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Liver Tissue Inducible Nitric Oxide Synthase (iNOS) Expression and Lipid Peroxidation in Experimental Hepatic Ischemia Reperfusion Injury Stimulated With Lipopolysaccharide: The Role of Aminoguanidine

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Background. **Hepatic ischemia-reperfusion (HIR) is a severe condition that is seen after hepatic arterial injury and in hepatic grafts in living donor transplantation. HIR not only causes liver injury by lipid peroxidation, but also stimulates systemic and portal endotoxemia. Also, lipopolysaccharide (LPS) induces hepatic injury mediated by inducible nitric oxide synthase (iNOS). There is little knowledge on the role of specific iNOS inhibitors in prevention of HIR injury followed by LPS administration. The aim of this study on a LPS induced HIR model was to investigate the effect of aminoguanidine (AG) administration on hepatic tissue iNOS expression and lipid peroxidation when given before or after LPS.**

Methods. **Six groups were designed; A: Sham, B: HIR, C: HIR + AG, D: HIR + LPS, E: HIR + LPS + AG, F: HIR + AG + LPS. No substance was given to the rats in Group A and B. HIR injury was induced with vascular occlusion for 45 min and reperfusion for 45 min. Drugs were given intraperitoneally 10 min before reperfusion. Serum and tissue analysis for myeloperoxidase (MPO), and malondialdehyde (MDA), and tissue NA^+/K^+ adenosine 5′triphosphatases (ATPase) and tissue iNOS staining were performed. Permission for this study was obtained from the local Ethics Committee.**

Results. **The level of MPO, MDA, and iNOS staining scores in Group B were significantly higher than Group A and ATPase was lower in Group B ($P < 0.05$). Contrary to results in Group C, results of MPO, MDA, and iNOS staining scores of Group D was higher than Group B ($P < 0.05$); however, although iNOS in Group C was lower than Group B, the difference was not significant ($P > 0.05$). MPO and MDA levels of Groups E and F were significantly lower than Group D. Level of ATPase in Group F was significantly different from Groups D and E. iNOS scoring was low in Group F compared with Group D ($P < 0.05$). MDA, MPO, and iNOS levels of Group F was lower than Group E, and ATPase of Group F was higher than Group E ($P < 0.05$).**

Conclusions. **The results of this study in a LPS induced HIR model showed that LPS after HIR aggravated HIR injury by increasing neutrophil activation and lipid peroxidation both in serum and liver tissue and iNOS in liver, and depleting energy in liver. AG, a selective iNOS inhibitor, ameliorated the negative effects of endotoxemia induced by LPS after HIR; however, energy depletion and iNOS expression in liver tissue were attenuated only when AG was administered prior to LPS. The findings of this study supported the hypothesis that LPS after HIR would aggravate HIR injury and AG would ameliorate this aggravated injury.**

Key Words: **aminoguanidine; inducible nitric oxide synthase; lipid peroxidation; lipopolysaccharide; hepatic ischemia reperfusion injury.**

INTRODUCTION

Hepatic ischemia-reperfusion (HIR) injury is a result of a series of complex mechanisms including free oxygen radicals (FORs) activated by energy depletion and failure of oxygen delivery to the vital tissues in the ischemic period. Decreased liver blood flow is observed

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during shock, major vascular trauma, hemorrhagic shock, and the late phase of sepsis. Vascular occlusions during major hepatic resections for hepatic tumors and transplantation surgery also lead to decreased hepatic blood inflow [1–3].

Activation of anaerobic metabolism during ischemia leads to degradation of adenine nucleotides to hypoxanthine forms, and xanthine dehydrogenase is converted to xanthine oxydase. With reperfusion, hypoxanthine is converted to xanthine by xanthine oxydase. During this conversion dangerous FORs, lipid peroxide radicals, lipid hydrogen peroxide, and other lipid degradation products are generated. This process is a self-stimulating cascade, which results in dangerous cellular and tissue damage [4, 5]. The results of several studies suggest that strategies to decrease FOR production or to increase FOR degradation may have beneficial effects to protect organ damage after reperfusion [6].

Nitric Oxide (NO) is a biologically active agent that controls leukocyte endothelial interactions and smooth muscle relaxation. Several studies have reported on the positive and negative effects of NO and the controlling enzyme of NO production, nitric oxide synthase (NOS), in several clinical conditions including thermal injury, sepsis, shock, and ischemia-reperfusion (I/R) injury [7]. Most of these studies are focused on the inducible isoform of NOS (iNOS). iNOS is an enzyme that is reported to be inhibited in selective and/or non-selective mechanisms. Some authors reported positive clinical outcomes after iNOS inhibition in different experimental organ I/R injury models, but other authors have reported negative results after iNOS inhibition. Aminoguanidine (AG) is reported to be a selective iNOS inhibitor, and has been studied in experimental intestinal and HIR models [8–10].

Endotoxemia, which can be induced by lipopolysaccharide (LPS) experimentally, may complicate HIR injury. Endotoxemia with/without HIR injury leads to hepatic failure. Increased and prolonged tumor necrosis factor-alpha (TNF-α) response is shown in LPS induced experimental HIR injury in which increased liver and lung injuries have been observed [10, 11]. These results may partly explain the mechanisms of multiorgan failure, which can be seen after hepatic resections, major hepatic vascular injuries, transplantation surgery, and hypovolemic shock.

HIR not only causes liver injury by lipid peroxidation but also stimulates systemic and portal endotoxemia [12, 13]. Also, LPS induces hepatic injury mediated by iNOS [12]. Thus, endotoxemia may complicate HIR injury. It has been reported that AG attenuated either HIR induced or LPS stimulated liver injury [12, 14]. However, no data exists about the effect of AG on LPS stimulated HIR injury. In the light of above data, we hypothesized that LPS would aggravate HIR injury and AG had a potential to ameliorate this aggravated injury. We aimed in this study to investigate the effects of AG administration on hepatic tissue iNOS expression and tissue lipid peroxidation in LPS induced experimental HIR injury.

METHODS

The experiments were performed in adherence to National Institutes of Health guidelines on the use of experimental animals. Seventy-two male, Wistar rats, weighing from 150 to 220 g, were housed at constant temperature with 14/10 h periods of light and dark exposure. Animals were allowed access to standard rat chow and water ad libitum during an acclimatation period of at least 5 d prior to use in these experiments. Approval of the Institutional Ethics Committee was obtained. The experiments were performed in the Animal Experimental Laboratory of the Institution.

Surgical Procedures and AG and LPS Administrations

Rats were anesthetized with intramuscular ketamine 50 mg per kg and xylazine 7 mg per kg. The chest and abdomen were shaved and each animal was fixed in a supine position on the operating table. The abdomen was cleaned with 1% polyvinyl iodine and when dry, the operating field was covered with a sterile drape and median laparotomy was performed. There were six experimental groups (A, B, C, D, E, and F) and 12 rats in each group.

Sham operation was performed on the rats in Group A. The sham operation consisted of mobilization of the hepatic pedicle only. The rats in this group were sacrificed 90 min after the procedure. HIR injury was induced in Groups B, C, D, E, and F (HIR, HIR + AG, HIR + LPS, HIR + LPS + AG and HIR + AG + LPS groups, respectively) by hepatic pedicle clampage using a vascular clamp. After an ischemia period of 45 min the vascular clamp was removed. A reperfusion period was maintained for 45 min.

Group B was established to detect the prime changes on immunohistochemical and biochemical parameters in HIR, and served as a control group for AG and LPS administered groups (Groups C and D, respectively). We injected 100 mg per kg AG intraperitoneally (i.p.) in the rats in Group C and 10 mg per kg LPS i.p. in the rats in Group D 10 min prior to declampage. Groups C and D were designed to demonstrate the effects of AG and LPS on HIR, respectively. We expected AG and LPS to ameliorate and deteriorate the effects of HIR respectively. Group D represented LPS stimulated HIR injury and control for Groups E and F.

In Group E, 10 mg per kg LPS i.p. injection was followed by 100 mg per kg AG i.p. 10 min prior to reperfusion. In Group F, 100 mg per kg AG injection i.p. was followed by 10 mg per kg LPS i.p. 10 min prior to reperfusion. In Groups E and F both AG and LPS were administered with an interval of 1 min between injections of either of two drugs. Groups E and F represented the protective and therapeutical effects of AG in LPS stimulated HIR injury, respectively.

After inducing the HIR injury and administering drug injections, the rats were re-anaesthetized with intramuscular ketamine (25 mg per kg). After obtaining blood by cardiac puncture, the liver was harvested through a midline incision. Malondialdehyde (MDA) and myeloperoxidase (MPO) activities of serum and liver tissue, iNOS expression, and Na^+/K^+ adenosine 5′triphosphates (ATPase) activity of liver tissue were studied to evaluate the effects of aminoguanidine in a model of LPS induced HIR injury. The liver tissue iNOS expression was illustrated immunohistochemically. The pathologist was blinded to which group an animal belonged.

The comparison of Group A with Group B was made to determine if HIR caused an injury in liver tissue. Group B was compared with Groups C and D to determine whether AG attenuated HIR injury and induction of LPS deteriorated HIR injury. Group D was compared with Groups E and F to define whether AG caused amelioration in liver injury caused by LPS administration after HIR. Com-

parison of Group E with Group F was made to evaluate whether AG administration was more effective prior to or after LPS induction.

We tried to show that HIR caused an injury in liver and, as expected, AG attenuated, whereas LPS deteriorated HIR injury. We also tried to find out whether AG, which was expected to attenuate HIR injury, would ameliorate HIR injury deteriorated by LPS stimulation.

Lipid Peroxides Assays

MDA Determination

The levels of serum and tissue lipid peroxidation products as thiobarbituric acid (TBA)-MDA adducts were measured spectrophotometrically by the method described by Yagi [15]. The results were expressed as nmol of MDA formed per mL of serum and nmol of MDA per gram of tissue.

The Determination of MPO

The determination of serum and tissue MPO activity depends on the fact that it reduces 0-dianozidine. Reduced *o*-diazidine was measured 410 at nm by spectrophotometer [16]. The MPO activity was expressed as u/L for serum and u/g for tissue.

Determination of Na^+/K^+ ATPase Activities

After the animals were killed, the liver tissue was removed as rapidly as possible. Ten percent homogenates of the tissue was prepared in 0.3 M sucrose containing 1 mM magnesium by homogenizing for 90 s using a Teflon pestle (clearance 0.25–0.38 mm) at 1000 rpm. To remove the debris, the suspension was then centrifuged at 1000 rpm for 15 min. ATPase activities were determined on the resulting supernatants by measuring rate of liberation of inorganic phosphate (Pi) from disodium ATP incubation; media were made up as described previously [17, 18]. Incubation medium for NA^+/K^+ ATPase was as follows incubation medium (mM): $MgCl_2$ 6, KCl 5, NaCl 100, EDTA 0.1, tris-HCl buffer pH 7.4.

After preincubation for 5 min at 37°C, disodium ATP was added to each tube to reach a final concentration of 3 mM. The blank sample containing no enzyme, the standard and the unknowns were incubated at 37°C for 30 min. The reactions were stopped by putting the samples on ice. Inorganic phosphate was determined on 1 mL aliquots of the incubated mixtures by the addition of lubrol-molybdate solution followed by vortexing and then measuring at 340 nm. All assays were done in triplicate and run with enzyme and reaction blanks. Samples were compared for phosphate content with standards of KH_2PO_4. Specific activities were calculated as nmol Pi/mg protein/h.

All reagents were of Analar grade unless otherwise stated. Disodium ATP was obtained from Sigma Chemical Co. Ltd. Protein content was determined according to the method described by Lowry *et al.* and bovine serum albumin was used as a standard [19].

Histological Evaluation

Immunohistochemical Assay

The extracted liver tissue were fixed in 10% phosphate-buffered formalin and subsequently embedded in paraffin. Five-μm thick sections were prepared from the paraffin blocks and liver tissue was used to quantify the iNOS expression. Avidin-biotin complex immunoperoxidase staining system was used. Primary antibodies used were anti-iNOS, a synthetic peptide derived from the extreme C-terminus of the human iNOS protein (Zymed Laboratories, San Francisco, CA), dilution 1:1000. The sections were deparaffinized in xylene through ethanol to phosphate-buffered saline (pH 7.2). To block endogenous peroxidase activity, 3% hydrogen peroxide was applied for 30 min. The slides incubated in citrate buffer were heated in a microwave oven for 5 min. After waiting 20 min, they were removed, and ultra V block (Lab Vision) was added. Primary antibody for iNOS was applied and incubated overnight in a moist chamber at 4°C. The slides were subsequently incubated in biotinylated goat anti-polyvalent (Lab Vision) for 10 min, and in streptavidin peroxidase (Lab Vision) for 20 min. Finally, AEC substrate system (Lab Vision) was applied for about 3 min. After incubation, the sections were rinsed with distilled water and tap water. The tissue was counterstained with Mayer's hematoxylin. All slides were covered with a coverslip after mounting in buffered glycerin.

Morphometric Determination

iNOS protein was observed in the cytoplasm of parenchymal cells of liver. iNOS protein expression in the groups was assessed according to the intensity of staining; scores 0: <2% or absent, 1: 2%–10% weak, 2: 10%–50% intermediate, and 3: >50% strong [20].

Statistical Analysis

Biochemical values were described as mean ± standard deviation (SD) values. Statistical differences for serum and tissue MDA, serum and tissue MPO activity, and tissue ATPase activity values were evaluated using one-way analysis of variance followed by Schefe post hoc test. Histopathological examinations were presented as median and 25% to 75% (percentiles) in parenthesis. Comparisons of iNOS staining scores were analyzed using the Kruskal-Wallis test followed by the Dunn test. P-values < 0.05 were considered as statistically significant. Box-plot graphics were used to show medians of liver tissue iNOS scores.

RESULTS

Serum and Tissue MPO

Mean ± SD value of serum MPO in Groups A, B, C, D, E, and F were 1.42 ± 0.04, 2.23 ± 0.09, 1.43 ± 0.09, 3.12 ± 0.26, 2.90 ± 0.20, and 2.63 ± 0.25, respectively (Table 1). Mean ± SD values of liver tissue MPO in Groups A, B, C, D, E, and F were 1.36 ± 0.18, 4.94 ± 0.35, 1.42 ± 0.17, 6.75 ± 0.30, 3.41 ± 0.30, and 2.68 ± 0.33, respectively, (Table 1). The descriptive statistics of serum and liver tissue MPO are presented in Fig. 1. Serum MPO of Group B was significantly higher than Group A ($P < 0.05$). Serum MPO of Group B was significantly higher than Group C, and significantly lower than Group D ($P < 0.05$). Serum MPO of Group E was lower than Group D but the difference was not statistically significant ($P > 0.05$). Serum MPO of Group F was significantly lower than Groups D and E ($P < 0.05$). HIR and LPS after HIR significantly increased serum MPO activity; LPS administration after HIR increased serum MPO more than HIR alone. AG administration attenuated serum MPO both in HIR and LPS administered HIR. AG was significantly effective to lower serum MPO of LPS after HIR when given prior to LPS.

Liver tissue MPO of Group B was significantly higher than Group A ($P < 0.05$). Liver MPO of Group B was significantly higher than Group C, but lower than Group D ($P < 0.05$). Level of liver MPO in Group D was significantly higher than Groups E and F ($P < 0.05$),

TABLE 1

The Liver Tissue iNOS Scores*, and Mean ± SD Values of Serum and Liver Tissue MPO, Serum and Liver Tissue MDA, and Liver Tissue ATPase Levels in the Groups

	Group A (Sham)	Group B (HIR)	Group C (HIR+AG)	Group D (HIR+LPS)	Group E (HIR+LPS+AG)	Group F (HIR+AG+LPS)
Liver iNOS Score	0,5 [0 to 1]	2 [2 to 3]	1,5 [1 to 2]	4 [3 to 4]	3 [3 to 3]	2 [1 to 3]
Serum MPO (U/mL)	1.42 ± 0.04	2.23 ± 0.09	1.43 ± 0.09	3.12 ± 0.26	2.90 ± 0.20	2.63 ± 0.25
Liver MPO (U/g)	1.36 ± 0.18	4.94 ± 0.35	1.42 ± 0.17	6.75 ± 0.30	3.41 ± 0.30	2.68 ± 0.33
Serum MDA (U/mL)	9.65 ± 0.64	17.03 ± 2.06	12.76 ± 0.56	20.14 ± 0.58	16.04 ± 1.99	13.66 ± 0.95
Liver MDA (U/g)	12.26 ± 0.27	21.52 ± 1,75	14.67 ± 1.76	32.83 ± 0.65	21.93 ± 2.91	14.73 ± 1.74
Liver ATPase (nmolPi/mgPro/h)	73.96 ± 0.32	48.64 ± 8.21	68.08 ± 1.51	38.50 ± 3.42	29.74 ± 3,43	45.51 ± 10.03

* The immunohistochemical liver tissue iNOS expression scores of each group is presented as median, with interquartile range in brackets.

and also Group F was lower than Group E. HIR and LPS administration increased liver MPO. LPS administration after HIR increased liver MPO more than HIR alone. AG attenuated liver MPO in HIR and LPS administration. AG was more effective to lower liver MPO of LPS after HIR when given prior to LPS.

Serum and Tissue MDA

Mean ± SD value of serum MDA in Groups A, B, C, D, E, and F were 9.65 ± 0.64, 17.03 ± 2.06, 12.76 ± 0.56, 20.14 ± 0.58, 16.04 ± 1.99, and 13.66 ± 0.95, respectively, (Table 1). Mean ± SD value of liver tissue MDA in Groups A, B, C, D, E, and F were 12.26 ± 0.27, 21.52 ± 1.75, 14.67 ± 1.76, 32.83 ± 0.65, 21.93 ± 2.91, and 14.73 ± 1.74, respectively (Table 1). The descriptive statistics of serum MDA, liver MDA, are presented in Fig. 2.

Serum MDA of Group B was significantly higher than Group A ($P < 0.05$). Serum MDA of Group B was significantly higher than Group C ($P < 0.05$), but lower than Group D ($P < 0.05$). Level of serum MDA in Group D was higher than Groups E and F ($P < 0.05$); also, Group F was lower than Group E ($P < 0.05$). HIR and LPS after HIR significantly increased serum MDA activity. AG attenuated serum MDA in HIR and LPS after HIR. The decrease of serum MDA in LPS induced HIR was more when AG was administered prior to LPS.

Liver tissue MDA of the Group B was significantly higher than Group A ($P < 0.05$). Liver MDA of Group B was significantly higher than Group C, but lower than Group D ($P < 0.05$). The level of liver MDA in Group D was higher than Groups E and F ($P < 0.05$). Liver MDA of Group E was higher than Group F ($P < 0.05$). HIR and LPS after HIR increased liver MDA. LPS admin-

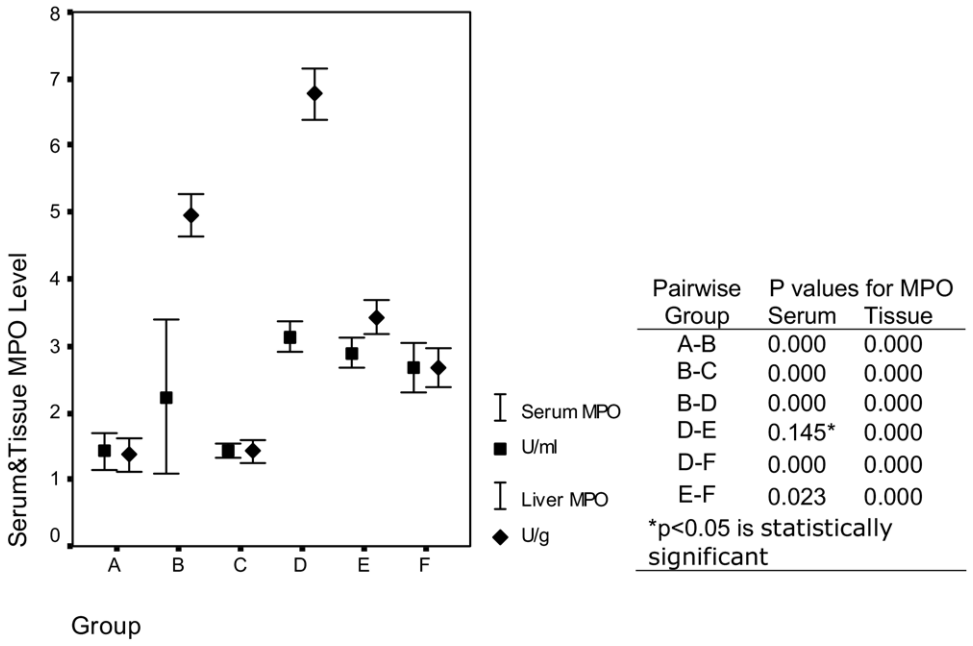
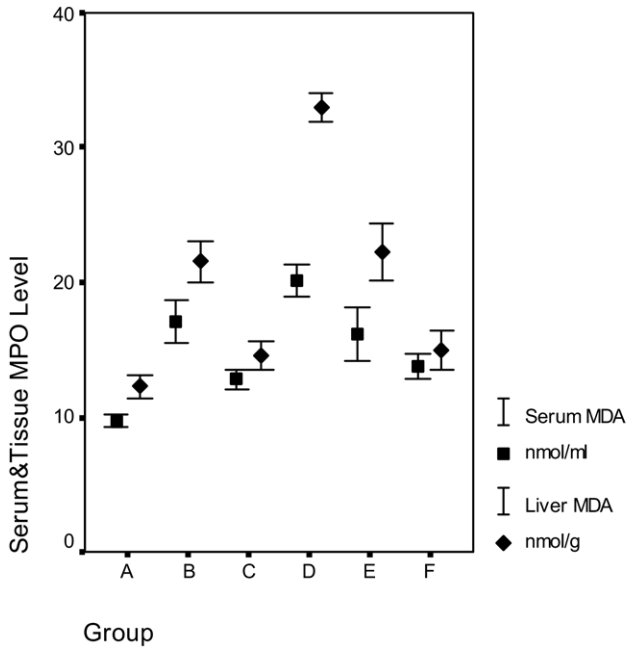


FIG. 1. The descriptive statistics of serum and liver tissue MPO and the significance levels of pair wise comparisons of groups according to Schefe test.



Pairwise Group	P values for MDA Serum	Tissue
A-B	0.000	0.000
B-C	0.000	0.000
B-D	0.000	0.000
D-E	0.000	0.000
D-F	0.000	0.000
E-F	0.003	0.000

P<0.05 is statistically significant

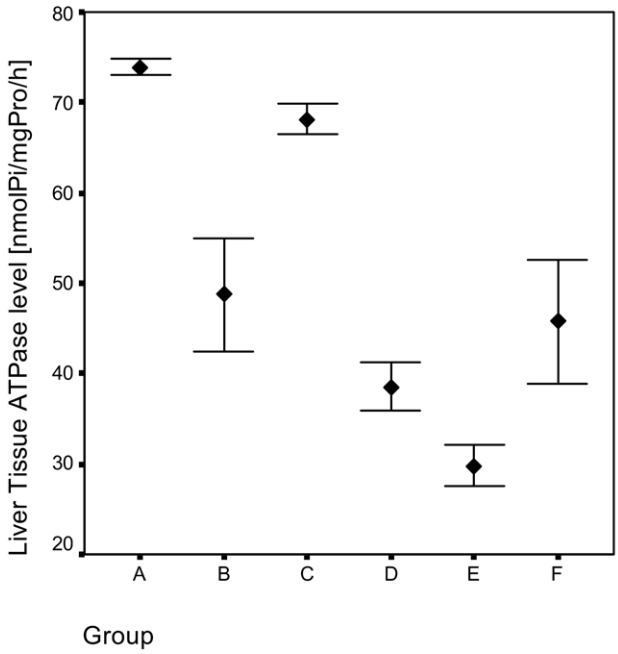
FIG. 2. The descriptive statistics of serum and liver tissue MDA and the significance levels of pair wise comparisons of groups according to Schefe test.

istration after HIR increased liver MDA more than HIR alone. AG attenuated liver MPO in HIR and LPS after HIR. The decrease of liver MDA in LPS induced HIR was more when AG was administered prior to LPS.

Tissue Na^+/K^+ ATPase

Mean ± SD value of liver ATPase in Groups A, B, C, D, E, and F were 73.96 ± 0.32, 48.64 ± 8.21, 68.08 ± 1.51, 38.50 ± 3.42, 29.74 ± 3.43, and 45.51 ± 10.03, respectively (Table 1). The descriptive statistics of liver ATPase is presented in Fig. 3.

Liver tissue ATPase of the Group B was significantly lower than Group A ($P < 0.05$). Liver ATPase of Group B was significantly higher than Group D, but lower Group C ($P < 0.05$). Level of liver ATPase in Group D was higher than Group E and significantly lower than Group F ($P < 0.05$). However, the difference between



Pairwise Group	P values for Tissue ATP'ase
A-B	0,000
B-C	0,000
B-D	0,004
D-E	0,120
D-F	0,022
E-F	0,000

*P<0.05 is statistically significant

FIG. 3. The descriptive statistics of liver ATPase and the significance levels of pair wise comparisons of groups according to Schefe test.

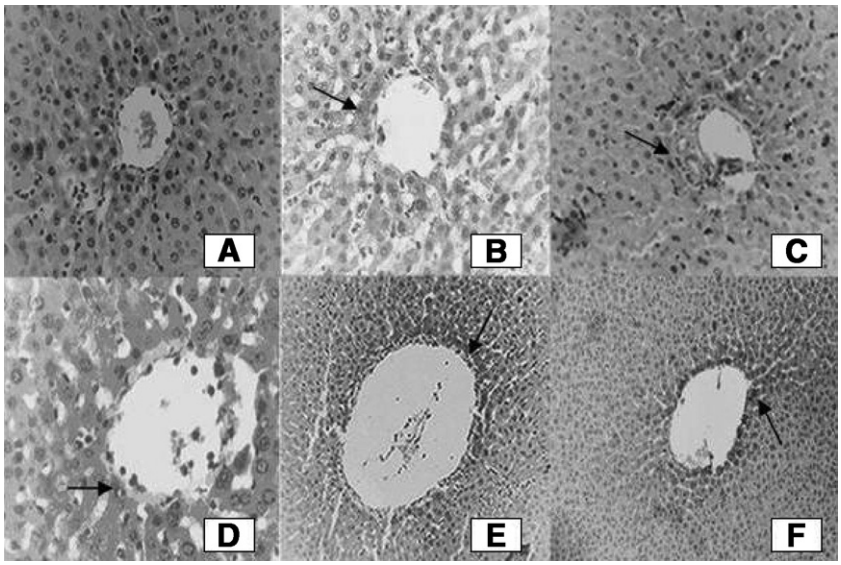


FIG. 4. (A)–(F) Representative of iNOS activity in Groups A, B, C, D, E, F, respectively in the immunohistochemical examination of liver tissue.

Groups D and E was not statistically significant ($P > 0.05$). Liver ATPase level in Group F was higher than Group E ($P < 0.05$).

Liver ATPase activity were decreased with HIR, and further decrease in liver ATPase activity was observed with LPS after HIR. Liver ATPase activity was increased by AG administration in HIR, although a significant increase in liver ATPase was noted with AG administration before LPS comparable to AG administration after LPS in LPS after HIR.

Immunohistochemical Liver iNOS Expression in the Groups

The median and interquartile ranges for the liver tissue iNOS expression scores in rats are presented in the Table 1. Group A: very little iNOS staining was detected around the central venules of the lobules; 0,5 [0 to 1]. Group B: the number of iNOS stained cells were increased; 2 [2 to 3]. Group C: there was sparse iNOS staining; 1,5 [1 to 2]. Group D: there was an intense iNOS staining; 4 [3 to 4]. Group E: there was an intense iNOS staining; 3 [3 to 3]. Group F: there was sparse iNOS staining; 2 [1 to 3], (Table 1, Fig. 4a–f).

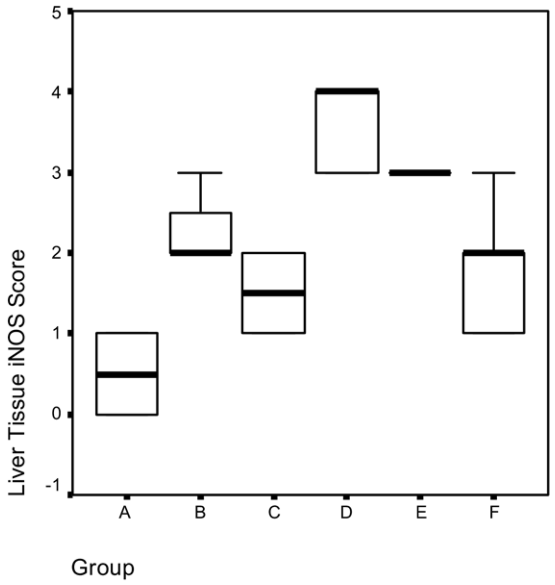
Liver iNOS expression score in Group A was significantly lower than Group B ($P < 0.05$). The liver iNOS expression score of Group B was significantly lower than Group D ($P < 0.05$). Although liver iNOS expression score of Group C was lower than Group B, the difference was not significant ($P > 0.05$). The liver iNOS score of Group D was higher than Groups F ($P < 0.05$). The liver iNOS score of Group E was higher than Group F ($P < 0.05$, Fig. 5).

The immunohistochemical examination with iNOS demonstrated that HIR significantly increased liver iNOS staining and also LPS increased iNOS staining in HIR. Despite decrease in iNOS staining score when AG was administered in HIR, the difference was not significant. AG attenuated liver iNOS expression in HIR after LPS when given prior to LPS.

DISCUSSION

HIR injury, which is seen after major hepatic vascular injury, major hepatic resections, and hepatic transplantation surgery, is the result of a series of complex interactive mechanisms. Energy depletion in the hepatic ischemia period results in transmembrane active transport failure, especially in the endothelial and Kupffer cells. The major problem in HIR injury is the prolongation and aggravation of this local energy depletion by activation of local and systemic inflammation after reperfusion [21].

HIR injury causes delayed hepatic regeneration and hepatic failure due to severe hepatocellular damage. Ischemia in hepatic tissue induces local hypoxia, which activates inflammatory cells, including Kupffer and T-cells, and induces increased FOR production. Activation of the inflammatory cascade causes increased production and secretion of prostaglandins and cytokines, including PAF, IL-1, IL-6, IFNγ, and GM-CSF. Cytokines induce increased expression of adhesion molecules in the endothelial cells affected by hypoxia. The cascade is followed by increased neutrophil transmigration and irreversible cellular damage, which is followed by local and systemic tissue and organ failure. This complex and multifactor pathophysiologic process including FOR, cytokines, PAF NO, and ET is controlled by transcriptional regulation of the mediators. Nuclear factor kappa B (NF-κB) is reported to



Pairwise Group	P values for Liver iNOS Score
A-B	0.000
B-C	0,085*
B-D	0,000
D-E	0,602*
D-F	0,000
E-F	0,002

*p<0.05 is statistically significant

FIG. 5. The differences between medians of groups in the immunohistochemical examination of liver tissue with iNOS staining.

control transcription of iNOS induced cytokines (TNF-α), chemokines, and adhesion molecules (intercellular adhesion molecule-1). Activation of NF-κB in liver tissue results in increased synthesis of proinflammatory cytokines and adhesion molecules, which causes tissue injury by increased FOR and neutrophil activation [22–30].

Increased oxidative stress induced by HIR injury and endotoxemia is the result of an imbalance between oxidative and antioxidative activity. Oxidative activity is increased to high levels that are impossible to neutralize with endogenous antioxidative activity. Oxidative stress is responsible for the early and late phase pathological changes in HIR injury. Xanthine oxydase in Kupffer's and renal tubular epithelium is activated by oxidative stress induced by hypoxia, and this activation is prolonged and increased with reperfusion. Lipid peroxidation causes lysis of cellular membranes. This cascade is reversed experimentally in septic shock, burns, and HIR injury by antioxidants [25–27]. Removal of oxidative stress in these conditions is the primary intervention to decrease tissue injury. Superoxide dismutase, catalase, glutathione, α-tocopherol, and carotene are the known endogenous antioxidants, but none of these endogenous antioxidants are sufficient to compensate FOR activity in I/R and septic shock. Several antioxidants have been studied and reported to decrease oxidative stress in hepatic and renal tissue in experimental obstructive jaundice, septic shock, and I/R models. Caglikulekci and coworkers reported decreased lipid peroxidation and oxidative stress in LPS induced obstructive jaundice by antithrombin III and N-acetylcysteine [20, 31, 32].

AG needs to be incorporated into the guanido group of hydrazine linked L-arginine to be activated. AG not only inhibits iNOS in a selective manner but effects several enzymatic processes including nonenzymatic glucolization, inhibition of diamine oxydase (causes in vivo inactivation of histamine in pulmonary arteries) and causes prostacyclin secretion.

Recently, the role and effect of NO and iNOS in HIR injury have begun to be studied, but in our literature search we observed no specific data on the role of AG in LPS induced experimental HIR injury in the rat [33–35]. In our study, a short period of ischemia was followed by a short period of reperfusion. The in vivo selective inhibitory effect of AG administered just before LPS on nitric oxide production in the rat liver tissue has been demonstrated with electron paramagnetic resonance [36]. Also in our study, AG and LPS was administered within a short interval in Groups E and F before reperfusion to test and evaluate if we could observe a similar effect of AG in LPS after HIR on lipid peroxidation and iNOS expression or not. The differences we observed in our study between the Groups E and F can be explained by the presence of some expression of iNOS in the liver tissue due to natural exposure of the rats to some inflammatory agents [37].

Na^+/K^+ ATPase is an integral membrane enzyme, which is known to maintain the electrochemical gradients for ions Na^+ and K^+ across the cell membrane. Thus, Na^+/K^+ ATPase regulates the active transport across the plasma membrane for bile acid and water from sinusoidal to canalicular membranes by regulating the concentrations of these ions, and their gradients. The enzyme is also in close relationship with submembrane microfilaments and is involved in cellular structure via this way. Since Na^+/K^+ ATPase plays a key role in the physiology and structure of hepato-

cytes, it could be considered as an indicator of liver tissue viability and hepatic function [38].

In our study, HIR and LPS after HIR decreased liver ATPase. Although LPS administration deteriorated, AG ameliorated liver ATPase in HIR. Deterioration with LPS can be explained by the fact that endotoxemia leads to an exaggerated oxidative stress and energy depletion due to an exaggerated inflammatory response. Higher tissue ATPase activity with AG can be explained not only with increased liver tissue viability but also decreased NO levels due to iNOS inhibition. Although a significant increase in ATPase level with AG administration when given before LPS in LPS after HIR was noted, AG did not lead to ATPase level increase when given after LPS in LPS after HIR. These may be explained as due to dose and/or time interval insufficiency of our AG administration to reverse the effect of triggered inflammatory agents with LPS [36, 39].

HIR injury induces systemic inflammatory responses affecting chiefly pulmonary, renal, and the gastrointestinal systems. This effect is the result of activation of systematic inflammatory cells triggered by the cytokines released from activated local inflammatory cells. In this cascade, local and systemic tissue injury is seen because of enhanced lipid peroxidation. Serum and tissue MDA levels are used as a quantitative indicator of oxidative stress and lipid peroxidation. Increased levels of tissue MDA levels were reported in several experimental I/R injury models [40–42].

In this study, both serum and liver tissue MDA levels were increased by HIR, and LPS caused more increment when administered after HIR. Contrary to LPS, AG decreased MDA level of HIR injury. The results suggested that LPS induced endotoxemia after HIR aggravated lipid peroxidation more than HIR alone, whereas AG maintained an attenuation of lipid peroxidation in HIR. Also, AG produced amelioration in lipid peroxidation aggravated by LPS induced endotoxemia after HIR, and it was more effective when given prior to LPS. This data shows the protective effect of AG against increased lipid peroxidation due to LPS induced endotoxemia after HIR injury.

Neutrophils, as the primary potential site of FOR production, play a major role in the development of oxidative stress. MPO levels are used as an index of neutrophil infiltration, and were shown to be increased in several inflammatory events including experimental I/R injury, burns, and sepsis [43, 44]. The results of MPO obtained in this study showed that HIR gave rise to increased intensity of neutrophils both in serum and liver tissue, and contrary to LPS administration, which caused more increment than HIR, AG provided a decrement in neutrophils when administered in HIR. Besides this, in endotoxemia aggravated HIR, AG decreased MPO levels, which were likely due to decreased activation of FOR producing neutrophils. These results show the attenuation of oxidative stress induced by endotoxemia in experimental HIR injury. Neutrophils and Kupffer cells probably play a key role in this effect. In experimental intestinal I/R injury, AG was shown to attenuate hepatic microcirculation defects, energy deficiency, and lipid peroxidation. These effects were observed probably because of decreased postcapillary and postsinusoidal leukocytes, circulating granulocytes, and significantly decreased CD 11b expression on the granulocytes [35, 45].

Increased iNOS gene transcription due to NF-κB activation was shown to result after HIR injury in the rats. NF-κB complex levels in ischemic liver tissue (composed of p50/p65 heterodimer and p50 homodimer) are found to be increased in the first hour of reperfusion. These elevations continue for 3 h, and begin to decrease after 5h. In time curve studies, iNOS mRNA expression is shown to begin in the first hour of reperfusion, and to increase for the next 5 h. Increased up-regulation of iNOS mRNA expression in liver tissue was shown to coincide with increased iNOS isoenzyme and NF-κB binding activity. These results show the activation of NF-κB in HIR injury induced oxidative stress and the major role of NF-κB in iNOS gene induction [46–48].

Increased NO production and iNOS expression in HIR injury and in LPS induced endotoxemia causes the inhibition of mitochondrial respiratory cascade enzymes and gluconeogenesis. Increased TNF-α release was found in LPS plus HIR injury compared with LPS induced endotoxemia alone and HIR injury alone. LPS plus HIR injury causes more severe hepatic and pulmonary injury [49]. This may explain the development of multiple organ failure in some hepatic resections, hepatic transplantation, complex vascular surgery, and hypovolemic shock. In the present study, liver tissue iNOS staining was increased by HIR, and endotoxemia aggravated this result. Despite the aggravating effect of endotoxemia, AG attenuated liver iNOS staining in LPS after HIR when administered prior to LPS.

The results of this study showed the attenuation of liver tissue iNOS expression and lipid peroxidation in HIR injury by the inhibition of iNOS by AG. LPS after HIR aggravated HIR injury by increasing neutrophil activation and lipid peroxidation both in serum and liver tissue, and iNOS in liver, and depleting energy in liver. AG, a selective iNOS inhibitor, ameliorated the negative effects of endotoxemia induced by LPS after HIR; however, energy depletion and iNOS expression in liver tissue were attenuated only when AG was administered prior to LPS. The findings of this study supported the hypothesis that LPS after HIR would aggravate HIR injury and AG would ameliorate this aggravated injury.

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