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A Novel Antioxidant Non-steroidal Anti-inflammatory Agent Protects Rat Liver against Ischemia-Reperfusion Injury

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Abstract. *Liver ischemia followed by reperfusion is an important and common clinical event. A major mechanism is leukocyte adhesion to endothelium followed by release of reactive oxygen metabolites. The aim of this study was to determine the effects of a novel antioxidant ethylenediamine derivative with anti-inflammatory properties (compound IA) on an imitated clinical setting of acute hepatic ischemia-reperfusion injury. Eight groups of rats were subjected to a model of hepatic ischemia that was produced by occluding for 30 min the portal vein and hepatic artery. At the end of ischemia, compound IA was administered intravenously and the clamps were removed allowing reperfusion for 60 min or 24 h. The effect of compound IA was evaluated by histopathological examination, lipid peroxidation and plasma levels of liver enzymes. Administration of compound IA resulted in significantly less histological damage in liver tissue after 30-min ischemia followed by 60-min and 24-h reperfusion. Ischemia followed by 60 min of reperfusion increased lipid peroxidation compared to the sham-operated and the non-ischemic group. This increase was attenuated in the group treated with compound IA. Serum enzyme levels were significantly higher in the reperfusion groups compared to the non-ischemic groups and diminished after treatment. Compound IA exerted a protective effect on hepatic reperfusion injury in rats. Compound IA is believed to act by means of its potent antioxidant and anti-inflammatory activities.*

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Post-ischemic hepatic injury can present after hypovolemic shock, major surgery, liver transplantation, trauma and other forms of severe physiological stress (1). Reactive oxygen intermediates generated following reperfusion seem to be responsible for at least part of the reperfusion injury (2). Increased formation of superoxide radical leads to the generation of hydrogen peroxide which can further produce cellular damage. These oxygen-related products can interact in the presence of suitable transition metal catalysts to form the highly toxic hydroxyl radical and other oxidizing species, all of which are toxic to cells by producing lipid peroxidation of the cell membranes (3).

Rat liver contains xanthine dehydrogenase, but this is only slowly converted to oxidase during ischemia, although both enzymes can be released into the systemic circulation from damaged rat liver and may contribute to extra-hepatic damage (4). Other sources of oxidative stress in reperfused livers include increased superoxide anion production by damaged mitochondria, adherence to the liver vascular endothelium of circulating neutrophils and activation of macrophages (Kupffer cells) present in the liver itself. Endothelial damage early in liver reoxygenation may involve activation of Kupffer cells, whereas neutrophils and xanthine oxidase may contribute to later damage (4). In addition, mildly toxic reactive oxygen species such as superoxide radical released by adherent neutrophils react with intracellular iron in endothelial cells and hepatocytes to produce highly toxic free radicals, particularly hydroxyl radical. Once formed, these toxic free radicals react with the endothelial cells and hepatocytes resulting in cell injury or death (5).

In order to eliminate the damage produced by the reactive oxygen species in the reperfused liver, two strategies are referred to in the literature (6). In the first, specific enzymes such as superoxide dismutase and glutathione peroxidase decrease the oxidative stress injury induced by oxygen free radicals, while in the second, radical generation is prevented. Many studies have been conducted into this matter and agents such as allopurinol, N-acetyl-cysteine, tocopherol and others have been tested (3, 7, 8). However, in only a few of these studies was the agent administered following ischemia and before reperfusion, simulating the likely use of a protective agent in the clinical setting of acute ischemia-reperfusion injury (9).

The compound IA [5-(2-amino-ethylamino)-1-phenyl-2-pentanone] is a novel non-steroidal anti-inflammatory agent with basic character (thus causing fewer side- effects than the commonly used non-steroidal anti-inflammatory drugs) (10). It is known that free radicals, lipid peroxides and arachidonic acid metabolites are closely related to mechanisms of injury (11). It was found that compound A possesses significant anti-inflammatory activity, reducing carraggeenan rat paw edema (12). It has also been shown that this compound inhibits non-enzymatic lipid peroxidation of rat liver microsomal lipids. Furthermore, it was found to be a very potent scavenger of hydroxyl radicals and to interact with the stable 1,1-diphenyl-2-picrylhydrazyl free radical (DPPH), a property that

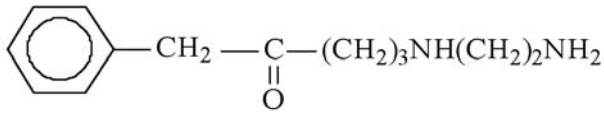

Figure 1. *The chemical structure of compound IA.*

Table I. *Influence of treatment with compound IA on the extent of tissue damage on individual histological features. Groups: (1) control , sham-operated; (2) control, 30-min ischemia; (3) control, 30-min ischemia and 60-min reperfusion; (4) control, 30-min ischemia and 24-h reperfusion; (5) IA, sham- operated; (6) IA, 30-min ischemia; (7) IA, 30-min ischemia and 60 min reperfusion; (8) IA, 30-min ischemia and 24 h reperfusion.*

	Histological features		
Group	Hepatocellular necrosis	Poymorphonuclear infiltration	Vascular congestion
1	0	0	0
2	0	0.5	0
3	0	2.5 [a]	1.5 [a]
4	2 [a]	2 [a]	2 [a]
5	0	0	0
6	0	0.5	0
7	0	0.5	0
8	0 [b]	0.5 [b]	0 [b]

[a] Statistically significant difference compared to the corresponding sham groups at $p<0.05$ (Kruskall-Wallis test).
[b] Statistically significant difference compared to the corresponding control groups at $p<0.05$ (Mann-Whitney test).

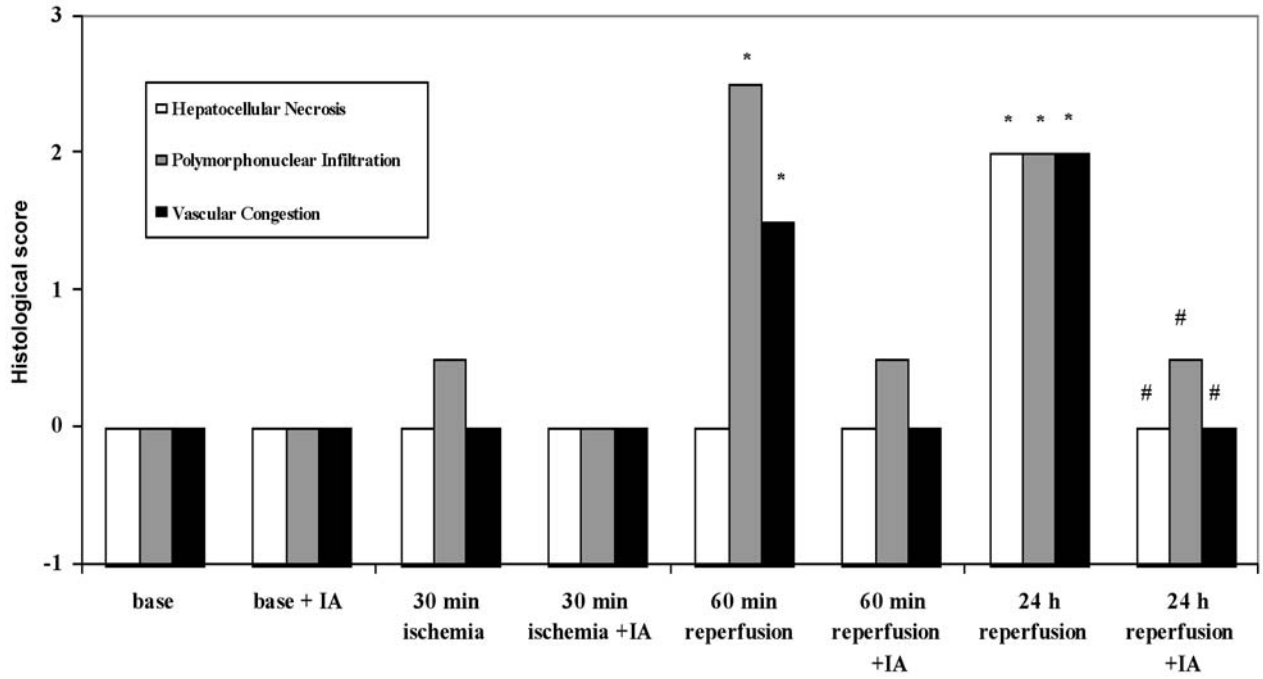

Figure 2. *Influence of treatment with compound IA on the extent of hepatic tissue damage on individual histological features. The values represent the median of 6 animals per group. *Statistically significant difference compared to the corresponding sham groups at $p<0.05$ (Kruskall-Wallis test). # Statistically significant difference compared to the corresponding control groups at $p<0.05$ (Mann-Whitney test).*

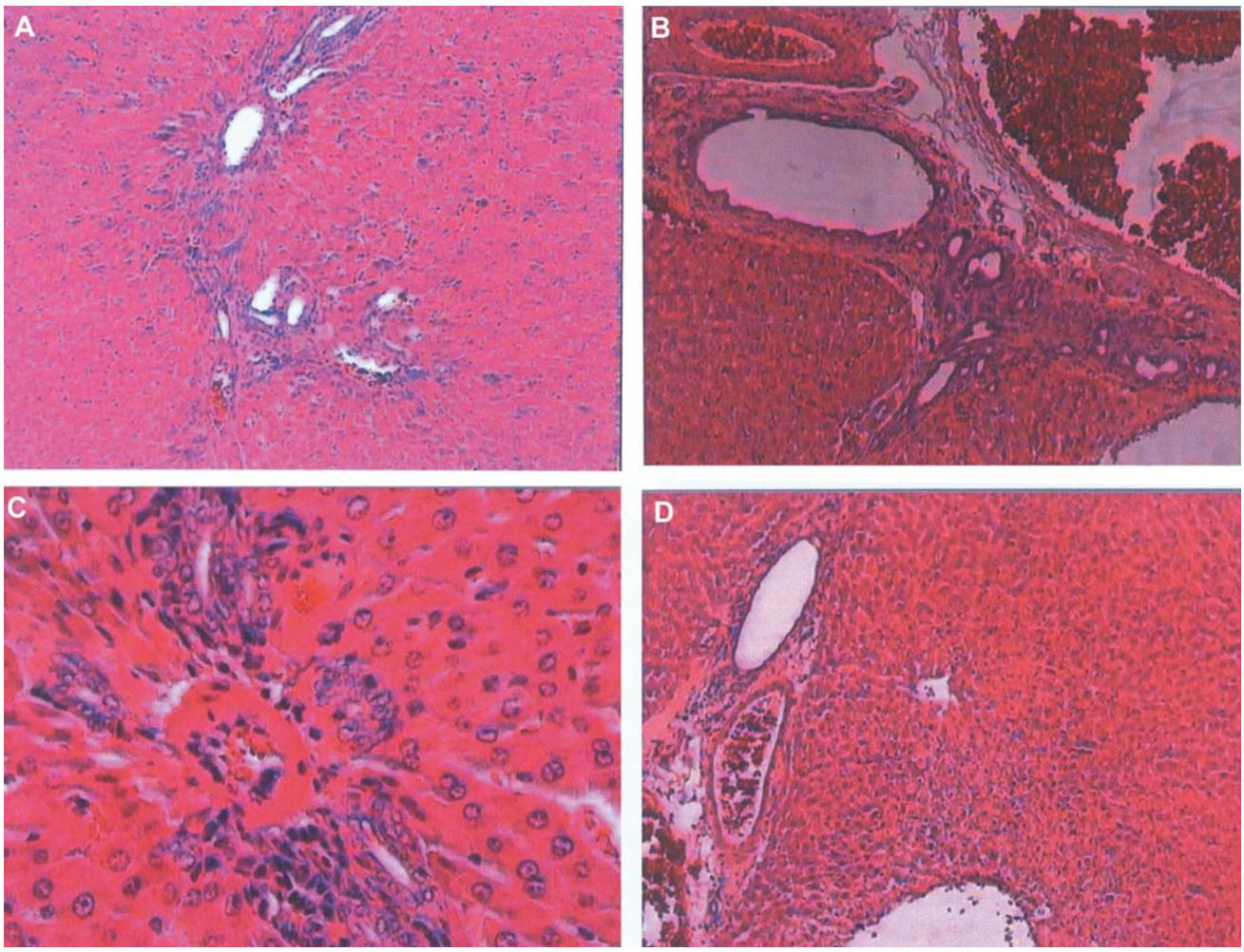

Figure 3. *A. Microscopic aspect of rat liver submitted to 30-min ischemia followed by 60- min reperfusion. Sparse, diffuse granulocytic infiltrates among hepatocytes and slightly dilated vessels were observed (eosin-haematoxylin staining, magnification x10). B. Microscopic aspect of rat liver submitted to 30-min ischemia followed by 24-h reperfusion. Extensive areas of necrosis, vessel dilation and hyperhaemia and dense granulocytic infiltrates were observed (eosin-haematoxylin staining, magnification x20). C. Microscopic aspect of rat liver submitted to 30-min ischemia followed by 60- min reperfusion and treated with IA. Thickening of the vascular walls with evident perivascular inflammatory infiltrates. Absence of necrosis and of multinucleated cells was observed (eosin-haematoxylin staining, magnification x40). D. Microscopic aspect of rat liver submitted to 30-min ischemia followed by 24-h reperfusion and treated with IA. Scanty granulocytes, located around vessels (when present), without necrosis were observed (eosin-haematoxylin staining, magnification x 20).*

expresses the reducing activity of the compound and its ability to scavenge N-centered radicals (12). The above compound did not show any superoxide anion radical scavenging activity (10).

Recently, compound IA has been shown to have a beneficial effect on the repair process of intestinal mucosa after transient mesenteric ischemic reperfusion. Its mechanism was considered to be mediated *via* its potent antioxidant properties as it decreased the concentration of malondialdehyde of small intestineμ tissue (13).

The present study was carried out to evaluate the effects of compound IA on ischemia-reperfusion injury in the rat liver. Histopathological examination, malondialdehyde content and liver enzymes were assayed to measure the effect of this agent on its protective ability against ischemia-reperfusion injury of the liver.

Materials and Methods

Materials. The synthesis of compound IA has already been described in detail (12). The chemical structure of compound IA is shown in Figure 1.

Forty-eight adult male Wistar rats weighing 280-320 g were obtained from the Pasteur Institute, Athens, Greece. The rats were acclimatized to our laboratory conditions for 1 week prior to use

in experiments and housed individually in stainless steel cages at a constant temperature (28˚C) and a 12-h day / night cycle. The rats were fed commercial rat chow and had water *ad libidum*.

All experimental procedures described below were approved by the Animal Care Committee in accordance with European Union legislation and the Greek law on experimentation (160, A-64, May 1991).

Experimental protocol. Animals were divided into 8 groups consisting of 6 rats each: (1) control , sham-operated; (2) control, 30-min ischemia; (3) control, 30-min ischemia and 60-min reperfusion; (4) control, 30-min ischemia and 24-h reperfusion; (5) IA, sham-operated; (6) IA, 30-min ischemia; (7) IA, 30-min ischemia and 60-min reperfusion; (8) IA, 30-min ischemia and 24-h reperfusion. The rats were anesthetized with ketamine hydrochloride (80mg/kg) and xylazine (16mg/kg), administered intramuscularly. A jugular venous cannula was inserted for fluid and drug administration.

The dose for compound IA was 0.3 mmol/kg, which is the same as that used for the determination of anti-inflammatory activity (carrageenan experiments) and the same for evaluation of its protective activity against mesenteric ischemia-reperfusion injury. The LD_{50} of compound IA in rats is 0.86 mmol/kg intraperitoneally [12]. The control agent was normal saline.

Surgical technique. Through a midline abdominal incision using aseptic technique, the portal vein and the hepatic artery were isolated. In groups **(1)** and **(5)** the tested agent was administered intravenously and animals were sacrificed after a 60-min period. In groups **(2)**, **(3)**, **(4)**, **(6)**, **(7)** and **(8)** both isolated vessels were occluded using microsurgical clamps for 30 min. Just before the end of this period of ischemia, the substance was administered intravenously and then the clamps were removed. In groups **(3)** and **(7)** a 60-min period of reperfusion followed the ischemia period. In groups **(4)** and **(8)** a 24-h period of reperfusion followed the ischemia period. At the end of the operations all animals were exsanguinated, blood samples were obtained for measurement of liver enzymes and malondialdehyde levels, and tissue samples from the liver were subjected to histopathological evaluation. Furthermore, at the end of the experiment, all animals appeared normal both macroscopically and by autopsy.

Histopathological assessment. At the end of the operations all animals were exsanguinated. The medial lobe of the liver was removed, fixed in formalin and submitted to pathological process after staining using hematoxylin-eosin. A single pathologist, blinded to the treatment each animal had received, then evaluated the process. Three randomized power fields were analyzed and graded on a scale of 0-3 (0: normal; 1: minimal alterations of under 25%; 2: moderate alterations of 25-50%; 3: severe alterations of over 50%) and separate scores were obtained for each of the following: hepatocellular necrosis (hepatocytes with microscopic aspects of necrosis such as nuclear pyknosis, karyorrhexis and karyolysis without pericellular inflammatory infiltrate), infiltration of polymorphonuclear cells and vascular congestion (vascular dilation demonstrating erythrocytes within the centrilobular veins) (7).

Assay of lipid peroxidation. Blood samples were taken at the end of the operation of each group. Malondialdehyde concentration was determined spectrophotometrically at 586 nm and expressed as μM as previously described (14).

Briefly, 0.65 ml of 10.3 mM N-methyl-2-phenyl-indole in acetonitrile was added to 0.2 ml of blood plasma. After vortexing for 3-4 sec and adding 0.15 ml of HCl 37%, the samples were mixed well, closed with a tight stopper and incubated at 45˚C for 60 min. The samples were then cooled on ice, centrifuged and the absorbance measured spectrophotometrically at 586 nm. A calibration curve of an accurately prepared standard MDA solution (from 2 to 20 nmol/ml) was also run for quantitation. Measurements of each group were performed in triplicate and standard deviation was less than ± 10%.

Enzymatic assay. At the end of each experiment, blood samples were collected in order to obtain the levels of alanine aminotransferase (ALT), serum aspartate aminotransferase (AST) and lactate dehydrogenase (LDH), using a multianalyzer (Datebehring Dimension AR). Measurements of each group were performed in triplicate and standard deviation was less than ± 10%.

Statistical analysis. All variables are represented using the number of rats (n), mean value (mean) and standard deviation (s.d) for biochemical markers and the median for histological values. Comparisons of biochemical markers and histological variables between groups were performed by means of the two-sample Student's *t*-test (or Welch-test in case of unequal s.d.'s) and Mann-Whitney test, respectively. Comparisons of time measurements for each biochemical marker and histological variable were performed using the One factor with no repeated measurements, ANOVA model (Scheffe test for pairwise multiple comparisons) and Kruskall-Wallis test (Mann-Whitney test for pairwise multiple comparisons), respectively. All tests are two-sided with 95% significance level. Statistical analysis was carried out using the statistical package SPSS vr 8.00 (Statistical Package for the Social Sciences) at the 0.05 level of significance.

Results

Histology. The histological features for each group are summarized in Table I. Furthermore, Figure 2 illustrates hepatocellular necrosis, polymorphonuclear infiltration and vascular congestion in liver specimens of all groups. Sham-operated groups (**1** and **5**) did not show any damage as expected. Group **4** (control, 24-h reperfusion) had significantly more hepatocellular necrosis ($p<0.05$) than group **1** (control, sham-operated). On the contrary, the difference in hepatocellular necrosis between groups **8** (IA, 24-h reperfusion) and **5** (IA, sham-operated) was of no statistical significance ($p>0.05$). Furthermore, at 24-h reperfusion, control animals (group **4**) had significantly more hepatocellular necrosis ($p<0.05$) than the animals treated with IA (group **8**). At 60-min but also at 24-h reperfusion, only non-treated animals (groups **3** and **5**, respectively) showed significantly more polymorphonuclear infiltration and vascular congestion ($p<0.05$) than the corresponding non-ischemic (sham-operated) group (group **1**). At 60-min reperfusion, control animals (group **3**) had significantly more polymorphonuclear infiltration and vascular congestion ($p<0.05$) than animals treated with IA

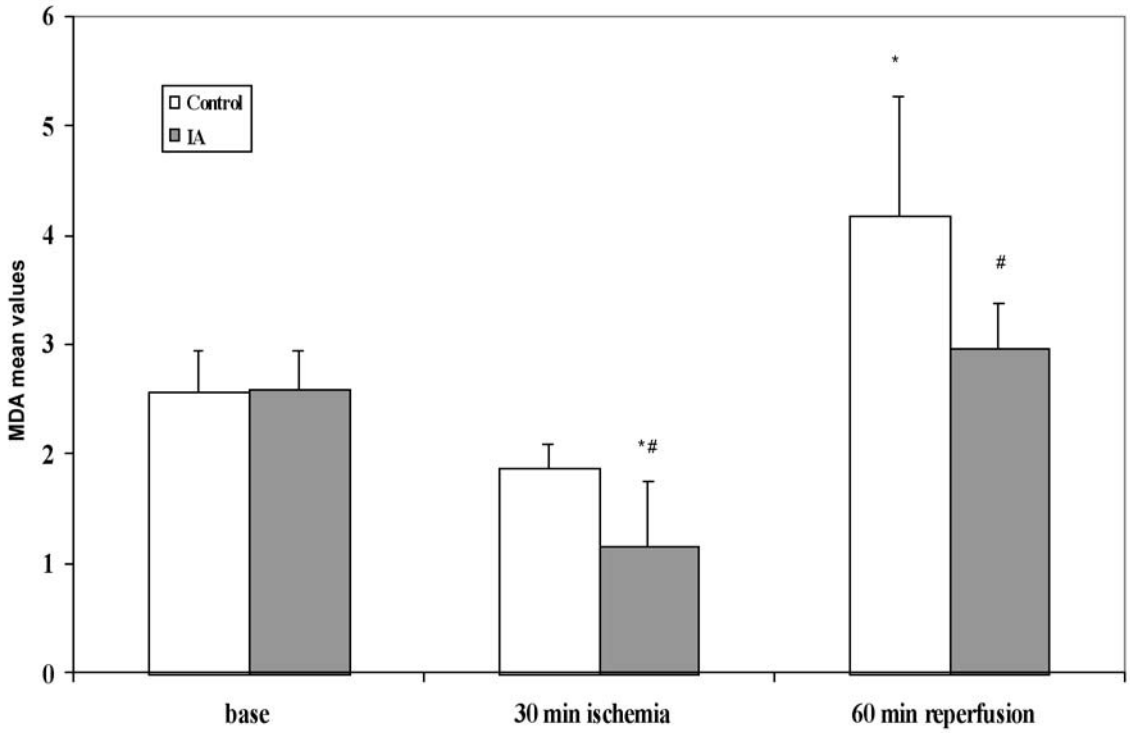

Figure 4. *Effects of IA on the MDA formation during ischemia reperfusion on baseline, after 30 min of ischemia and after 30 min of ischemia followed by 60 min of reperfusion * p <0.05 versus the corresponding baseline, # p <0.05 compared to the corresponding control group.*

(group **7**). Finally, at 24-h reperfusion, the comparison between non-treated (group **4**) and IA-treated animals (group **8**) showed significantly less vascular congestion ($p<0.05$) when IA was administered.

In histological sections of the groups under study (ischemia-reperfusion), we observed sparse, diffuse granulocytic infiltrates among hepatocytes in control group **3** that was subjected to 30-min ischemia followed by 60 min of reperfusion (Figure 3A). Extensive areas of necrosis, vessel dilation and hyperhaemia were observed in group **4** (60-min ischemia followed by 24-h reperfusion) (Figure 3B). When compound IA was administered, thickening of the vascular walls with evident perivascular inflammatory infiltrates and absence of necrosis and of multinucleated cells were observed (group **7**, 30-min ischemia followed by 60-min blood reperfusion + IA) (Figure 3C). In group **8** (30- min ischemia followed by 24-h reperfusion + IA) scanty granulocytes, located around vessels without necrosis were observed (Figure 3D).

Protective effect of compound IA against oxidative damage after hepatic ischemia- reperfusion. The production of malondialdehyde concentration was measured in groups **1**, **2**, **3**, **5**, **6** and **7** at the end of each experiment as an index of the development of lipid peroxidation. The effect of compound IA on the malondialdehyde concentration is illustrated in Figure 4. Sham-operated rats and those subjected to 30 min of ischemia showed low blood levels of MDA whether normal saline or compound IA was administered. In comparison with non-treated rats, compound IA significantly ($p<0.05$) attenuated the increase of MDA concentration in rats submitted to hepatic 30-min ischemia followed by 60 min of reperfusion (group **3** *vs.* group **7**). Moreover, at 60 min of reperfusion, MDA values of non-treated rats were significantly higher ($p<0.05$) compared to baseline (group **3** *vs.* group **1**), but IA-treated rats showed no ($p>0.05$) statistically significant difference (group **7** *vs.* group **5**). Furthermore, at the 30th minute of ischemia, the first minute of administration of compound IA resulted in a significant decrease in malondialdehyde levels compared to those of the corresponding control group ($p<0.05$ *vs.* group 2).

Measurement of AST, ALT and LDH levels. Comparisons of plasma levels of AST, ALT and LDH between control groups and groups treated with IA are represented in Figure 5, Figure 6 and Figure 7, respectively. The data obtained from non-ischemic (sham-operated) groups did not demonstrate statistical difference between the groups ($p>0.05$). AST values were significantly lower ($p<0.05$) in

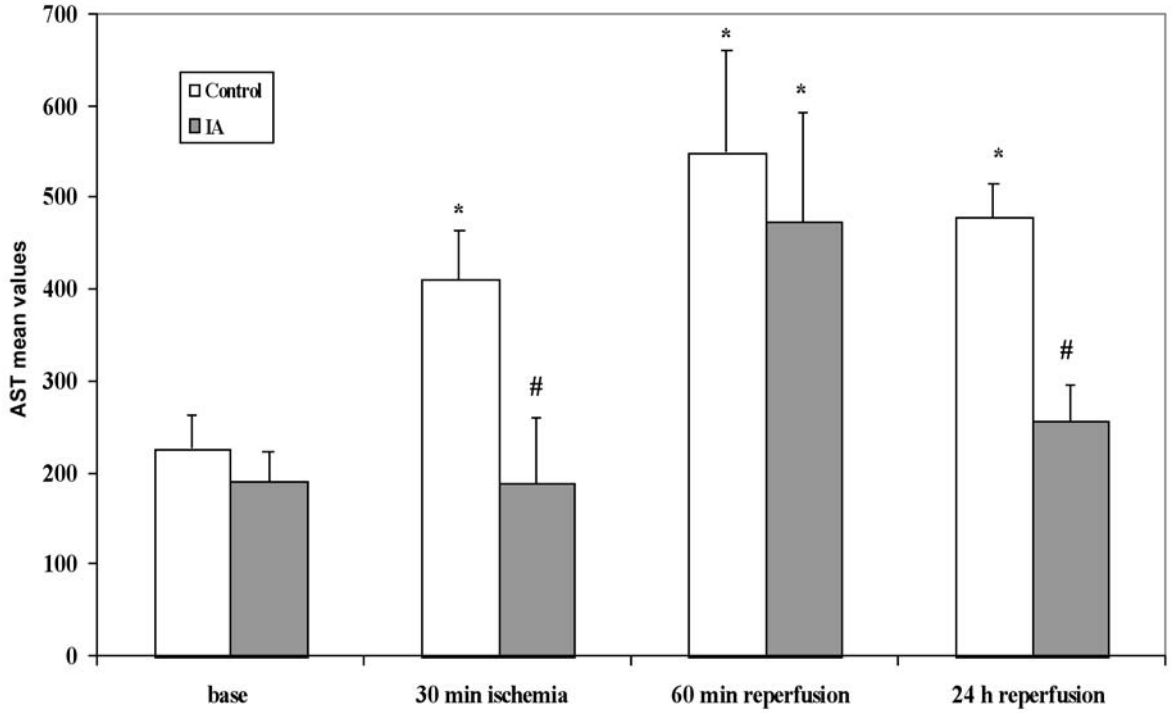

Figure 5. *Comparison between IA-treated and non-treated animals at baseline, after 30 min of ischemia, after 30 min of ischemia followed by 60 min of reperfusion and after 30 min of ischemia followed by 24 h of reperfusion, concerning AST values in plasma. * p <0.05 versus the corresponding baseline, # p <0.05 compared to the corresponding control group.*

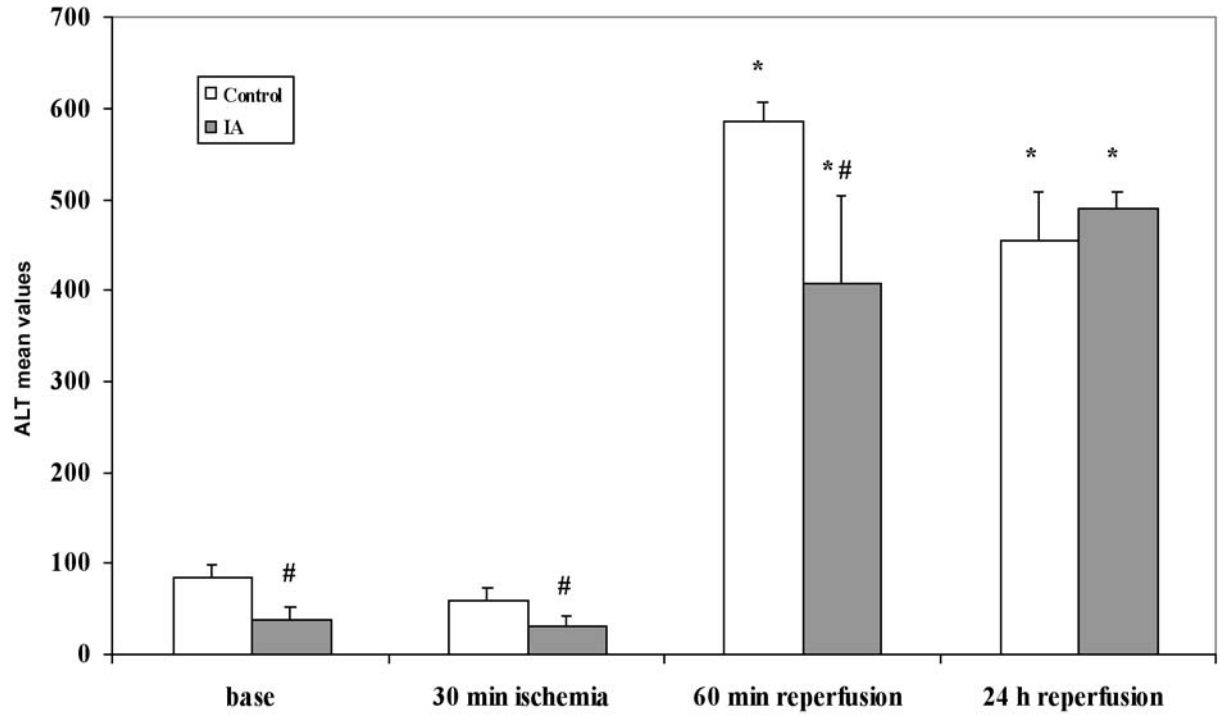

Figure 6. *Comparison between IA-treated and non-treated animals at baseline, after 30 min of ischemia, after 30 min of ischemia followed by 60 min of reperfusion and after 30 min of ischemia followed by 24 h of reperfusion, concerning ALT values in plasma. * p <0.05 versus the corresponding baseline, # p <0.05 compared to the corresponding control group.*

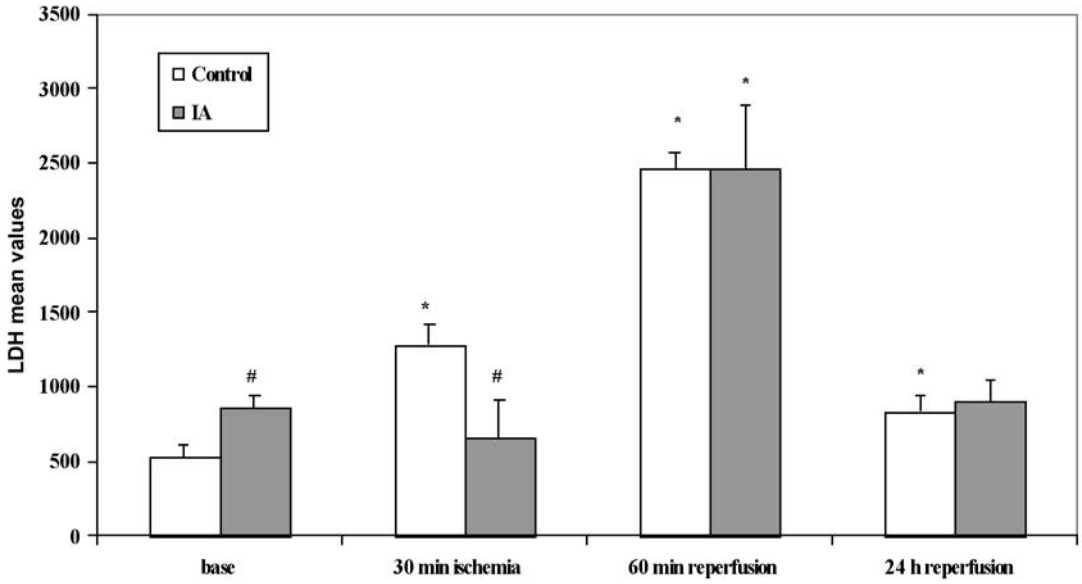

Figure 7. *Comparison between IA-treated and non-treated animals at baseline, after 30 min of ischemia, after 30 min of ischemia followed by 60 min of reperfusion and after 30 min of ischemia followed by 24 h of reperfusion, concerning LDH values in plasma. * p <0.05 versus the corresponding baseline, # p <0.05 compared to the corresponding control group.*

animals treated with IA than in the control group after 24 h of reperfusion (group **8** *vs.* **4**), as shown in Figure 5. ALT values were significantly lower ($p<0.05$) in animals treated with IA than in the control group, after 60 min of reperfusion (group **7** *vs.* **3**), as shown in Figure 6. LDH values did not demonstrate statistical difference ($p>0.05$) between controls and the IA-treated group either in the 60-min or in the 24-h reperfusion group (Figure 7). However, in the comparison between the non-ischemic (sham) group and the 24-h reperfusion group, LDH values were significantly higher ($p<0.05$) only in the non-IA-treated animals (group **4** *vs.* **1**) and were of no significant difference ($p>0.05$) in animals treated with IA (group **8** *vs.* **5**), as shown in Figure 7. All other comparisons between reperfusion groups and non-ischemic (sham) groups showed a significantly important increase in AST, ALT and LDH values in the reperfusion groups in both IA-treated (groups **7** *vs.* **5**, and **8** *vs.* **5**) and non-IA-treated groups (groups **3** *vs.* **1**, and **4** *vs.* **1**).

Discussion

It is well documented that oxygen free radicals (superoxide and hydroxyl), related species (hydrogen peroxide) and phagocytes (Knupffer cells and neutrophils) have been implicated in the pathogenesis of ischemia-reperfusion injury (15). Many studies have used xanthine oxidase inhibitors in order to improve ischemic injury and it has thus been proven that superoxide anions produced by the xanthine oxidase enzymic system have a role in postischemic injury. However, the source of oxygen free radicals in the liver remains controversial. It has been demonstrated that the xanthine-xanthine oxidase pathway has a very limited role in oxygen free radicals production in hepatic ischemia-reperfusion (16). On the other hand, it has been shown that cellular damage of the liver by ischemia followed by reperfusion is assumed to be at least in part due to lipid peroxidation in biomembranes, which results in mitochondrial dysfunction as well as damage to energy metabolism and cellular calcium homeostasis (17, 18).

Compound IA had no effect on superoxide anion scavenging activity as was observed by using the xanthine-xanthine oxidase system that generates superoxide anions (10). On the other hand, compound IA has been shown to inhibit lipid peroxidation completely at a concentration of 25 μM, with an IC_{50} value of 18 μM, after 45 min of incubation. DL-(a) tocopherol, that was used as a reference compound, was found to become inactive at a concentration of 0.5 mM (19).

In the present study, we showed that treatment with compound IA resulted in a significantly beneficial effect on total hepatic ischemia followed by reperfusion by reducing the histological damage. Moreover, compound IA resulted in significantly less tissue necrosis, polymorphonuclear infiltration and vascular congestion after 30 min of ischemia followed by 24 h of reperfusion. There is an indication that

oxygen free radicals play a major role in producing the microvascular and parenchymal cell damage associated with reperfusion of ischemic tissues (20-22). Compound IA attenuated the inflammatory infiltrates in hepatic parenchyma after 30 min of ischemia and 60 min of reperfusion and scanty granulocytes, located around vessels without necrosis, were observed after 30 min of ischemia and 24 h of reperfusion when IA was administered.

It has been suggested that cellular damage caused by ischemia-reperfusion in liver can be explained by lipid peroxide formation, which could support the assumption that liver cell injury would be improved by modifying free radical metabolism and subsequent lipid peroxidation (23). In this study, compound IA protected the liver from oxidative damage after 30-min ischemia followed by 60 min of reperfusion by inhibiting lipid peroxidation, as measured by malondialdehyde levels. This strong inhibition can be considered one of the mechanisms of action of compound IA in the protection during total hepatic ischemia reperfusion.

Plasma aspartate aminotransferase, (AST), plasma alanine aminotransferase (ALT) and lactate dehydrogenase (LDH) were measured in order to assess the damage of the hepatic parenchyma (3). In the operated groups, the plasma levels of ALT, AST and LDH were significantly increased at 60 min and 24 h of reperfusion. It is of particular interest that treatment with compound IA showed protective effects on the plasma level of liver enzymes after 60 min (ALT) and 24 h (AST) of reperfusion.

Oxygen free radicals derived from the xanthine oxidase pathway, mainly hydroxyl radicals, seem to exert an important role in the injury induced by hepatic ischemia in rats (24). Another potential source of oxygen radicals are activated polymorphonuclear leukocytes, which accumulate after reperfusion in the capillaries and venules of the tissue. Stimulation of neutrophils induces the release of large amounts of reactive oxygen species that accumulate in the tissues after reperfusion, thus mediating microvascular damage (25). After administration of compound IA, hepatic parenchyma was free of inflammatory infiltrates after 30 min of ischemia and 60-min reperfusion, and no necrosis or inflammatory infiltration was observed in the portal spaces. Compound IA has been shown to be an anti-inflammatory agent since it reduces carraggeenan rat paw edema (12). The fact that it combines antioxidant and anti-inflammatory activities should be considered as a beneficial property in one molecule.

In conclusion, the administration of compound IA just before reperfusion in an imitated clinical setting of acute ischemia-reperfusion injury resulted in a significant reduction of histological damage. Furthermore, it resulted in a significant decrease in lipid peroxidation and plasma level of liver enzymes. We suggest that the protective effect of compound IA may be attributed to its significant antioxidant and anti-inflammatory activities. As the same compound has proved to be protective against mesenteric ischemia-reperfusion injury in rats when administered just before reperfusion, it may be used as a potential therapeutic agent for the treatment of post-ischemic injury.

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