Effects of L-arginine and L-NAME on ischemia-reperfusion in rat liver[1]

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ABSTRACT

PURPOSE: To evaluated the effects of L-arginine (a NO donor) and L-NAME (N^{w}-nitro-L-arginine methyl ester – a NOS inhibitor) on ischemia-reperfusion in rat livers.

METHODS: One hundred fifty two male Wistar rats were divided into four groups: control (simulated surgery); hepatic IR; pretreatment with L-arginine plus hepatic IR; and L-NAME plus hepatic IR. The hepatocellular damage was evaluated at the first, third and seventh days after the procedures through the alanine-aminotransferase (ALT) and aspartate-aminotransaminase (AST) levels, as well as histopathological features: vascular congestion (VC); steatosis (STE); necrosis (NEC); and inflammatory infiltration (INF). The mortality rate was also evaluated.

RESULTS: The pretreatment with L-NAME significantly worsened the AST levels after hepatic IR ($p<0.05$) at first day and L-arginine demonstrated an attenuating effect on ALT levels at seventh day ($p<0.05$). Furthermore, the administration of L-arginine was able to reduce the VC and STE in the seventh day after hepatic IR ($p<0.05$). The analysis of the mortality rates did not demonstrate any difference between the groups. Nevertheless, there was not effect of L-arginine and L-NAME on the mortality of the animals.

CONCLUSION: L-arginine/NO pathway has a role in the hepatic IR because the pretreatment with L-arginine partially had attenuated the hepatocellular damage induced by hepatic IR in rats.

Key words: Liver. Reperfusion Injury. Ischemia. Arginine. NG-Nitroarginine Methyl Ester. Rats.

Introduction

In the liver, the hepatic ischemia-reperfusion (IR) may cause significant tissue damage in different clinical conditions, such as in surgeries of liver resection by the Pringle maneuver; liver transplantation; and in hypoxia situations, such as shock (septic, hypovolemic or cardiogenic), cardiac surgery with cardiopulmonary bypass and abdominal compartment syndrome[1-4]. Several types of cells and inflammatory mediators have been implicated in the pathophysiology of hepatic IR, standing out the involvement of reactive oxygen species (ROS), nitric oxide (NO), as well as the participation of the complement system, cytokines and chemokines, intercellular adhesion molecule, metalloproteinases, and genetic transcription factors. All those elements interact among themselves, resulting in the secondary injury due to hepatic IR[5,6].

The NO is a colorless gas, relatively unstable, with short half-life (around four seconds), having reduced water solubility, being a free radical that has an unpaired electron that may suffer several oxidation and reduction reactions[7]. It is a product from L-arginine amino acid through the action of the nitric oxide synthase enzyme (NOS)[1]. The NOS enzyme may be inhibited by some analogs of L-arginine, being the most studied ones the L-NMMA (N^w-monomethyl-L-arginine), the L-NA (N^w-nitro-L-arginine), the L-NAME (N^w-nitro-L-arginine methyl ester), and the aminoguanidine[8]. The L-NAME inhibitor needs hydrolysis of the methyl ester radical by cellular esterases to become the functional inhibitor L-NA[8]. In the liver there are basically two types of NOS: the cNOS (constitutive) and the iNOS (inducible)[1,4,9]. The cNOS is expressed constitutively, its activity being dependent of calcium and calmodulin, producing small amounts of NO[1,4,9]. On the other hand, iNOS is independent from calcium and is not found constitutively in liver tissue, but it is expressed in some situations, such as the inflammatory response, trauma, infection and IR, being synthesized, mainly in hepatocytes, Kupffer cells and endothelial cells, producing large amounts of NO[2,9].

It is believed that NO has an ambiguous role during IR, depending on its concentration. Thus, during initial periods of hepatic ischemia, the small amounts of NO produced by sinusoidal endothelial cells would act as a mitigating factor of tissue injury through its vasodilator activity and counter-balancing the endothelin activity[10]. With iNOS activation large NO amounts are produced, there could be a tendency for deleterious effects due to the production of peroxynitrite radicals ($\bullet ONOO^-$)[11]. Thus, our study was proposed to study the importance of the via L-arginine/NO through the effects of modulating agents of NO production (L-arginine and L-NAME) on hepatic IR in rats.

Methods

All the experiments were performed according to the Institutional Animal Care and Use of the Federal University of Health Sciences of Porto Alegre (UFCSPA).

One hundred and fifty two Wistar rats, males, adults, with body mass between 220 and 330g were used. The animals were reared in the *vivarium* of the Division of Pharmacology and Toxicology of the UFCSPA. The animals were kept in plastic cages in groups of four animals in each cage and received standard rodent food and water *ad libitum*, with light cycle by artificial lightning between 7:00 am and 7:00 pm and temperature kept around 22ºC. The animals were randomized in four groups: control (C): submitted to simulated surgery; ischemia-reperfusion (IR): rats who received 1 mL/kg of saline solution (NaCL 0.9%), intraperitoneally (ip) 20 minutes before hepatic IR; L-arginine (L-ARG): pre-treatment with L-arginine (200 mg/kg), via ip, 20 minutes before hepatic IR; L-NAME: animals pre-treated with L-NAME (20 mg/kg), via ip, 20 minutes before hepatic IR. The animals were kept fasting during six hours before the procedures been anesthetized with ketamine (50 mg/kg) and xylazine (10 mg/kg) via ip.

Hepatic ischemia-reperfusion

After preparing the animals and anesthesia induction, longitudinal laparotomy was performed, followed by section of the falciform ligament and, by delicate digital maneuvers, the median hepatic lobe was rebated in cranial direction, allowing the exposition of the hepatic pedicle. The hepatic ischemia was obtained by selective clamping, comprising the pedicle of median and left lobes (corresponding to 70% of the liver) using delicated clamps during a period of 45 minutes and releasing the clamp to reperfusion[12]. The simulated surgery consisted in the delicate exposition of the hepatic pedicle, leaving humid gauzes in physiological solution lightly warmed to cover the visceral content, with the abdominal cavity exposed during 45 minutes. After this period of time, the suture of the abdominal wall in one plan was performed using unabsorbed suture followed by their recovery, with biochemical analyzes being made posteriorly.

Measurement of aminotransferases

For this experiment, 112 rats were used and the determination of serum levels of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) was made by colorimetry

in spectrophotometer, using kits obtained commercially (Labtest - Sistemas para Diagnostico LTDA, Brazil), used in the Laboratory of Biochemistry of the Course of Pharmacology and Toxicology of the Federal University of Health Sciences from Porto Alegre (UFCSPA). The enzymes were evaluated on the 1st, 3rd and 7th day of reperfusion.

Histopathological study

A random sample of 63 animals used in the experiment of hepatic enzymes analysis was applied in the study of histopathological changes. After each group procedures, a resection of hepatic tissue sample was performed, being prepared according to the previous description[12] to analysis in optical microscopy by a pathologist, who had no knowledge about the groups to which the analyzed tissues belonged. The following histopathological caracteristics were evaluated in the 1st, 3rd and 7th day of reperfusion: vascular congestion (VC): vascular dilatation and presence of erythrocites inside the central vein; hepatic steatosis (STE): lipid vacuolar accumulation inside the hepatocits; hepatic necrosis (NEC): hepatocyte with necrosis aspect; such as pyknosis, karyorrhexis, karyolysis; and inflammatory infiltrate (INF): accumulation of inflammatory cells – neutrophils, Kupffer cells. The quantification of the analyzed characteristics was expressed in crosses (0 to 3+), found by the average of three microscopic fields, random, evaluated under 200 times magnification, considering: 0 (lack of changes); 1+ (light changes – until 25% of the analyzed field); 3+ (severe changes – more that 50% of the analyzed field).

Perioperative mortality

Forty different rats were subdivided in the same four groups previouslly described, with 10 animals each one, with death being observed during seven consecutive days after the procedures. At the analysis end, the surviving animals were sacrified with sodium thiopental 150mg/kg given ip.

Statistical analysis

The data received statistical treatment using the software SPSS 17.0 (Statistical Package to Social Sciences for Windows) where, as decision criterion, was adopted the significance level (a) of 5%. Results concerning serum levels of ALT and AST were expressed in average ± standard deviation, being posteriorly analyzed by variance analysis (ANOVA), followed by Dunnett's test T3[13]. The data obtained referring to histopathological studies were expressed by median and statistically analyzed by Kruskal-Wallis test followed by Dunn's test. Mortality was expressed in percentage and its variability between groups was analyzed by chi-squared test[13].

Results

In the first day of liver reperfusion, AST levels were significantly higher in the group IR (324.5±85.6 UI/L) when compared to group C (156.1±50.8 UI/L; p=0.003). The group L-NAME also showed AST levels statistically higher to animals of groups C (p<0.001) and IR (p=0.034). Besides, animals from group L-ARG demonstrated AST levels significantly lower than the ones from group L-NAME (243.6± 129.7 versus 443.5 ± 38.9 UI/L; p=0.005). In the third day analysis of liver reperfusion, there was no difference statistically significant in AST levels between the groups (p<0.05). In the 7th day it was detected that AST values in group C were significantly lower than groups IR (p=0.034) and L-NAME (p=0.005). In the group L-ARG there was no significant difference in those levels when compared to group IR (93 ± 31.8 versus 115 ± 43.9 UI/L; p>0.05) (Figure 1).

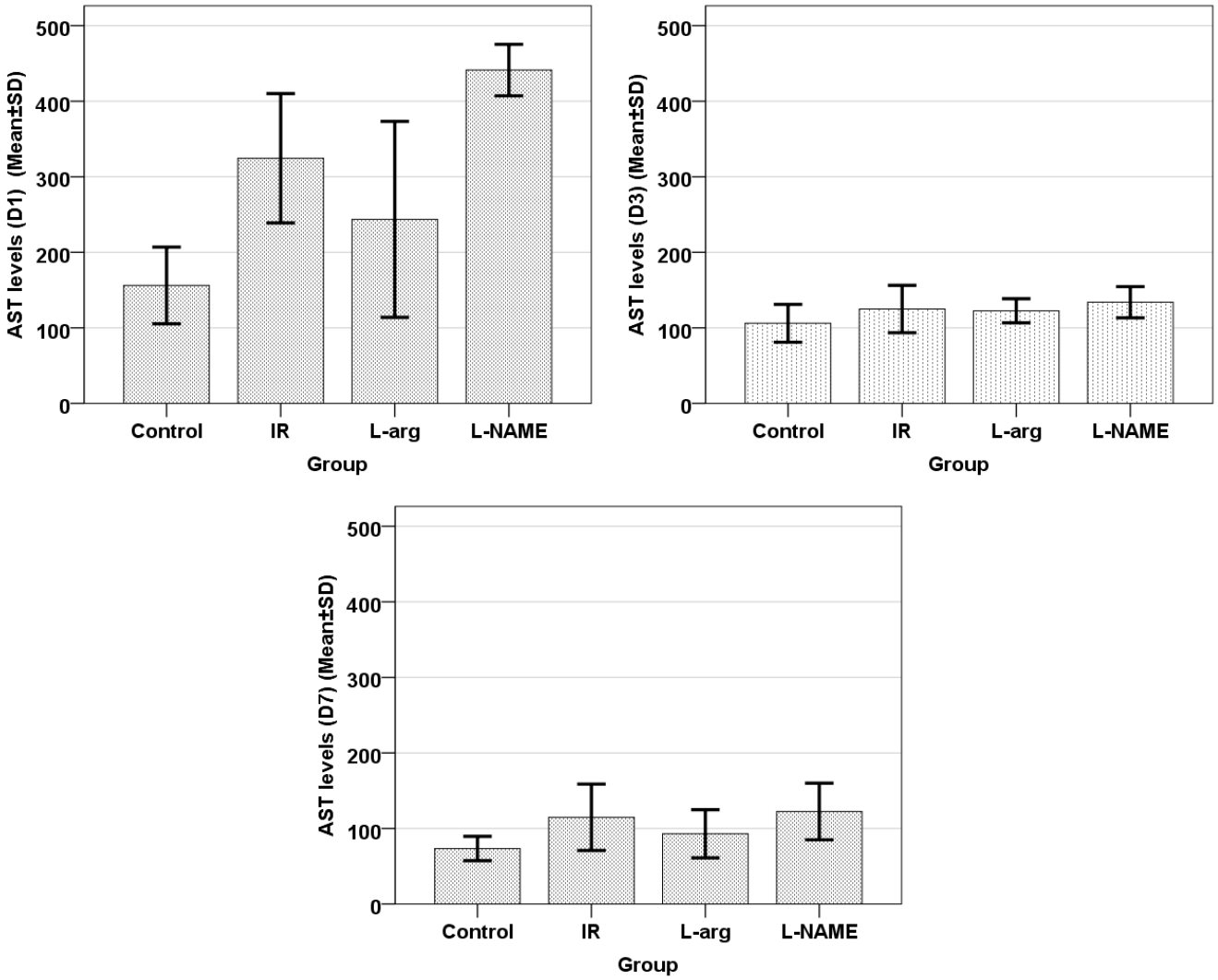

FIGURE 1 - Aspartate aminotransferase levels (AST) in rats submitted to simulated surgery (control); hepatic ischemia-reperfusion (I-R); pretreatment with L-arginine + I-R (L-ARG); and pretreatment with L-NAME + I-R (L-NAME) in different reperfusion periods: **A**) 1st day; **B**) 3rd day; and **C**) 7th day. Statistical difference when compared to control (*) and when compared to the other groups ($^{\#}$); $p<0.05$.

The ALT levels of the group IR (326.7±121.4 UI/L), group L-ARG (286.3±79.3 UI/L), and group L-NAME (349.3±51.5 UI/L) were significantly higher when compared to the group C (107±32.6 UI/L) on the first day after the procedures ($p<0.05$). In the 3rd day after procedures, groups L-NAME (183±68.7 UI/L) and IR (129±52.3 UI/L) had significantly higher levels of ALT when compared to group C (69 ± 23.1 UI/L; $p<0.05$). In the 7th day after procedures, group IR (84.8±28.7 UI/L) and group L-NAME (98.3±12.2 UI/L) had higher levels of ALT when compared to the group C (72±19.8 UI/L; $p<0.005$). Furthermore, the group L-ARG demonstrated significantly lower levels of ALT when compared to group L-NAME (69.1 ± 28.2 versus 98.3 ± 12.2 UI/L; $p<0.05$) (Figure 2).

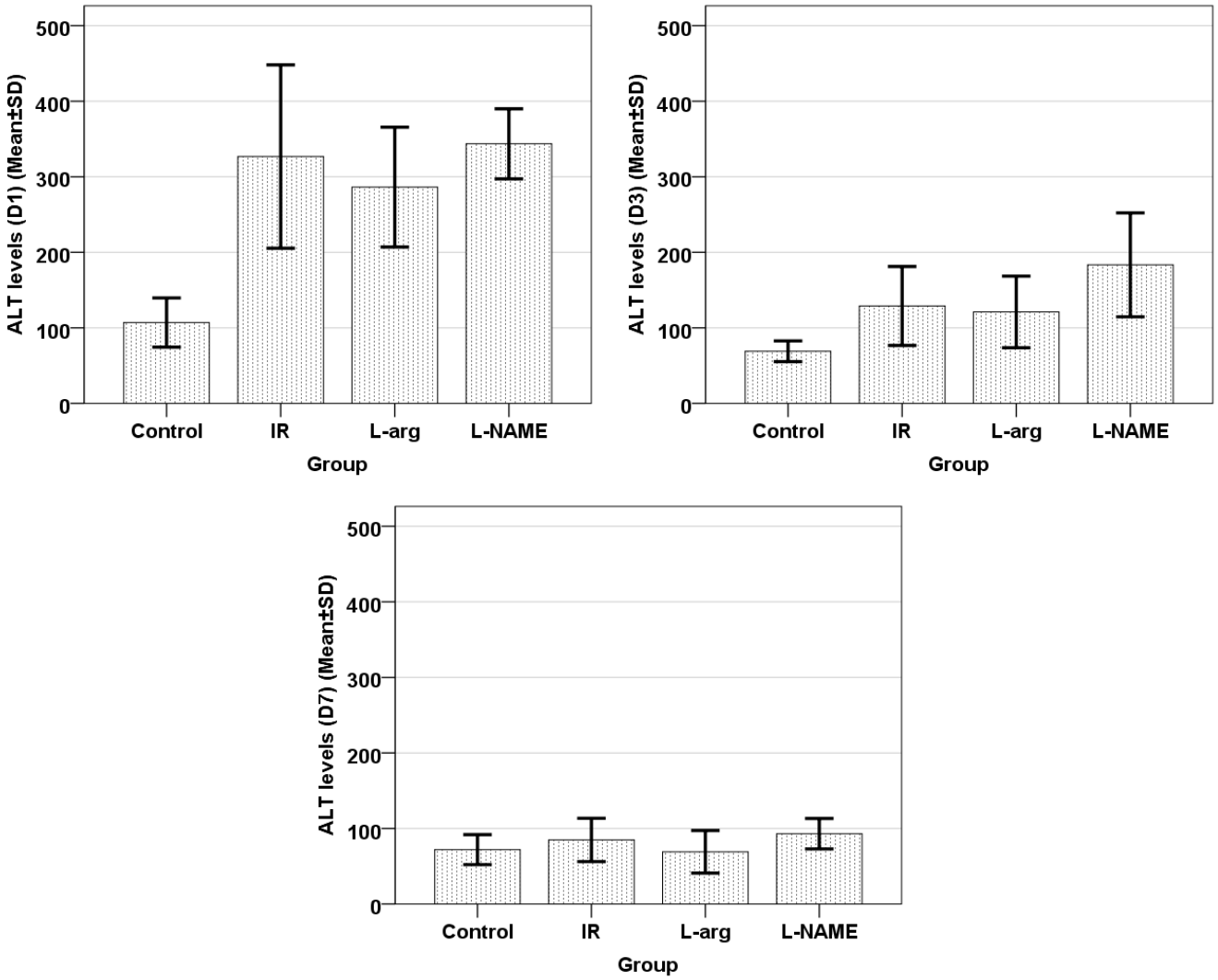

FIGURE 2 - Alanine-aminotransferase (ALT) levels in rats submitted to simulated surgery (control); hepatic ischemia-reperfusion (I-R); pre-treatment with L-arginine + I-R (L-ARG); and pre-treatment with L-NAME + I-R (L-NAME) in different reperfusion periods: **A**) 1st day; **B**) 3rd day; and **C**) 7th day. Statistical difference when compared to control (*) and L-NAME groups (**); $p<0.05$.

Concerning histopathological changes (VC, STE, NEC and INF), the results are summarized in Table 1. In the first day after the procedures, a significant increase in the level of INF in group IR was observed when compared to other groups ($p<0.05$). Besides, the group L-ARG had a trend to reduce the NEC rate when compared to group IR ($p>0.05$).

TABLE 1 - Result of histopathological changes of rats liver submitted to hepatic ischemia-reperfusion, pretreated or not with L-arginine or L-NAME.

GROUPS	1st. day				3rd. day				7th. day			
	VC	STE	NEC	INF	VC	STE	NEC	INF	VC	STE	NEC	INF
Control	0	0	0	0	1	0	0	0	1	0	0	0
IR	2	0	1	2^{*}	$3^{\#}$	0	0	1	$3^{\#}$	$2^{\#}$	0	0
L-ARG	1	0	0	0	2,5	0	0	0	0^{+}	0^{++}	0	0
L-NAME	2	0	1	0	$3^{\#}$	0	0	0	$3^{\#}$	0	0	0

Values represented by the median. VC: Vascular congestion; STE: steatosis: NEC: hepatic necrosis; INF: inflammatory infiltrate. * $p<0.05$ when compared to other groups, $^{\#}$ $p<0.05$ when compared to control group; $^{+}$ $p<0.05$ when compared to groups IR and L-NAME; and $^{++}$ $p<0.05$ when compared to group IR.

During analysis of the 3rd day of reperfusion, a significant increase in the rate of VC in animals from groups IR and L-NAME when compared to group C ($p<0.05$) was found. As in the 1st day after procedures, no STE was seen in the samples of liver tissue in the groups.

Results from the 7th day after procedures showed a significant increase in the rate of VC and STE in animals of group IR when compared to group C ($p<0.05$). Those two parameters were attenuated in group L-ARG when compared to group IR ($p<0.05$) (Figure 3). Rats treated with L-NAME had a higher rate of VC when compared to animals of group C ($p<0.05$). No differences were seen between groups in relation to parameters INF and NEC.

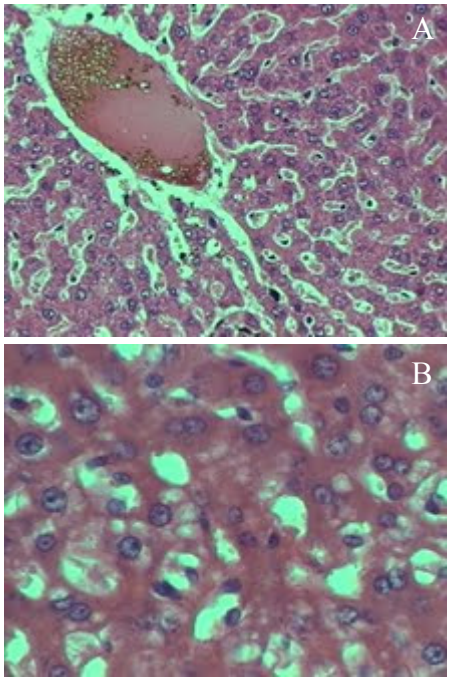

FIGURE 3 – Effect o L-arginine in histopathological aspects in rats livers submitted to hepatic IR. **A**) Areas of hepatic steatosis and vascular congestion of rat submitted to hepatic IR without pre-treatment (x200). **B**) Attenuation of steatosis areas and vascular congestion in rats pretreated with L-arginine (x400). Hematoxylin-eosin.

There was no death in animals from C group. There were seven deaths during the period in other groups; therefore, a global mortality rate of 17.5%. From those deaths, two happened in the IR group; one in the L-ARG group; and four in the group L-NAME, with mortality rates of 20%, 10% and 40% in those groups, respectively. The death of six animals (87.5% of deaths) happened during the first 24 hours after the procedure, all of them during or just after the surgery. The death that did not happen in the immediate perioperative period was an animal from the group L-NAME, that was observed in the 2nd day after the procedure. Analyzing mortality by each group separately, no significant difference between them was seen ($c^2_{calc}=2.000$; $p=0.519$).

Discussion

The L-arginine/NO pathway seems to be relevant during the IR process in several organs such as the liver[9], kidney[14], heart[15], skeletal muscle[16], intestine[17] and brain[18]. As previously described, the NO seems to have an ambiguous role during hepatic IR, because it may act both as a protector of the lesion due to its vasodilator effect, antiplatelet and scavenger of ROS and as a deleterious agent by interaction with superoxide radicals, forming the radical •ONOO-[4,9]. To elucidated the role of the L-arginine/NO pathway in hepatic IR, most of the authors are using, in experimental studies, drugs that modulate the production of NO, such as donors (e.g. sodium nitroprusside and FK-409), precursors (e.g. L-arginine), and inhibitors of NO production (e.g. L-NAME)[19-22]. The basic design of those studies is the previous use of those drugs and subsequent analysis about the treatment response in several indicators of hepatic tissue injury after IR.

In this study, we demonstrated a significant increase in levels of aminotransferases after hepatic IR, when compared to control group in all analyzed periods, except in the 3rd day of reperfusion, when AST levels did not show differences between the groups. Shimamura *et al*.[23] observed a significant increase and maximum of AST levels after 12 hours of reperfusion in a model of hepatic IR, with return to basal level from the 3rd day of reperfusion. Other authors also evidenced a significant increase in aminotransferases levels after hepatic IR[22,24,25].

There was a significant increase of AST levels in animals pre-treated with L-NAME, when compared to untreated group IR, in the first day of reperfusion. Those findings may be compared with other studies[9,21]. Cottart *et al*.[26] demonstrated that hepatic lesion parameters (levels of aminotransferases and hyaluronic acid) worsened with nonselective inhibition of NOS (with L-NA). In another study, Morisue *et al*.[20] observed that L-NAME significantly increased the levels of AST, being maximum in 2 hours of reperfusion, while aminoguanidine reduced those levels.

Peralta *et al*.[21] observed a significant increase in levels of aminotransferases that were exacerbated by the use of L-NAME and attenuated with L-arginine. However, there was no correlation between hepatocyte lesion and degree of oxidative stress, because L-NAME had a tendency to reduce the levels of malondialdehyde (a product of oxidative stress) and increase myeloperoxidase activity. On the other hand, Lin *et al*.[25] demonstrated a protective effect of L-NAME in hepatic IR and a deleterious effect of L-arginine, concluding that the expression of NOS and the increase in production of NO and hydroxyl radicals have deleterious effects during hepatic IR.

A significant protective effect of L-arginine was observed in ALT levels in the 7th day of reperfusion, when compared to group L-NAME. Some authors also demonstrated a beneficial effect of L-arginine with significant attenuation of AST and ALT levels after IR[22,24,26-28]. Tuncer *et al*.[29] also demonstrated a reduction of hepatocyte injury, by the reduction of aminotransferase and gamma-glutamyl-transpeptidase levels with the pretreatment with L-arginine.

Results of this study evidenced a higher grade of INF in rats of group IR when compared with the other groups in the 1st day. The rate of VC in animals submitted to hepatic IR treated or not with L-NAME was superior when compared to control group on the 3rd and 7th day after procedures. Besides, VC and STE rates were significantly reduced with pretreatment with L-arginine in the analysis made on the 7th day. In another study performed among us, it was demonstrated that hepatic IR caused an increase of NEC and STE levels, being attenuated by allopurinol, an inhibitor of ROS production[12]. The parameters used in this study (VC, STE, NEC, and INF) were also studied by other authors in experimental models of hepatic IR[9,19]. In rabbits, it was observed a significant reduction of VC, INF, STE and NEC on livers after IR[22].

Previously, in another study performed among us, it was demonstrated that pretreatment with L-arginine was able to reduce INF in a model of renal IR in rats[14]. In the work of Shimamura *et al*.[23] both L-arginine as well as FK 409 attenuated the rate of VC, NEC and INF after hepatic IR. Those findings were corroborated by Chattopadhway *et al*.[24] who demonstrated a reduction of NEC rate, atrophy, hemorrhage and hepatic degenerative changes secondary to IR. In another study, L-arginine was able to attenuate the leukocyte infiltration in a model of hepatic IR in rats[19]. Tuncer *et al*.[29] also demonstrated a reduction in the histological injury rate induced by hepatic IR and administration of L-arginine.

The global mortality rate of animals in our study was 17.5% (seven deaths in the total of 40 animals). We did not observed any death in animals of the control group. After the statistical analysis, no significant difference in mortality between groups was observed. With L-NAME pre-administration, there was an increase in mortality rate, being higher than in other groups, however without statistical significance. On the other hand, there was only one death in the group treated with L-arginine (10%). Those results are comparable to the ones of other studies. Morisue *et al*.[20] demonstrated that the mortality of rats from group IR was 18.4%, while L-NAME administration significantly increased the mortality rate (57.3%). According to these authors, the deaths may be correlated to a microcirculatory insufficiency and multiorgan failure. The higher number of deaths in animals treated with L-NAME is due, at least in part, to hemodynamic alterations caused by the drug, like systemic and splanchnic vasoconstriction[19]. In another study, Shimamura *et al*.[23] observed a mortality rate of 70% in group IR, while in the animals treated with L-arginine or FK-409 it was null during a 14 days follow-up. Rivera-Chavez *et al*.[28] demonstrated that both L-arginine and nitroprusside reduced the mortality rate of animals after hepatic IR when compared to the untreated group in 7 days after the procedures; however, iNOS inhibition did not altered the survival rate when compared to group IR.

Several therapeutic strategies have been proposed to attenuate the tissue injury caused by tissue IR, such as use of antioxidant drugs (e.g, a-tocopherol, N-acetylcysteine and allopurinol), modulation of superoxide dismutase activity, control in production of inflammatory mediators, use of immunosuppressive, among others[2-4,30,31]. In this context, the modulation of L-arginine/NO pathway may also have an effect on the tissue injury caused by IR. In most of the performed studies, it was observed that donors as well as precursors of NO had a beneficial effect in tissue IR, acting, basically, in the increase of NO bioavailability, promoting vasodilatation of the microvasculature, inhibition of aggregation and platelet adhesion, as well as reduction of interaction between leukocytes and endothelial surface, thus reducing the inflammatory activity in the spot[19,23,24,26-29].

Although some controversial results[25] many works demonstrate a beneficial effect of NO during hepatic IR[9,21,22,24,26-29,32]. The results of this study indirectly pointed to a trend of a protective effect of NO in hepatic IR in rats. This effect was not absolute, probably due to the ambiguous effect of NO that, depending on the moment of the tissue IR analysis, may be beneficial or deleterious. Notwithstanding the fact that NO level was not directly measured, we believe that L-arginine effect is due to the increase in its production, although there are few information about the amount of NO produced after L-arginine administration in normal conditions.

Conclusion

An incomplete protective effect of L-arginine and a partial deleterious effect of L-NAME in rats submitted to hepatic IR, probably due to modulation in NO production.

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