLR4) antagonist eritoran tetrasodium attenuates liver ischemia and reperfusion injury through inhibition of high-mobility group box protein B1 (HMGB1) signaling. Mol Med 2015; 20: 639-648.

42) Sun Y, Pu L-Y, Lu L, Wang X-H, Zhang F, Rao JH. N-acetylcysteine attenuates reactive-oxygen-species-mediated endoplasmic reticulum stress during liver ischemia-reperfusion injury. World J Gastroenterol 2014; 20: 15289-15298.

43) Baumann J, Ghosh S, Szakmany T, Jancso G, Ferencz A, Roth E, Bogar L. Short-term effects of N-acetylcysteine and ischemic preconditioning in a canine model of hepatic ischemia-reperfusion injury. Eur Surg Res 2008; 41: 226-230.

44) Sarıtaş TB, Korkmaz M, Sevimli A, Sarıtaş ZK. Comparison of the effects of gabapentin and pregabalin on wound healing in rats: Effect of gabapentinoids on wound healing. Int Wound J 2016; 13: 748-753.

45) Kawano T, Eguchi S, Iwata H, Yamanaka D, Tateiwa H, Locatelli FM, Yokoyama M. Pregabalin can prevent, but not treat, cognitive dysfunction following abdominal surgery in aged rats. Life Sci 2016; 148: 211-219.