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ORIGINAL ARTICLE

Study of the protective effect of ischemic and pharmacological preconditioning on hepatic ischemic reperfusion injury induced in rats

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Abstract

Background and Aim: Hepatic ischemia reperfusion injury is the main cause of liver failure following liver surgery, so an effective method is needed to prevent or reduce this hepatic injury. The aim of the present study is to investigate the potential effect of ischemic preconditioning versus pharmacological preconditioning with lisinopril or verapamil for protection against hepatic ischemia reperfusion injury induced in rats.
Methods: Rats were divided into six groups. Group I served as control untreated. Rats of group II were subjected to laparotomy without induction of ischemia reperfusion. Ischemia reperfusion by ligation of the portal trait for 30 min, followed by reperfusion for 2 h, was performed in rats of groups III–VI. Ischemic preconditioning was performed for rats of group IV before induction of ischemia reperfusion. Lisinopril and verapamil was given daily for 3 days before induction of ischemia reperfusion in groups V and VI, respectively. Serum level of liver transaminases and liver malondialdehyde content were measured, and hepatic histopathological examination was assessed.
Results: Induction of ischemia reperfusion resulted in significant elevation of liver transaminases and liver malondialdehyde content associated with significant hepatic histopathological injury that were significantly improved by ischemic preconditioning, lisinopril, or verapamil treatment. Verapamil showed the most significant improvement compared with ischemic preconditioning or lisinopril treatment.
Conclusion: Ischemic preconditioning and pharmacological preconditioning by lisinopril or verapamil can protect against hepatic ischemia reperfusion probably through inhibition of oxidative stress and neutrophil infiltration. The most potent protection is demonstrated by verapamil treatment.

Introduction

Hepatic ischemia reperfusion (IR) injury may be associated with many situations, such as hemorrhagic shock, hepatic trauma, liver resection, liver transplantation, or even biliary tract operations that need temporary ligation of the hepatoduodenal ligament.[1] Hepatic IR injury may result in liver dysfunction or even loss of function, so searching for an effective preventive and treating method is mandatory.[2]

Ischemic preconditioning is a brief period of ischemia followed by reperfusion, which makes the tissues resistant to injury resulting from prolonged ischemia and reperfusion. The phenomenon of ischemic preconditioning has been recognized as one of the most potent mechanisms to protect against myocardial,[3] cerebral,[4] renal,[5] and retinal ischemic injury.[6]

Oxidative stress and calcium accumulation have an important role in the progression of hepatic IR injury and apoptosis. Hepatic reperfusion is associated with reactive oxygen species (ROS)-induced mitochondrial dysfunction, lipid peroxidation, and damage of endothelial cells and integrity of the microvasculature.[7] Intracellular Ca^{2+} overload during oxidative stress can activate Ca^{2+}-dependent enzymes, leading to cell death or apoptosis.[8] Thus, drugs that modulate calcium homeostasis and prevent oxidative stress may play a role in the prevention of hepatic IR injury.[9]

Lisinopril is an angiotensin-converting enzyme inhibitor (ACEI) that results in decreased plasma angiotensin II, vasopressor activity, and aldosterone secretion, so it is used in treatment of hypertension and congestive heart failure.[10] Angiotensin II has been shown to stimulate expression of proinflammatory chemokines and increase ROS production,[11] so lisinopril may ameliorate inflammation and oxidative stress occurred with reperfusion.[12] Lisinopril has been shown to have a protective effect against renal oxidative stress.[13]

Verapamil is a voltage-dependent calcium channels blocker. It causes relaxation of the vascular smooth muscle and vasodilatation, so it is used in treating high blood pressure and angina

pectoris.[14] By preventing the calcium influx, it may help in the decrease of progression of IR injury.[1] It has been detected to protect against renal IR induced in rats.[15]

The aim of the present study is to record the possible protective effect of ischemic preconditioning versus pharmacological preconditioning by lisinopril or verapamil on hepatic IR injury induced in rats and to investigate their effect on oxidative stress and activation of inflammatory cells.

Methods

Drugs. Verapamil (El Nasr Pharmaceutical Chemicals Company, Cairo, Egypt) and Lisinopril (Multi-Apax for Pharmaceutical Industries, Cairo, Egypt): dissolved in distilled water immediately before use.

Kits for measurement of tissue malondialdehyde (MDA) (Bio-diagnostic Company Giza, Egypt), Kits for measurement of serum levels of aspartate aminotransferase (AST), and alanine aminotransferase (ALT): (Integra-400, Roche, Germany).

Animals used. A total of 96 adult healthy male Wistar rats weighing 200–250 g were used. Animals were housed for at least 2 days before experiments under a 12 h light/dark cycle. Food and water were provided ad libitum. The study protocol was approved by the Institutional Reviewer Board of institute of ophthalmology, and the animal experiments were carried out in accordance with the ethical guidelines of animal welfare.

Experimental design. Rats were classified into six groups (16 rats each):

Group I (control untreated group). Rats of this group were received 1 mL distilled water orally for three consecutive days and were maintained alive until the end of the experiment.

Group II (sham group). Laparotomy was performed on the rats of this group, the liver was manipulated, the animal's abdomen was covered with plastic wrap to prevent dehydration, and the animals were maintained alive until the end of the experiment.

Group III (ischemia/reperfusion group). Rats of this group were subjected to hepatic ischemia for 30 min followed by reperfusion for 2 h.[16]

Group IV (ischemic preconditioning group). Rats of this group were subjected to hepatic ischemia for 10 min followed by reperfusion for 10 min before induction of prolonged ischemia/reperfusion as in group III.[16]

Group V (lisinopril treated group). Rats of this group were received lisinopril in a dose of 10 mg/kg/day orally for three consecutive days before prolonged ischemia/reperfusion as group III, with the last dose administered 1 h prior to ischemia.[17]

Group VI (verapamil treated group). Rats of this group received verapamil in a dose of 10 mg/kg/day orally for three consecutive days before prolonged ischemia/reperfusion as group III, with the last dose administered 1 h prior to ischemia.[18]

Methods

Induction of hepatic ischemia/reperfusion. Rats were starved for 12 h before the operation, were anesthetized, and a midline laparotomy was performed. The portal vein, hepatic artery, bile duct, and caudate hepatic lobe were freed by blunt dissection, with the hepatoduodenal ligament separated. The portal triad—portal vein, hepatic artery, and bile duct—was clamped with a mini-artery clamp for 30 min, followed by reperfusion for 2 h. Appropriate clamping was confirmed by visual inspection of the ischemic lobes. During the period of hepatic ischemia, the animal's abdomen was covered with plastic wrap to prevent dehydration. Rats of this group served as a model for hepatic ischemia/reperfusion injury.[16]

Assessment of liver enzymes (AST and ALT). After the end of the experiment, all rats were sacrificed by decapitation. Blood samples were taken for assessment of serum level of liver enzymes.

Tissue sampling for lipid peroxidation. Eight rats from each group were sacrificed by decapitation, and their livers were rapidly isolated. Isolated livers were placed into Petri dishes containing an ice-cold isolation medium, consisting of 125 mM KCl, 15 mM Tris, and pH 7.4; 20% homogenates were prepared with a Potter–Elvehjem homogenizer set at a standard velocity (500 r.p.m.) for determination of tissue MDA content. Malondialdehyde reacts with thiobarbituric acid producing a thiobarbituric acid reactive substance (TBARS), a pink chromogen, which is measured spectrophotometrically at 532 nm.[19]

Histopathological examination. Liver tissue specimens were fixed in 10% formol saline and then trimmed off, washed, and dehydrated in ascending grades of alcohol. The dehydrated specimens were then cleared in xylene, embedded in paraffin blocks, and sectioned at a thickness of 4–6 μm thick. The obtained tissue sections were deparaffinized using xylol and stained using hematoxylin and eosin (HE) for histopathological examination through the electric light microscope according to Bancroft *et al*.[20]

Hepatic histological damage and hepatocellular necrosis were evaluated according to.[21] The hepatic histological damage scale consists of four degrees (G0–G3): grade 0 indicates minimal or no evidence of injury; grade 1 indicates mild injury with cytoplasm vacuolization and focal nuclear pyknosis; grade 2 indicates moderate to severe injury with extensive nuclear pyknosis, loss of intercellular borders, and mild to moderate neutrophil infiltration; and grade 3 indicates severe injury with disintegration of hepatic cords, hemorrhage, and severe polymorphonuclear cells (PMN) infiltration.

The statistical methodology. Data were coded and entered using the statistical package SPSS version 22 (Armonk, NY, USA). Data were summarized using mean ± SD. Comparisons between groups were conducted using ANOVA with multiple comparisons post hoc Tukey test.[22] *P*-values less than 0.05 were considered statistically significant.

Results

Rats that were subjected to sham operation (group II) did not show any significant difference in any of the tested parameters compared with control untreated rats (group I).

Table 1 The mean serum level (mean ± SD) of aspartate aminotransferase (AST) and alanine aminotransferase (ALT) in different groups

Groups	AST (U/L)	ALT (U/L)
Group I (control untreated group)	126.38 ± 3.852	78.250 ± 5.849
Group II (sham group)	129.63 ± 2.669	88.250 ± 4.590
Group III (ischemia/reperfusion group)	514.38 ± 25.834†	467.38 ± 20.894†
Group IV (ischemic preconditioning group)	373.25 ± 10.096†,‡	327.63 ± 15.014†,‡
Group V (lisinopril-treated group)	291.38 ± 13.522†,‡,§	247.13 ± 14.197†,‡,§
Group VI (verapamil-treated group)	210.88 ± 18.627†,‡,§,¶	183.38 ± 7.150†,‡,§,¶

Values are expressed as mean ± SD for 16 animals in each group.
†Statistically significant as compared to control untreated group (group I) ($P < 0.05$).
‡Statistically significant as compared to ischemia/reperfusion group (group III) ($P < 0.05$).
§Statistically significant as compared to ischemic preconditioning group (group IV) ($P < 0.05$).
¶Statistically significant as compared to lisinopril-treated group (group V) ($P < 0.05$).

Biochemical results. Induction of hepatic IR in rats of group III resulted in significant elevation of the mean serum level of AST and ALT and significant elevation in liver MDA compared with control untreated group (group I) ($P < 0.05$) (Tables 1,2).

Ischemic preconditioning induced in rats of group IV before induction of I/R caused significant decrease in the mean serum level of AST and ALT that was associated with significant lowering in liver MDA compared with the IR group (group III) ($P < 0.05$) (Tables 1,2).

Treatment of rats for 3 days before induction of I/R with either lisinopril (group V) or verapamil (group VI) caused significant decrease in the mean serum level of AST and ALT and significant lowering in liver MDA compared with IR group (group III) and ischemic preconditioning group (group IV) ($P < 0.05$). Rats treated with verapamil showed the most significant improvement in the mean serum level of AST and ALT and hepatic MDA compared with rats treated with lisinopril ($P < 0.05$) (Tables 1,2).

Histopathological results. Histopathological examination of the liver tissue obtained from all control untreated rats (group I) showed normal hepatic lobules that were made up of radiating strands of polygonal cells vertical to central vein. The cells had prominent round nuclei and eosinophilic cytoplasm (Fig. 1). Sinusoids were lined by a discontinuous layer of fenestrated endothelial cells with fine arrangement of Kupffer cells (Grade 0) (Fig. 2). The portal area showed normal histological structure of the bile duct, portal vein, and hepatic artery (Fig. 3).

Table 2 The mean level (mean ± SD) of liver malondialdehyde (MDA) in different groups

Groups	MDA (nmol/g)
Group I (control untreated group)	18.500 ± 3.071
Group II (sham group)	19.250 ± 2.375
Group III (ischemia/reperfusion group)	44.625 ± 3.701†
Group IV (ischemic preconditioning group)	31.125 ± 2.031†,‡
Group V (lisinopril-treated group)	25.125 ± 2.232†,‡,§
Group VI (verapamil-treated group)	21.250 ± 1.488†,‡,§,¶

Values are expressed as mean ± SD for eight animals in each group.
†Statistically significant as compared to control untreated group (group I) ($P < 0.05$).
‡Statistically significant as compared to ischemia/reperfusion group (group III) ($P < 0.05$).
§Statistically significant as compared to ischemic preconditioning group (group IV) ($P < 0.05$).
¶Statistically significant as compared to lisinopril-treated group (group V) ($P < 0.05$).

Liver tissue sections obtained from all rats subjected to IR (group III) revealed severe injury with disintegration of hepatic cords. Hemorrhage and severe PMN infiltration were detected. Coagulative necrosis of hepatocytes was seen in the centrilobular and midzonal areas. Neutrophilic inflammatory cells infiltrated the necrotic area and presented in hepatic sinusoids (Fig. 1). Venous thrombi were occasionally present. The hepatic sinusoids were engorged with blood, and there was marked hyperplasia of Kupffer cells (Grade 3). The portal trait showed dilatation of portal vein and hepatic artery with neutrophilic infiltration (Figs 2,3).

Microscopic examination of liver tissue sections obtained from 75% of rats subjected to ischemia preconditioning before hepatic I/R (group IV) revealed moderate to severe injury of hepatocytes, with extensive nuclear pyknosis and loss of intercellular borders. There were numerous numbers of binucleated cells and deeply eosinophilic apoptotic bodies. Mild to moderate neutrophil infiltration was seen between the hepatic cords in addition to marked dilatation of hepatic sinusoids engorged with blood (Grade 2) (Fig. 1). The portal trait showed dilatation of the portal vein and leukocytic infiltration, mainly by neutrophils (Fig. 2). Grade 3 was recorded in 25% of animals (Fig. 3).

The histopathological features of Grade 2 were recorded in 62.5% of rats treated with Lisinopril for 3 days before induction of hepatic IR (group V) (Fig. 3); 37.5% of rats of group V showed mild injury of hepatocytes with granularity of cytoplasm and focal nuclear pyknosis scattered in-between hepatic cords as deeply eosinophilic apoptotic bodies. Less number of neutrophilic infiltrations was noticed (Grade 1) (Fig. 1). The portal trait showed dilatation of portal vein with mild leukocytic infiltration (Fig. 2).

All rats treated with verapamil for 3 days before induction of hepatic IR (group VI) showed the least histopathological features of injury (Grade 1) (Figs 1–3).

Discussion

Hepatic vascular control is used to provide a bloodless operative field and prevent hemorrhage during liver resection or transplantation, which has increased in the recent years. IR injury may be

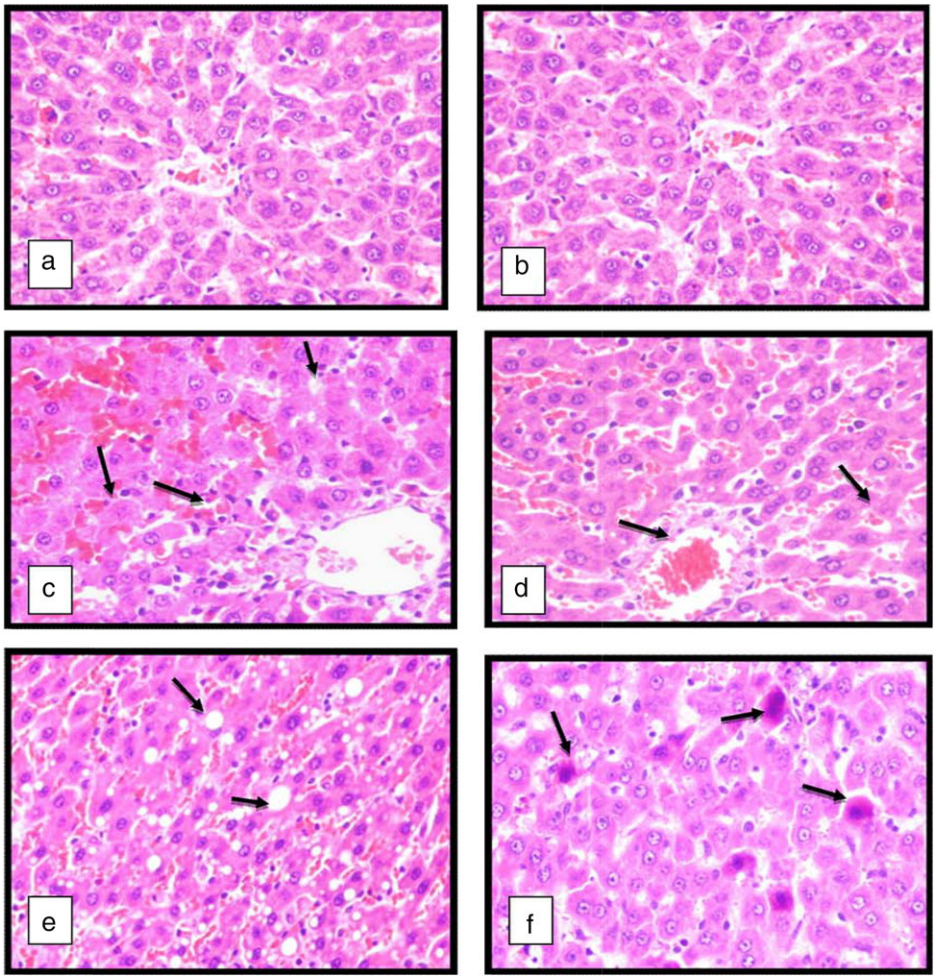

Figure 1 Histopathology of hepatic lobules (H&E ×400). (a) Group I (control untreated group): normal histological structure (Grade 0). (b) Group II (Sham group): normal histological structure (Grade 0). (c) Group III (ischemia/reperfusion group): hemorrhage, neutrophils infiltration, and centrilobular coagulative necrosis of hepatocytes (arrow) (Grade 3). (d) Group IV (ischemic preconditioning group): numerous number of binucleated cells and deeply eosinophilic apoptotic bodies (arrow) (Grade 2). (e) Group V (lisinopril-treated group): vacuolation of hepatocytes (arrow) and neutrophilic infiltration (Grade 2). (f) Group VI (verapamil-treated group): focal nuclear pyknosis scattered between hepatic cords (arrow) (Grade 1).

the main cause of primary graft dysfunction after liver transplantation.[16]

MDA results from lipid peroxidation of polyunsaturated fatty acids. ROS degrades lipids forming MDA, which is a reactive aldehyde that reacts with deoxyadenosine and deoxyguanosine in DNA causing toxic stress in cells. MDA is used as a biomarker to measure the level of oxidative stress in tissues.[23]

In the present study, induction of IR in group III resulted in significant elevation of the mean serum level of AST, ALT, and liver MDA that were associated with significant histopathological evidence of hepatocytes necrosis compared with control untreated group (group I).

The results of the present study are in agreement with Pérez *et al.*[24] who detected the effect of hepatic I/R on the elevation of hepatic MDA. Glanemann *et al.*[25] and Liu *et al.*[26] described the histopathological insults that are caused by hepatic I/R injury.

Several factors may share in injuries associated with hepatic IR, such as anaerobic metabolism, mitochondria dysfunction, oxidative stress, intracellular calcium overload, Kupffer cells activation, cytokines, and chemokines. During hepatic ischemia, the metabolic pattern is shifted to anaerobic, the redox process of the hepatocytes is blocked, and intracellular ATP is rapidly depleted. After reperfusion, the pH values are restored to normal, leading to the activation of proteases and phospholipases and further worsening the damage of tissues and organs.[27]

Cytokines such as interleukin 1 (IL-1), tumor necrosis factor-α (TNF-α), vascular endothelial growth factor (VEGF), and hepatocyte growth factor (HGF) are released from activated Kupffer cells, and neutrophils promote leukocyte activation, aggravating liver damage.[28] IL-1β upregulates leukocyte aggregation and adhesion by activating NF-κB macrophage inflammatory protein (MIP)-2. IL-12 and IL-23 stimulate TNF-α production, signal transducer, and activate transcription (STAT)-4 and CD4 T cells to produce IL-17.[29]

In the present study, ischemic preconditioning before the induction of IR in group IV resulted in a significant decrease in

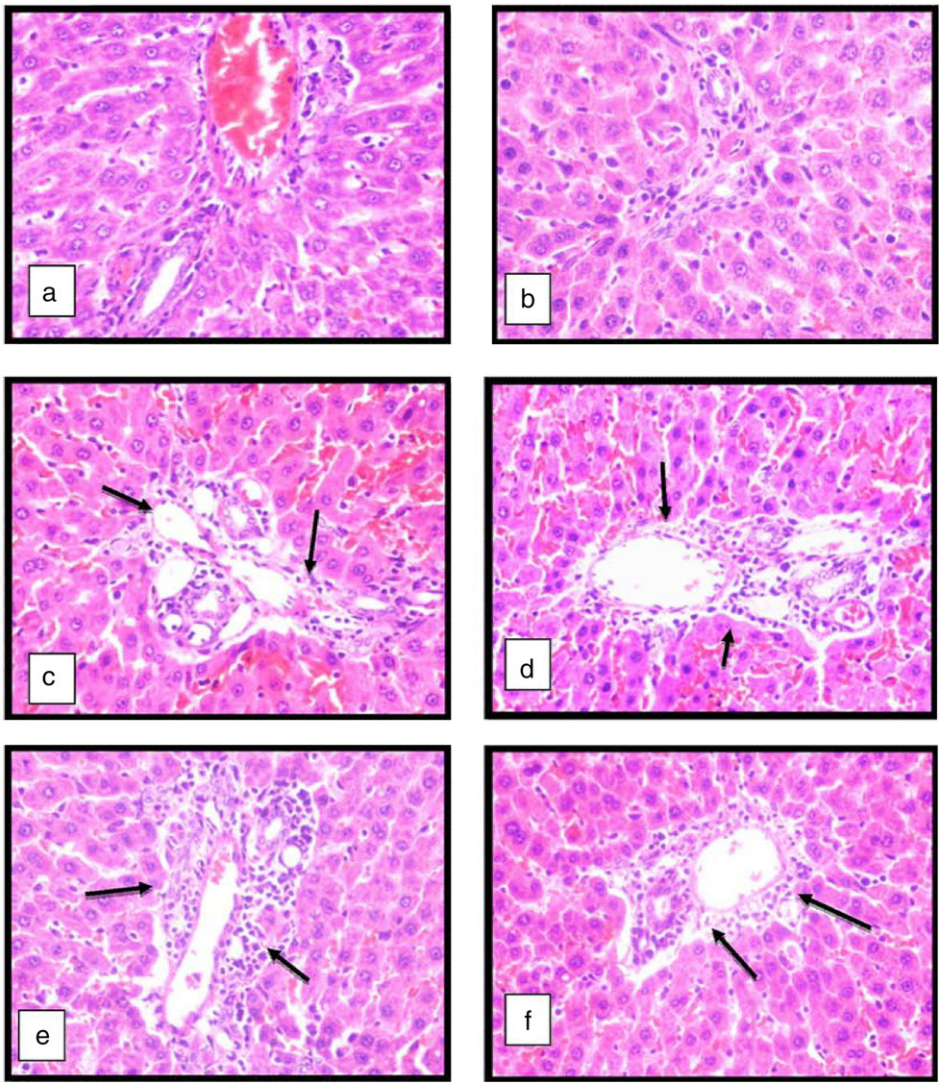

Figure 2 Histopathology of portal trait (H&E ×400). (a) Group I (control untreated group): normal histological structure. (b) Group II (sham group): normal histological structure. (c) Group III (ischemia/reperfusion group): dilatation of both portal vein and hepatic artery with neutrophilic infiltration (arrow). (d) Group IV (ischemic preconditioning group): dilatation of portal vein (arrow) and neutrophil infiltration. (e) Group V (lisinopril treated group): leukocytic infiltration mainly neutrophils (arrow). (f) Group VI (verapamil-treated group): dilatation of portal vein with mild leukocytic infiltration (arrow).

the mean serum level of AST, ALT, and significant lowering in liver MDA that was associated with a significant improvement of the histopathological evidence of hepatocytes necrosis and inflammatory cells infiltration compared with the IR group (group III).

The results of the present study are matched with Glanemann *et al.*[25] and Kuo *et al.*[30] who described the protective effect of IP against hepatic I/R injury. Liu *et al.*[26] detected that IP can protect against hepatic I/R injury through increased hemeoxygenas-1 (HO-1). Peralta *et al.*[31] demonstrated the ability of IP to reduce Kupffer cells activation and improve hepatic microcirculation.

During the short reperfusion phase of ischemic preconditioning, little amounts of ROS are generated that can activate redox-sensitive intracellular signaling pathways, which may protect the liver cells through the inhibition of cell death pathways and simulation of antioxidant.[32]

Ischemic preconditioning may improve liver microcirculation and reduce hepatocellular apoptosis and necrosis through stimulation of HO-1, inducible nitric oxide synthase (iNOS), adenosine receptor and hypoxia-inducible factor (HIF)-1 that activates several hypoxia-responsive genes.[30]

Stimulation of adenosine receptors can stimulate nitric oxide (NO) production, protein kinase C, and adenosine monophosphate-activated protein kinase (MAPK) that increase hepatocytes tolerance to ischemic insults and stimulate their regeneration.[33] NO can inhibit TNF-α, interleukin-1β (IL-1β), and oxidative damage by increasing the antioxidants. The HO-1 product carbon monoxide can activate p38 MAPK.[34]

In the present study, rat treatment with lisinopril for 3 days before induction of I/R (group V) resulted in a significant decrease in the mean serum level of AST and ALT, significant lowering in

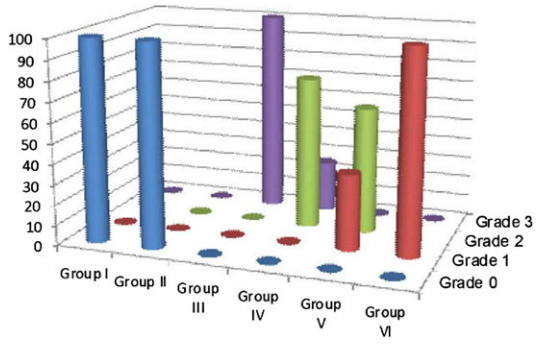

Figure 3 Percentage of occurrence of different histopathological grades (0–3) in hepatic tissue (each group contained eight rats). Group I: control untreated group. Group II: sham group. Group III: ischemia/reperfusion group. Group IV: ischemic preconditioning group. Group V: lisinopril-treated group. Group VI: verapamil-treated group. Grade 0: minimal or no evidence of injury; Grade 1: mild injury with cytoplasm vacuolization and focal nuclear pyknosis; Grade 2: moderate to severe injury with extensive nuclear pyknosis, loss of intercellular borders, and mild to moderate neutrophil infiltration; Grade 3: severe injury with disintegration of hepatic cords, hemorrhage, and severe PMN infiltration.[21] ■, Grade 0; ■, Grade 1; ■, Grade 2; ■, Grade 3.

liver MDA that was associated with profound improvement of the histopathological evidence of hepatocytes necrosis, and inflammatory cellular infiltration compared with IR group (group III) and ischemic preconditioning group (group IV).

The results of the present study were previously recorded by Morsy[17] who showed that lisinopril can protect against hepatic I/R injury in rats. The protective effects are associated with a reduction of the lipid peroxidation level and enhancement of nitric oxide availability. Yirmibesoglu[35] recorded the ability of lisinopril to improve the serum level of liver transaminases after hepatic IR injury induced in rats through decreased endothelin-1 levels.

The protective effect of lisinopril may be due to decreased oxygen-free radical formation,[12] increase nitric oxide[36], and decrease in the expression of inflammatory mediators.[37]

The suppressive effect of lisinopril on inflammatory mediators may be through the inhibition of bradykinin degradation or the stimulation of peroxisome proliferator-activated receptor gamma (PPARγ).[38] Freise *et al.*[39] correlated the protective effects of ACEI to the effect of bradykinin in the induction of endothelium synthesis of antiaggregatory, cytoprotective, and anti-inflammatory prostacyclins. PPARγ activation inhibits the release of TNF-α, IL-1, and IL-6 by macrophages.[40]

Thus, lisinopril is the only ACEI that does not depend on hepatic metabolism, it is preferred in the treatment of hypertensive patients before and after liver transplantation.[41]

In the present study, rats treated with verapamil before the induction of I/R (group VI) resulted in a significant decrease in the mean serum level of AST and ALT, significant lowering in liver MDA that was associated with significant improvement of the histopathological evidence of hepatocytes necrosis, and inflammatory cells infiltration compared with the IR group (group III), the ischemic preconditioning group (group IV), and the lisinopril-treated group (group V).

The previous results were similar to those of Messiha and Abo-Youssef[18] who demonstrated that verapamil administration improved serum ALT and AST levels and markedly corrected the oxidative stress biomarkers, which were associated with marked improvement of histopathological findings in rats subjected to hepatic I/R. Chin *et al.*[42] detected that diltiazem can protect against hepatic IR induced in rats, indicated by an increase tissue blood flow.

The hepatoprotective effect of verapamil could be attributed to its calcium channel blocking activity. It blocks the calcium influx to hepatocytes and prevents mitochondrial calcium overload.[43] Verapamil can attenuate chemo-attractant release by Kupffer cells after hepatic I/R[44] and has an antioxidant effect.[45]

Similarly, Pronobeshi *et al.*[46] described that amlodipine can protect against hepatic IR injury induced in rats and decrease MDA level in liver through increased mitochondrial antioxidants, superoxide dismutase, and catalase.

Calcium channel blockers may reduce hepatic necrosis and apoptosis after I/R indirectly through increased B-cell lymphoma 2 (Bcl-2) expression by inhibiting Ca^{2+} efflux into mitochondria.[47]

In conclusion, pharmacological preconditioning by lisinopril or verapamil showed more significant protection than ischemic preconditioning against hepatic IR injury. The verapamil-induced liver protection was found to be more effective than lisinopril-induced protection. The degree of protection of either ischemic or pharmacological preconditioning is correlated with intense inflammatory cellular infiltration and lipid perioxidation.

Further studies are needed to investigate the effect of different types of calcium channel blockers and angiotensin-converting enzyme inhibitors on hepatic IR. Further studies are also needed to examine these effects in different animal species, with different doses and durations and study of possible side effects of these drugs, especially in presence of hepatic insult.

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