The effect of Liv-52 on liver ischemia reperfusion damage in rats

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Abstract

Background: Liver ischemia reperfusion (I/R) damage is frequently seen in clinical hepatobiliary surgeries and there is no effective treatment for it. Liv-52, known to have hepatoprotective effects, is a natural antioxidant drug licensed by Ministry of Health of India. Therefore, the aim of our study is to investigate the effect of Liv-52 on liver damage induced by I/R in rats.

Methods: Albino Wistar male rats were divided into three groups; liver I/R (IR), 20 mg/kg Liv-52 + liver ischemia reperfusion (LIR) and sham operation applied to control group (HG). Liv-52 was administered to the LIR group (n-6) one hour prior to I/R application and distilled water was given orally to IR (n-6) and HG (n-6) groups as a solvent. In animals, ischemia was determined as one hour, and reperfusion was identified as six hours.

Results: Increased levels of alanine aminotransferase, aspartate aminotransferase and lactate dehydrogenase, malondialdehyde, myeloperoxidase, and decreased levels of superoxide dismutase, and glutathione related enzymes caused by I/R application have been converged to healthy group level with Liv-52 treatment and the damage occurred in liver tissue has been improved histopathologically.

Conclusions: Liv-52 may be beneficial for preventing liver I/R damage in pre-surgery application.

Background

Hepatic portal occlusion operation or pringle maneuver interrupting portal vein and hepatic artery entries is temporarily performed to provide bleeding control during operations such as liver resection, transplantation and hepatobiliary surgery[1]. This method is widely used since it is clinically simple, practical and effective. However, the significant risk of this method is a liver damage afterwards due to ischemia reperfusion (I/R)[2]. The formation of excessive free radicals is responsible for the reperfusion damage and for the molecular oxygen that is presented in abundant amounts with arterial blood to the ischemic tissue[3]. Free oxygen radicals cause cell membrane lipids to oxidize and allow toxic products such as malondialdehyde (MDA) to form from lipids[4]. This information obtained from the literature suggests that I/R damage is a pathological process that begins with the asphyxiation of the tissues and continues with the production of free oxygen radicals[5]. Therefore, it is being thought that antioxidant drugs may be useful for preventing I/R damage. In this study, we will examine the effects of Liv–52 which is licensed as an ayurvedic medicine by the AYUSH department, a drug regulatory authority of the Indian Ministry of Health against I/R liver damage. Each tablet of Liv–52 contains medicinal plants extracts at specified doses: 65 mg of Capparis Spinosa, 65 mg of Cichorium Intybus, 33 mg of Mandur Bhasma, 32 mg of Solanum Nigrum, 32 mg of Terminalia Arjuna, 16 mg of Cassia Occidentalis, 16 mg of Achillea Millefolium and 16 mg of Tamarix Gallica[6]. It has been reported that Liv–52 protects liver against toxicity of ethanol by preventing the increase of lipid peroxidation and the reduction of antioxidants in rat liver tissue[7].

The information obtained suggests that Liv–52 may be useful during I/R treatment or may protect the liver tissue from I/R damage. There was no information on the protective effect of Liv–52 against liver I/R damage in the literature scan. For this reason, the aim of our study is to investigate the effect of Liv–52 on the liver damage induced by I/R in rats biochemically and histopathologically.

Methodology

Animals

The animals were obtained from Atatürk University Medical Experimental Application and Research Center. A total of 18 albino wistar male rats with the weight ranging between 250–270 grams (8–10 months) were used in the experiment. Animals were hosted in standard cages with 6 rats per cage and fed ad-libitum in groups. They were maintained in a 12:12-h light–dark (LD) cycle and at constant temperature (22°C ± 1°C). Animal experiments were performed in accordance with the National Guidelines for the Use and Care of Laboratory Animals and were approved by the local animal ethics committee of Ataturk University, Erzurum, Turkey (Ethics Committee Number: 77040475–000-E.1700216877, Dated:03.08.2017).

Chemicals

Of the chemical substances used for the experiments, thiopental sodium was provided by IE Ulagay-Turkey. Liv–52 was provided by Himalaya drug –India.

Experimental groups

Experimental animals were randomly divided into three groups, with 6 rats in each group as follows: liver ischemia reperfusion (IR), 20 mg/kg Liv–52+ liver ischemia reperfusion (LIR) and the sham group (HG).

Experimental procedure

The surgical interventions on rats were carried out under sterile conditions; anesthesia was provided by giving 25 mg/kg of intraperitoneal (ip) thiopental sodium and administering xylazine by inhalation at appropriate intervals. One hour before thiopental sodium anesthesia, a dose of 20 mg/kg of Liv–52 (n–6) was given to the LIR animal group orally with a catheter. Distilled water as solvent was treated to the IR and HG rat groups with the same method. After the injection of thiopental sodium, the rats were kept waiting for the appropriate surgery period to occur. The period when the animals are motionless in the supine position is considered to be an appropriate period for surgical intervention. During this process, all the rats were brought to supine position and laparotomy was performed by the 3,5–4 cm long vertical dissection of the abdomen's anterior portion. Later, an hour of ischemia and six hours of reperfusion were

performed by placing clamps on the hepatic artery, portal vein and gail ductus in order to create total hepatic ischemia (excluding the HG group). At the end of this period, blood samples were taken from the tail veins of the animals for the measurement of Alanine aminotransferase (ALT), Aspartate aminotransferase (AST) and Lactate dehydrogenase (LDH) activities. Later, rat groups were killed with high dosage of anesthesia (50 mg/kg i.p. thiopental sodium IE Ulagay-Türkiye) and their liver tissues were removed. Oxidant antioxidant parameters were determined from the removed tissues and the tissues were examined histopathologically. The biochemical results obtained from the LIR group were compared with the results obtained from IR and HG groups.

Biochemical analyzes

In this part, 0.2 mg of whole liver tissue was weighed for each liver. The samples were homogenized in ice with 2-mL buffers (consisting of 0.5% HDTMAB [0.5% hexa desil tri methyl ammonium bromide] pH: 6 potassium phosphate buffer for myeloperoxidase analyze, consisting of 1.15% potassium chloride solution for thio barbituric acid reactions (TBARS) analysis and pH: 7.5 phosphate buffer for the superoxide dismutase, total glutathione analysis. Then, they were centrifuged at 4 °C, 10.000 rpm for 15 minutes. The supernatant part was used as the analysis sample.

Serum ALT, AST and LDH measurements

Venous blood samples were collected into tubes without anticoagulant. Serum was separated by centrifugation after clotting and stored at −80 °C until assay. Serum AST and ALT activities as liver function tests, and LDH activity as a marker of tissue injury, were measured spectrophotometrically on a Cobas 8000 (Roche) autoanalyser using commercially available kits (Roche Diagnostics, GmBH, Mannheim, Germany).

MDA analysis

The concentrations of liver lipid peroxidation were determined by estimating MDA using the thio barbituric acid test[8]. The rat livers were rinsed with cold saline. The corpus mucosa was scraped, weighed, and homogenized in 10 ml of 100 g/L KCl. The homogenate (0.5 ml) was added to a solution containing 0.2 ml of 80 g/l sodium lauryl sulfate, 1.5 ml of 200 g/l acetic acid, 1.5 ml of 8 g/L 2-thiobarbiturate, and 0.3 ml distilled water. The mixture was incubated at 98°C for 1 h. Upon cooling, 5 ml of n-butanol:pyridine (15:l) was added. The mixture was vortexed for 1 min and centrifuged for 30 min at 4000 rpm. The absorbance of the supernatant was measured at 532 nm. The standard curve was obtained by using 1,1,3,3- tetramethoxypropane.

MPO analysis

The activity of myeloperoxidase (MPO) in the total homogenate was measured according to the method of Wei and Frenkel with some modifications[9]. The sample was weighed and homogenized in 2 ml of 50 mmol/L phosphate buffer containing 0.5% hexadecyltrimethyl ammonium bromide (HDTMAB) and centrifuged at 3500 rpm for 60 minutes at 4°C. The supernatant was used to determine MPO activity using 1.3 mL 4-aminoantipyrine–2% phenol (25 mM) solution. 25 mmol/L 4-aminoantipyrine–2% phenol solution and 0.0005% 1.5 mL H_2O_2 were added and equilibrated for 3–4 minutes. After establishing the basal rate, a 0.2 mL sample suspension was added and quickly mixed. Increases in absorbance at 510 nm for 4 minutes at 0.1-min intervals were recorded. Absorbance was measured at 412 nm using a spectrophotometer.

SOD analysis

Measurements were performed according to the method of Sun et al[10]. When xanthine is converted into uric acid by xanthine oxidase, superoxide dismutase (SOD) forms. If nitro blue tetrazolium (NBT) is added to this reaction, SOD reacts with NBT and a purple-colored formazan dye occurs. The sample was weighed and homogenized in 2 ml of 20 mmol/L phosphate buffer containing 10 mmol/L EDTA at pH 7.8. The sample was centrifuged at 6000 rpm for 10 minutes and the brilliant supernatant was used as assay sample. The measurement mixture containing 2450 µL measurement mixture (0.3 mmol/L xanthine, 0.6 mmol/L EDTA, 150µmol/L NBT, 0.4 mol/L Na_2CO_3, 1 g/l bovine serum albumin), 500 µL supernatant and 50 µL xanthine oxidase (167 U/l) was vortexed. Then it was incubated for 10 min. At the end of the reaction, formazan occured. The absorbance of the purple-colored formazan was measured at 560 nm. As more of the enzyme exists, the least $O2^-$ radical that reacts with NBT occurs.

tGSH analysis

The amount of GSH in the total homogenate was measured according to the method of Sedlak and Lindsay with some modifications[11]. The sample was weighed and homogenized in 2 mL of 50 mmol/L Tris–HCl buffer containing 20 mmol/L EDTA and 0.2 mmol/L sucrose at pH 7.5. The homogenate was immediately precipitated with 0.1 mL of 25% trichloroacetic acid, and the precipitate was removed after centrifugation at 4200 rpm for 40 min at 4 °C and the supernatant was used to determine GSH level. A total of 1500 µL of measurement buffer (200 mmol/L Tris–HCl buffer containing 0.2 mmol/L EDTA at pH 7.5), 500 µL supernatant, 100 µL DTNB (10 mmol/L) and 7900 µL methanol were added to a tube and vortexed and incubated for 30 min in 37°C. 5,5-Dithiobis (2-nitrobenzoic acid) (DTNB) was used as an chromogen and it formed a yellow-colored complex with sulfhydry groups. The absorbance was measured at 412 nm using a spectrophotometer (Beckman DU 500, USA). The standard curve was obtained by using reduced glutathione.

GPO analysis

GPO activity was determined according to the method of Lawrence and Burk[12]. After tissue homogenization, supernatant was used for GPO measurement. After the KH2PO4, EDTA, GSH, B-NADPH, NaN3, and GR addition, mixture was incubated. As soon as H2O2 was added, chronometer was on, and the absorbance at 340 nm was recorded for 5 min every 15 s.

GSHR analysis

GR activity was determined spectrophotometrically by measuring the rate of NADPH oxidation at 340 nm according to Carlberg and Mannervik method[13]. After tissue homogenization, supernatant was used for GR measurement. After the NADPH and GSSG addition, chronometer was on and absorbance was measured for 5 min by 30-min intervals at 340 nm spectrophotometric methods.

GST activity

GST activity was determined by Habig and Jakoby[14]. Briefly, the enzyme's activity was assayed spectrophotometrically at 340 nm in a 4-ml cuvette containing 0.1 M PBS (pH 6.5), 30 mM GSH, 30 mM 1-chloro–2,6-dinitrobenzene, and tissue homogenate.

Histopathologic examination

Liver tissues obtained from the rats were fixed in 10% formalin solution for 24 hours. After routine tissue processing, 4 micron thick sections were obtained from the paraffin blocks and were stained with Hematoxylin&Eosin. All sections were examined under the light microscope (Olympus BX 52, Tokyo, Japan) by two pathologists who do not know which treatment protocol is used.

Statistical analyses

The results obtained from the experiments are depicted as "mean ± standard error " (x ± SEM). Normality of the data have been tested with Shapiro-Wilk test. All the parameters showed normal distribution. The significance level of the inter-group difference was identified using one-way ANOVA test. Then, Fisher's post-hoc Bonferroni was performed. All statistical analyses were performed using "IBM SPSS Statistics Version 22" program and $p<0.05$ was considered significant.

Results

Biochemical results

Effect of Liv–52 supplementation and I/R on liver enzymes

As seen in Figure 1, the AST, ALT and LDH levels used in evaluating the liver functions were found to have statistically significant difference ($p<0.001$) between the healthy group, and I/R group, also between I/R group and Liv–52 group ($p<0.001$). While the difference between the healthy group and the Liv–52 group was not significant ($p>0.05$).

Effect of Liv–52 supplementation on lipid peroxidation and antioxidant status in liver tissue

As seen in Figure 2A, the liver MDA level increased with I/R application according to the healthy group ($p <0.001$). In the group treated with Liv–52, this rise due to I/R was suppressed and the level was decreased. The difference between I/R group and Liv–52 group was significant ($p<0.001$). There was no significant difference between healthy group and Liv–52 group ($p> 0.05$). MPO activity was increased due to I/R application according to the healthy group ($p <0.001$). In the Liv–52 group, MPO level almost decreased to the level of the healthy group ($p> 0.05$) and the difference between I/R group and Liv–52 group was significant ($p<0.001$) (Figure 2B). SOD activity decreased with I/R application according to healthy group ($p< 0.001$). Also the difference between I/R group and Liv–52 group was significant ($p<0.001$) and Liv52 suppressed the decrease in SOD activity and elevated SOD activity to nearly activity of the healthy group (Figure 2C).

Effect of Liv–52 supplementation on tGSH level and activities of glutathione-dependent enzymes in liver tissue

As shown in Figure 3A, tGSH level decreased with I/R application compared to the healthy group ($p <0.001$). Liv–52 increased the level of tGSH again. There is a significant difference between I/R group and the Liv–52 applied group ($p<0.001$).

GPO activity was 28,67±0,88 U/g protein in healthy group and it decreased with I/R application ($p <0.001$). This decline due to I/R was suppressed by the application of Liv–52 and increased again, there is a significant difference ($p<0.001$) between these two groups (Figure 3B).

As it can be seen in Figure 3C, liver GSHRd activity decreased in the I/R group according to the healthy group ($p<0.001$). This value increased by Liv–52 application and the difference between Liv–52 and the healthy groups was not significant ($p>0.05$) and the difference between I/R group and Liv–52 group was significant ($p<0.001$)

GST activity decreased with I/R application compared to the healthy group and there was a significant difference between these two groups ($p<0.001$). GST activity increased with Liv–52 application, and the difference between Liv–52 group and the healthy group was not significant ($p>0.05$) but there was a significant difference between I/R and Liv–52 groups ($p<0.001$) (Figure 3D).

Histopathological results

In Figure 4A, normal liver parenchyma, portal vein, artery, gail ductus and central vein of healthy group are seen. In the liver tissue of the I/Rapplied group, common hemorrhage, edema, dilated congested blood vessels, dilated congested sinusoids, cells showing balloon degeneration and polymorphic leukocytes are observed (Figure 4B, 4C). Near-normal liver tissue is observed in the group treated with Liv–52 except for slight sinusoidal dilation and congestion in the liver tissue (Figure 4D).

Discussion

Hemorrhage control procedures performed during surgical operations on blood-rich liver cause I/R damage[15]. In recent years, clinical researches made on the field of hepatic surgery have focused on how to reduce blood loss and I/R damage more safely[16]. Especially lately, the experimental application of antioxidants on liver I/R damage is also important for understanding the underlying mechanisms. In this study, the effect of the antioxidant featured Liv–52 against liver I/R damage has been investigated and our results showed biochemically and histopathologically that Liv–52 has protective effect on preventing oxidative stress and apoptotic tissue damage formed by liver I/R damage.

I/R damage leads to the release of enzymes such as ALT which is a specific indicator for cytolysis marker and hepatic parenchymal damage and AST which is a nonspecific marker[17] . Studies have shown that LDH levels reflecting ALT, AST, and acute liver damages are significantly increased compared to normal group after I/R application [2]. Similarly, in our study, I/R application significantly increased ALT, AST and LDH levels. It is known that Liv–52, a herbal formulation rich in phenolic compounds, significantly reduces infections caused by biological agents and liver damage formed after chemical toxins in humans and experimental animals [18, 19]. In literature, liver damage that is formed by isoniazide and rifampicin has been reported to reduce liver enzymes to levels close to the control group[20]. Similarly, in our study, increase in liver enzymes due to I/R damage decreased to the level of the healthy group with the application of Liv–52.

It is also known that MDA which is a marker of lipid peroxidation of Liv–52 and SOD levels which clear intracellular free radicals, are brought to the normal control group level by suppressing the increases in hepatotoxicity formed by tert-butyl hydroperoxide[19]. It has been suggested that liver ischemia affects mitochondrial energy synthesis and electron transport in the respiratory chain as a cause of hypoxia[21], thereby produces a large number of free oxygen radicals that trigger cell damage through lipid peroxidation in biological membranes[22].

The studies carried out have shown that I/R application increases oxidative stress parameters such as MDA as well as MPO in the liver and decreases SOD activity[23, 24]. In our study, I/R application increased MDA and MPO levels and decreased SOD activity as well, in accordance with the literature. The Liv–52 application reversed this situation and showed hepatoprotective effect. In literature, there has been no study showing the effect of Liv–52 on MPO. Our study indicates that Liv–52 alleviates the

inflammatory reaction in the rat's damaged liver tissue which is I/R-induced by reducing the hepatic MPO activity.

As is known, GSH which is an effective antioxidant that protects cells from the damage of the free radicals formed by I/R, is present in high concentrations in hepatocytes[25]. Deng et al. reported that the GSH value of the I/R group significantly decreased compared to the normal group and that the GSH value of the I/R + melatonin applied group significantly increased compared to the I/R group after reperfusion, at 2nd, 4th and 8th hours[2]. In a toxicity study, it was shown that Cu^{2+} application reduced GSH in HepG2 cells by 86% and that Liv 52 application significantly increased GSH levels in toxic conditions induced by Cu^{2+} by 74%. In our study, the significantly decreased level of GSH compared to I/R and healthy groups significantly increased with the application of Liv–52 in accordance with literature.

Reductions in the activities of GPO, GSHRd and GST which are glutathione-related antioxidant enzymes due to liver I/R damage have been reported in studies carried out[26, 27]. GPO, GSHRd, and GST activities in the I/R damaged liver tissue significantly decreased compared to the healthy group in our study, in accordance with literature. The Liv–52 application suppressed this decline and brought the value to a level close to the healthy group. In literature, it has been shown that Liv–52 significantly increased the levels of serum GPO, reduced glutathione and GST which had been reduced in the paracetamol-induced liver toxicity group[28]. This information supports our findings as well.

We also examined histopathologically the effects of Liv–52 on the liver damage induced by I/R. There are publications showing that I/R application causes degeneration in hepatocyte, vein and intercellular edema and congested sinusoidal damage in the rat liver histopathologically[29]. It has also been reported that, by the application of Liv–52, hepatocytes become normal histopathologically in the cadmium-induced hepatic toxicity and Liv–52 reverses cadmium-induced hepatic damage[30]. In our study, findings such as hemorrhage, edema, dilated congested blood vessels caused by I/R were reduced with the application of Liv–52 and near-normal liver tissue was observed.It can be seen with this information that Liv–52, which is known to have hepatoprotective effects previously, also has protective effects on liver I/R damage.

Conclusion

It has been understood that I/R application leads to liver dysfunction and oxidative liver damage. I/R application had changed the oxidant antioxidant balance in favor of oxidants. Liv–52 had prevented the I/R-associated hepatic dysfunction and inhibited the change of oxidant antioxidant balance of oxidants to avoid the deterioration. Based on the literature information and our experimental results, we can say that Liv–52 has a therapeutic effect potency that reduces hepatic damage induced by I/R.

Abbreviations

ischemia reperfusion (I/R); 20 mg/kg Liv–52 + liver ischemia reperfusion (LIR); control group (HG); malondialdehyde (MDA); Alanine aminotransferase (ALT); Aspartate aminotransferase (AST); Lactate dehydrogenase (LDH); Myeloperoxidase (MPO); superoxide dismutase (SOD); Glutathione s Transferase (GST); Glutathione reductase (GSHRd); Total glutathione (tGSH); Glutatione reductase (GSHR);

Declarations

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Not applicable.

Authors' contributions

OC, HE, FKC: conception and design of study

ABC, NK, AOB: acquisition of data

BS, HS: analysis and interpretation of data

RM: drafting the manuscript,

KP, EK: revising the manuscript critically for important intellectual content.

All authors have read and approved the manuscript

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Availability of data and materials

There is no data other than the data given in the article.

Ethics approval and consent to participate

This experiment was performed in accordance with the National Guidelines for the Use and Care of Laboratory Animals and the study was approved by the Animal Care and Use Committee of Ataturk University, Erzurum, Turkey (Ethics Committee Number: 77040475–000-E.1700216877, Dated:03.08.2017).

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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Figures

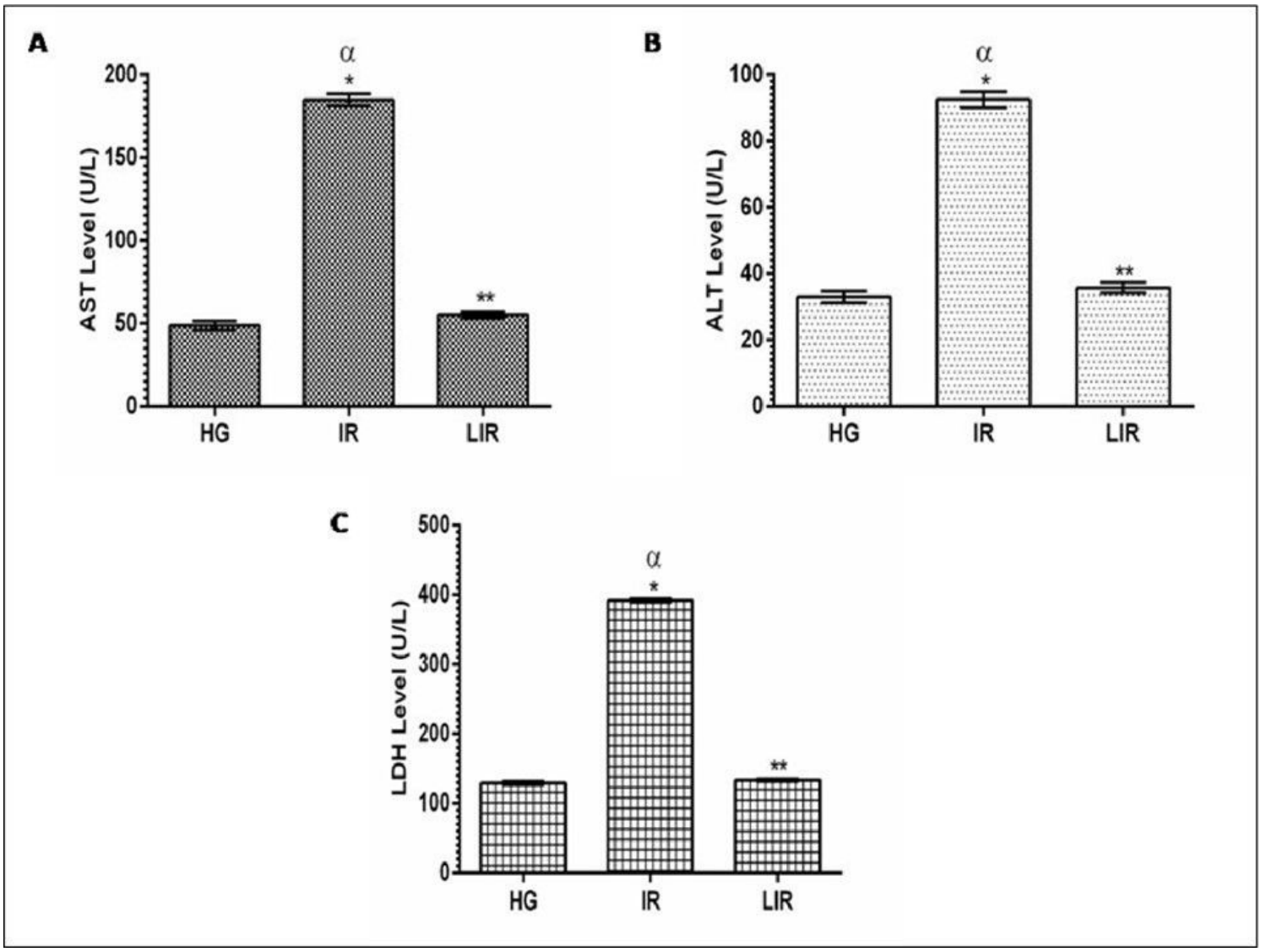

Figure 1

AST (A), ALT (B), LDH (C) levels of HG, IR and LIR groups. (n=6), * = P<0.001, ** = P>0,05 according to HG group.

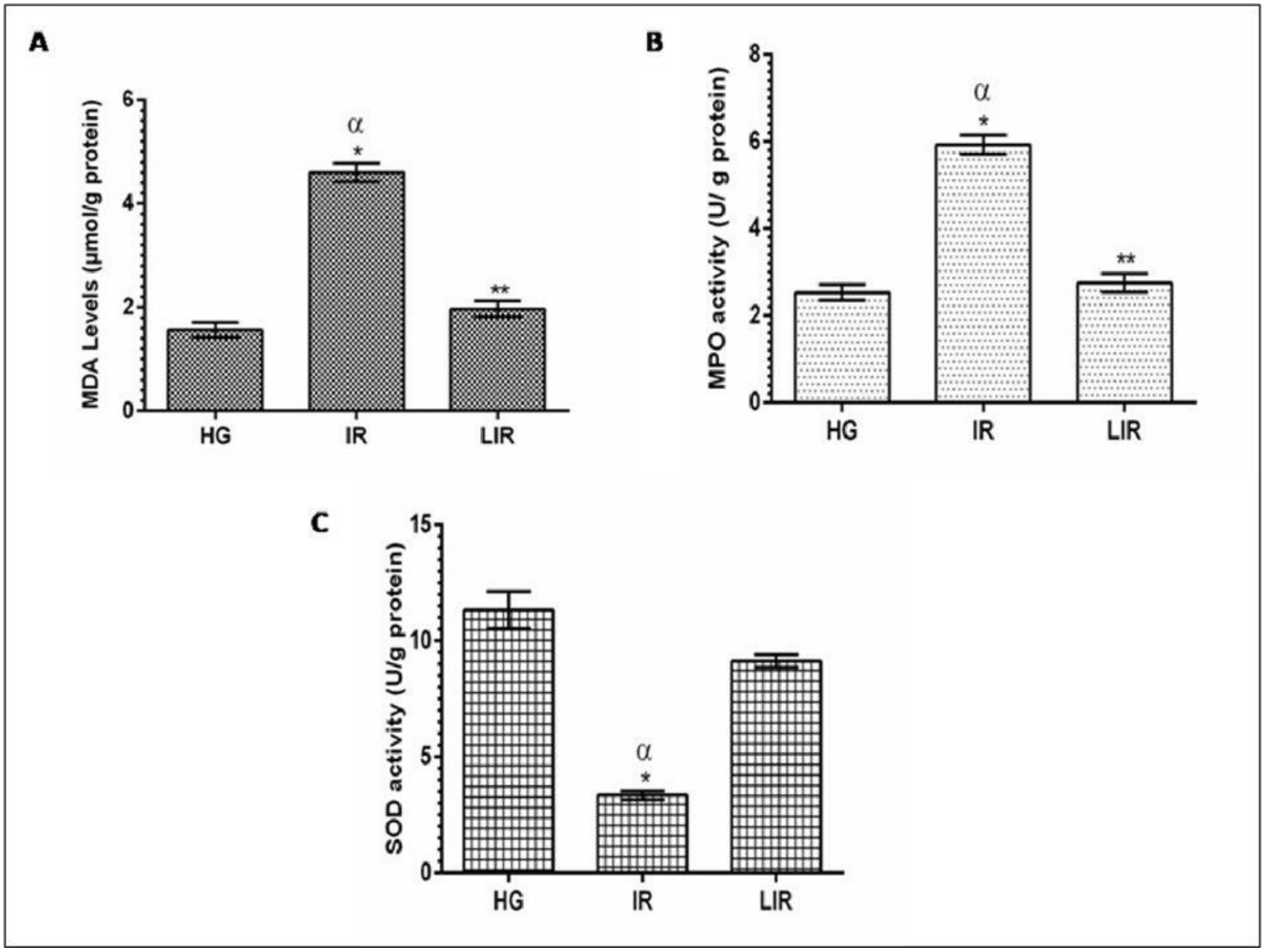

Figure 2

MDA levels (A), MPO activity (B), SOD activity (C) in the liver tissues of HG, IR and LIR groups. (n=6), * = P<0.001, ** = P>0,05according to HG group

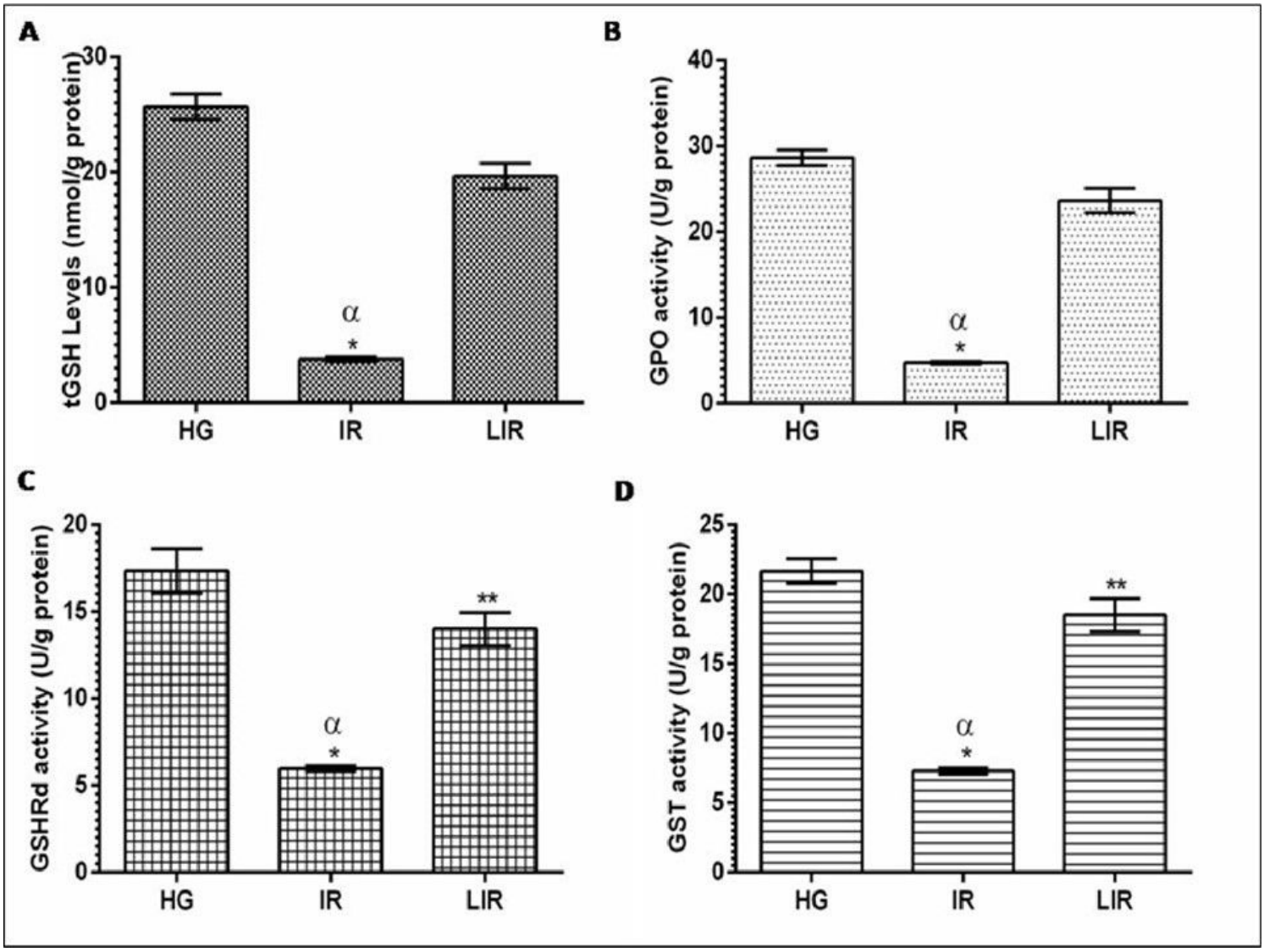

Figure 3

tGSH (A) level and activities of glutathione-dependent enzymes GPO (B), GSHRd (C), GST (D) in liver tissues of HG, IR and LIR groups. (n=6), * = P<0.001, ** = P>0,05 according to HG group

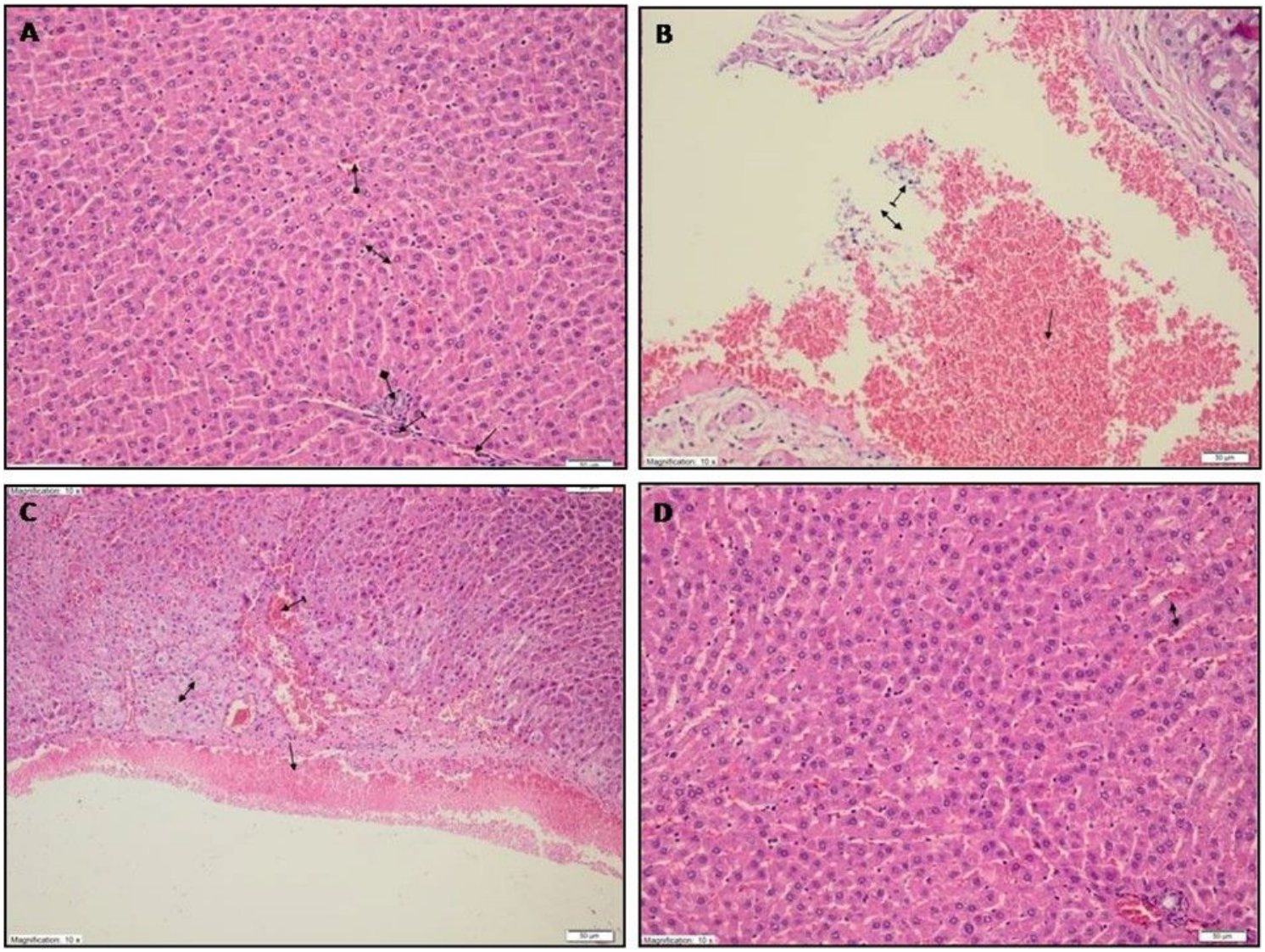

Figure 4

Histopathological findings of the liver tissues: A. Optical microscopic view of HG group: Healthy liver portal vein (straight arrow), artery (dashed arrow), gail ductus (square arrow), central vein (circular arrow), liver parenchyma (two-way arrow). B. Optical microscopic view of IR group: Hemorrhage (straight arrow), edema (two-way arrow) and polymorph (dashed arrow) are observed in the ischemic reperfusion applied liver tissue (HEX200). C. Optical microscopic view of IR group: Dilated congested blood vessels (straight arrow), dilate congested sinusoids (dashed arrow) and cells showing balloon degeneration (two way arrow) are observed in the ischemic reperfusion applied liver tissue (HEX 200) and D. Optical microscopic view of LIR group: Near-normal liver tissue is observed in the group treated with Liv-52 except for slight sinusoidal dilation and congestion in the liver tissue (HEX 200).