Proliferative Activity in Ischemia/Reperfusion Injury in Hepatectomized Mice: Effect of *N*-Acetylcysteine

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ABSTRACT

Background. Dysfunction of the liver after transplantation may be related to the graft size and ischemia/reperfusion (I/R) injury. *N*-Acetylcysteine (NAC) exerts beneficial effects on livers undergoing ischemia reperfusion. We sought to evaluate NAC modulation on reduced livers associated with I/R injury.

Methods. Male C57BL/6 mice of 8 weeks of age were divided into groups: 50% hepatectomy (G-Hep); NAC (G-Hep + NAC [150 mg/kg]) via vena cava 15 minutes before hepatectomy; ischemia (G-Hep + IR); NAC with hepatectomy (G-IR + Hep + Nac); and IR using 30 minutes selective hepatic occlusion and reperfusion for 24 hours. After 24 hours, the remaining liver was removed, for staining with hematoxylin and eosin or labeling by proliferating cell nuclear antigen. Blood was collected for biochemical evaluations. Significance was considered for $P \leq .05$.

Results. Aspartate aminotransferase was high in all studied groups reflecting the hepatectomy and intervention. injuries. However, when assessing alanine aminotransferase, which depicts liver function, induction of IR promoted a greater increase than hepatectomy ($P = .0003$). NAC decreased ALT activity in all groups, even in association with I/R ($P < .05$), reflecting a modulation of the injury. Necrosis resulting from IR was mitigated by NAC. The experimental model of 50% reduced live promoted regeneration of the hepatic remnant, which was accentuated by NAC, according to the total number of hepatocytes and PCNA values.

Conclusion. NAC preserved the remnant liver in mice and stimulates regeneration even after IR injury.

LIVER transplantation is a therapeutic resource for patients with irreversible liver failure; however, the shortage of organ donors requires innovative approaches to meet the demand. The broadening of clinical criteria concerning the suitability of donors, and the division of the liver to supply 2 recipients are alternatives to increase organ availability. Hepatectomy after division of the organ[1] induces a regeneration process in the lurer parenchyma.[1–7] The events resulting from hepatectomy are sequential, occuring over a week after the resection.[8] Moreover, the reduced amount of liver tissue increases portal blood flow. Thus, graft dysfunction after transplantation occurs frequently its intensity depends upon a combination of negative factors in addition to the ischemia and reperfusion (I/R) lesions already imposed on the transplanted organ.

Previous studies have sought to mitigate the damage caused by I/R using preconditioning protective strategies. Pharmacologic preconditioning has employed drugs to modulate I/R injury, among which is *N*-acetylcysteine (NAC). By increasing hepatic glutathione, which is a substrate for inactivation of foreign substances; by scavenger actions on oxygen reactive species; or by improving microcirculation,[9–11] NAC, commercially available low-molecular-weight thiol.[12,13] The therapeutic use of NAC has been established for acetaminophen toxicity hepatitis, wherein it protects the liver by increasing

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hepatic glutathione, which serves as a substrate to inactivate or combined with foreign substances.[12,14–18]

NAC, an amino acid derived from cysteine, has protective pulmonary effects,[16,19] as well as antioxidant and mucolytic actions acting to remove EROs.[17–19] Its use in clinical practice also occurs in situations of hemodynamic instability to protect the liver, lung, heart, and kidney tissues, which are compromised by ischemia followed by reperfusion. This amino acid is both an analogue and a precursor of intracellular glutathione synthesis.[17,20] It has protective effects on normothermic I/R liver lesions when injected before induction of ischemia, with maintained aerobic conditions.[8] Additionally, it acts as a direct antioxidant, by reacting with hydroxyl radicals, by improving blood flow in the microcirculation,[18,19,21] by inhibiting production of reactive nitrogen species,[19] and by the synthesis of proinflammatory cytokines.

The protective effect of NAC has already been reported in experimental models of fulminant hepatitis.[14,15] After a study of selective hepatic I/R in mice,[20] the authors observed NAC to inhibit activation of the expression of Toll-like receptors 2 and 4 in the liver, probably owing to the inhibition of nuclear translocation of nuclear factor-kappa B, which is activated by EROs. They also showed that NAC promotes morphologic and functional preservation of liver tissue during the ischemic phase,[18,20] decreasing the degree of hepatic congestion after reperfusion. due to improved blood flow in microcirculation[22] probably owing to antioxidant effects, thereby promoting greater bioavailability of nitric oxide (NO), resulting in vasodilation.[23] NAC has also been observed to reduce the occurrence of necrosis, apoptosis, and microvesicular steatosis.[18] Our laboratory has been investigating preconditioning with NAC[12,18] to mitigate I/R. We observed preservation of liver morphology in the ischemia phase and after reperfusion with decreased vascular congestion as well as necrosis, apoptosis, and microvesicular steatosis thereafter. Thus, considering that liver dysfunction after transplantation may relate to the size of the graft and the I/R lesions, and that NAC exerts beneficial effects on the hepatic parenchyma, we evaluated the modulation exerted by NAC on a reduced size liver organ with or without combined I/R lesions in mice.

METHODS

This study, approved by the Research Ethics Committee (0082/08), used 21 C57BL/6 male mice of approximately 8 weeks old that weighed 25–30 30 g obtained from the Institute of Pharmacy, UNIFESP. All animals were kept in individual cages in a temperature-controlled room with light/dark cycles of 12 hours.

The animals were fasted rom solids for 4 hours before the operative procedure. After receiving atropine (0.044 g/kg) as pretreatment mice were anesthetized with katamine and xylazine by intramuscular injection (70 and 10 mg/kg of body weight, respectively). After abdominal trichotomy and antisepsis with 70% alcohol, we hydrated the animal with saline solution (0.8 mL/10 g body weight), injected subcutaneously into the lateral region of the abdomen. The animals were kept in heating pad at 38°C throughout the procedure.

The animals were randomized into 4 groups after a median laparotomy from the xiphoid toward the tail: Hepatectomy (GHep); hepatectomy + NAC (GHepNac); hepatectomy associated with I/R (GHeplR): and hepatectomy associated with I/R and NAC (GHepNACIR).

We slowly injected NAC (150 mg/kg) NAC Protocol into the caudal vena cava at a dose 15 minutes before hepatectomy or ischemia. To perform selective hepatic I/R, animals underwent occlusion of the hepatic pedicle above the level of the right lateral branch segments, caudate lobe, and papillary process with a microsurgical vascular clamp for 30 minutes followed by 24 hours of reperfusion. After identification and isolation of the branches of the right lateral lobes, caudate, square, median, and papillary processes of the liver, we resected 50% of the liver parenchyma as adapted from the model described by Yadav et al.[24]

After the procedure the abdomen was closed in 2 layers using 6-0 wire. Under anesthesia. Twenty-four hours, later, blood samples were collected to assess aminotransferase levels with the remaining liver used for morphologic and morphometric analysis. Liver samples fixed in formalin 10% were kept in closed containers for 24

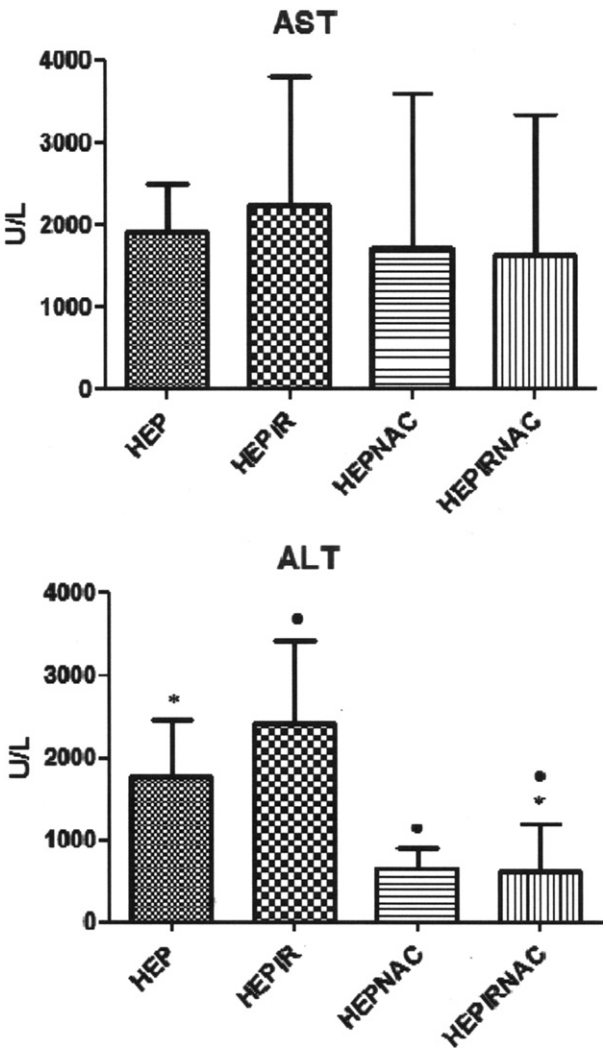

Fig 1. Values of the activity of asparate aminotransferase (AST) and alanine aminotransferase (ALT), expressed in U/L. Hep, hepatectomy; IR, ischemia/reperfusion; NAC, *N*-acetylcysteine. ANOVA *P = .0003; HepNAC, HepIRNAC < HepIR).

hours, dehydrated with alcohol solutions. After embedding in paraffin, the samples cut at 3 microns were stained with hematoxylin and eosin (HE). Thereafter samples were mounted on silanized slides for immunohistochemistry for proliferating cell nuclear antigen (PCNA) labeling.

The data were evaluated by analysis of variance with differences examined by Tukey's multiple comparison test. $P < .05$ was considered significant.

RESULTS

Aminotransferase activity was increased among all studied groups, showing the hepatectomy and I/R injuries ALT activity, which reflects hepatic function showed a greater increase when I/R was superimposed on isolated hepatectomy, but without significance ($P > .05$). NAC induced a decrease in alanine aminotransferase (ALT), including those that occurred in association with ischemia/reperfusion after hepatectomy ($P = .0003$), showing downregulation of the injury (Fig 1).

Morphologic and Morphometric Evaluation

Among the hepatectomy group (Hep), the liver parenchyma showed maintenance of the hepatic cords, central veins, and sinusoids. There were abnormal hyaline areas within the majority of hepatocytes, probably owing to fat accumulation. The hepatocytes were binucleate, with nuclei rich in euchromatin with ≥1 nucleoli. (Fig 2.1A and B). NAC did not modify the parenchyma architecture, but enhanced the steatosis (HepNAC Fig 2.3A and B).

Among hepatectomized animals with added I/R (HepIR), there were areas of heterochromatic, small nu-

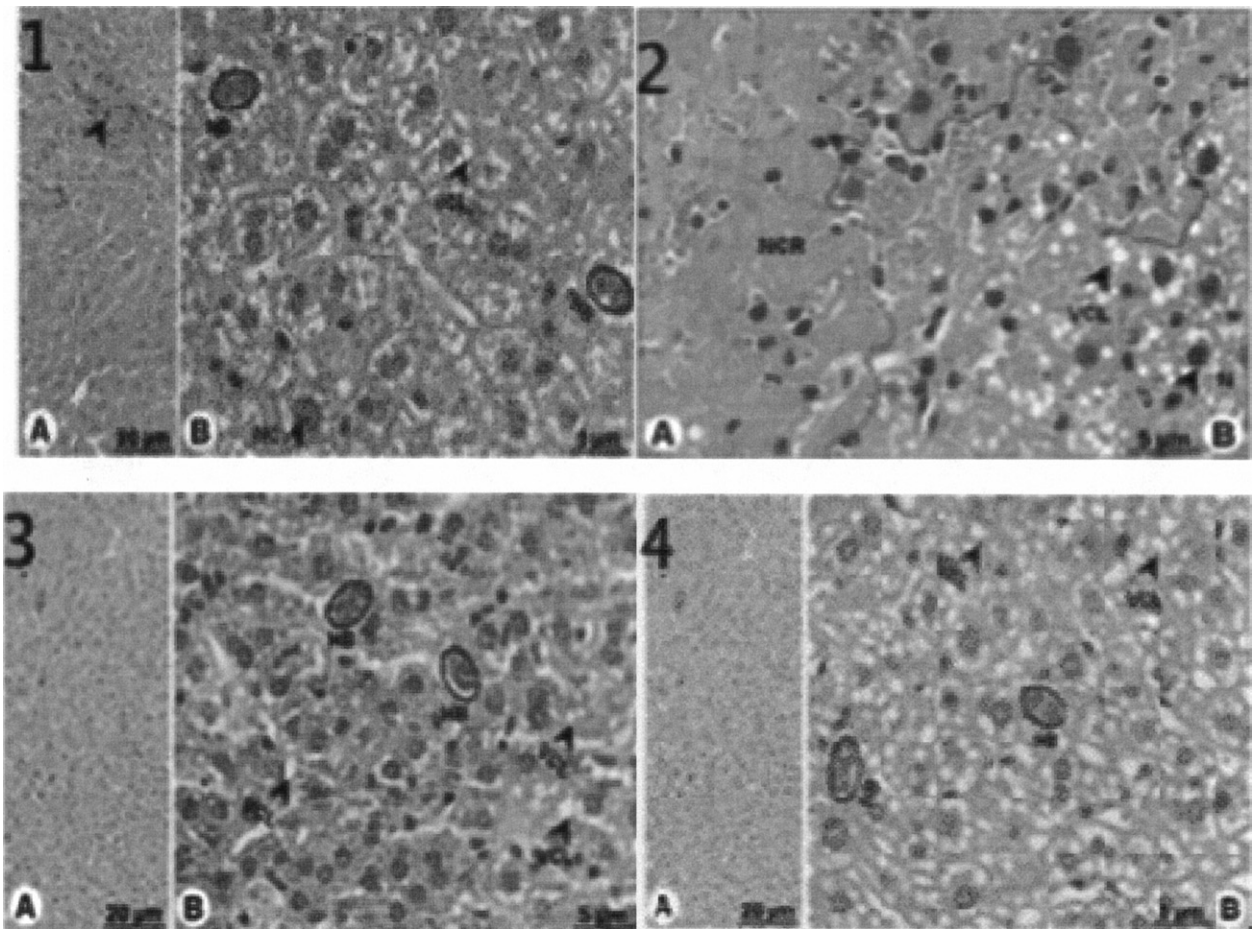

Fig 2. Photomicrography showing morphological aspects of the liver in all studied groups. **(1A)** Group Hep. Hepatic parenchyma without vascular congestion and rare picnotic nucleus (stain, HE; original magnification, × 100). **(1B)** Various binucleate hepatocytes (HB), with ≥1 nucleolus (NC) and cytoplasmic fatty vesicles (VCL; stain HE; original magnification, × 400). **(3A)** Group HepNAC Absence of vascular congestion and many nuclei (N) rich in heterochromatin (stain, HE; original magnification, × 100). **(3B)** Binucleate hepatocytes (HB) with intense amount of fatty vesicles (VCL; stain, HE; original magnification, × 400). **(2A)** Many picnotic nuclei (N), indicating cellular death (stain, HE; original magnification × 100). **(C2)** Necrosis (NCR) of the hepatic parenchyma (stain; HE; original magnification, × 100). **(2B)** Area of necrosis (NCR). **(C3B)** Area without necrosis with pinotic nuclei (N), indicating cellular death and fatty vesicles (VCL; stain, HE; original magnification, × 400). **(4A)** Group HepIRNAC Intense steatosis (stain, HE; original magnification, × 100). **(4B)** Large amount of fatty vesicle (VCL), binucleate hepatocytes (HB; stain, HE; original magnification, × 400).

clei, indicating low metabolic activity, as well as the presence of inflammatory infiltrates and areas containing hepatocytes with eosinophilic cytoplasm and pycknotic nuclei, indicating cellular degeneration and/or necrosis (Fig 2.2A and B). Addition of NAC (HeplRNAC), lead to lack of necrotic areas and inflammatory infiltrates, although intense fatty infiltrates, small nuclei, and nucleoli were evident (Fig 2.4A and B). Center veins diameters were similar among all groups, showing that there was no difference related to vascular congestion (Hep = 70 μm; HelpR = 66 μm; HepNAC = 64 μm; HeplRNAC = 52 μm; $P > .05$).

The proliferative activity in the remnant liver after 50% hepatectomy, was increased with NAC, even when associated with I/R, as observed by the total number of hepatocytes (Fig 3). However, the number of binucleate hepatocytes did not show a significant difference among the studied groups ($P > .05$).

Proliferative activity was enhanced using NAC associated with hepatectomy, as observed by the percentage of PCNA-labeled hepatocytes in all groups: Hep = 4.6%; HeplR = 5.2%; HepNAC = 8.3%; HepIR = 6.2% ($P < .05$, comparing the HepNAC group with the others).

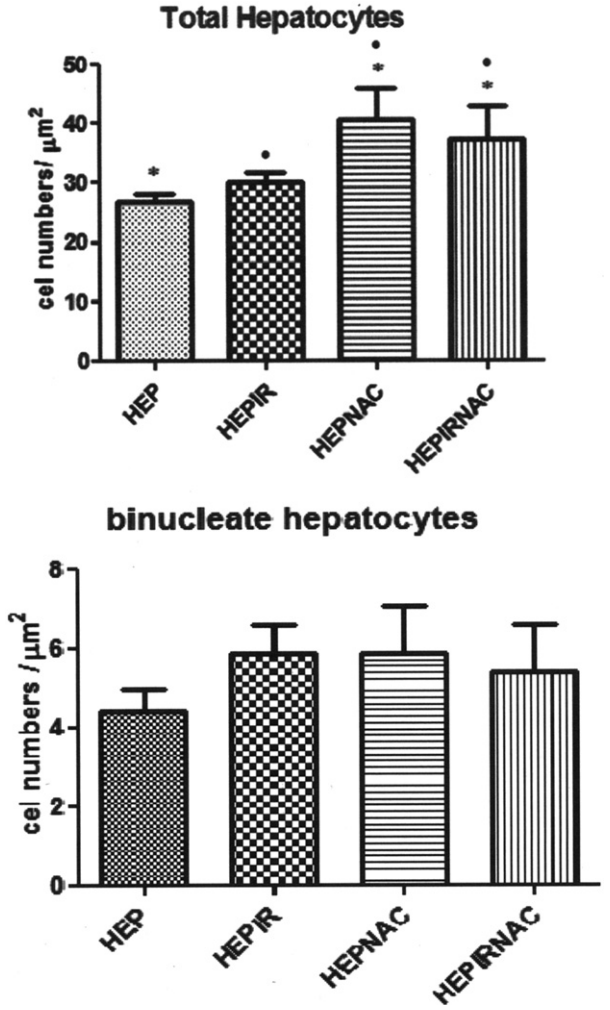

Fig 3. Total number of hepatocytes. Hep, hepatectomy; IPC, ischemic preconditioning; IR, ischemia/reperfusion; NAC, *N*-acetylcysteine. ANOVA: Hep, HepIR < HepNAC, HepIRNAC ($P < .0001$). Number of binucleate hepatocytes was similar in all groups ($P > .05$).

DISCUSSION

The complex mechanisms of liver injuries in clinical situations have lead to the development of protective strategies. As an experimental model, the rat has been partially replaced by mice which include genetically modified strains facilitating the study of mechanisms of injury and modulation.[25] Moreover, this mammal has liver metabolism comparable to humans, despite the anatomic peculiarities,[24] such as a multilobed liver.

In our study, NAC showed protective effects on hepatic enzymes with decreased ALT activity after hepatectomy even associated with an I/R injury, which was much higher than the posthepatecomy alone value. In a study assessing ischemia and reperfusion in mice, Yadav et al[24] observed a peak of transaminases after 6 hours with a fall at 24 hours. In a clinical study, Koneru et al[22] reported that the value changes did not show significance.

The extensive liver injury caused by I/R depends on the precondition of the liver and the duration of the ischemic insult. It can lead to liver or multiple organ failure.[26,27] The increased morbidity and mortality occurs in the state of anoxia or ischemic liver injury as in extensive resections and transplantation, or as a result of conditions that decrease blood flow to the liver, resulting in systemic hypoxia and poor perfusion.[26,27] Ischemia causes a proinflammatory state increasing tissue vulnerability during reperfusion leading to necrosis and apoptosis.[28]

Partial hepatectomy produces an essentially hyperplastic response with regeneration of cells and tissues over 3–14 days, resulting in restoration of glandular volume. The end result is influenced by the mechanisms controlling growth and cell proliferation. Even after hepatectomy the remnant liver still in the regenerative phase, can maintain liver functions necessary to sustain homeostasis at normal levels.[29]

Morphologic evaluation reveald necrosis in the remnant liver when subjected to I/R which was abolished with NAC treatment. These data in mice subjected to ischemic preconditioning corroborated the findings of decreased hepatic necrosis[12,14] and cell death by apoptosis.[12,30] NAC has been reported to decrease necrosis in livers of normal rats subjected to I/R[12]; however, scant data are available to address I/R associated with hepatectomy.

By morphometric analysis, we sought to evaluate the presence of stasis in the central lobular vein. Neither liver resection nor I/R promoted venous stasis in our model, probably owing to selective occlusion and prolonged reperfusion, allowing the repair of acute injury. Medeiros et al[9]

reported the maintenance of liver tissue architecture with the central lobular vein free of dilatation and unchanged sinusoids in the presence of NAC.

The evaluation of the total amount of hepatocytes, reflecting liver regeneration, showed that NAC stimulated hepatocyte hyperplasia in hepatectomized animals. The number of binucleate hepatocytes was increased in I/R when compared with hepatectomized animals, but without a significant difference. Tanou et al[31] studied posthepatectomy regeneration in mice, noting that after 24 hours there was increased regeneration with augmentation of cells positive for PCNA, corroborating our findings. However, when examining the hepatic regeneration state of binucleate hepatocytes this evidence is lost, probably owing to the occurrence of the division of hepatocytes, reflected by the total number of hepatocytes already advanced at the time of evaluation.

In conclusion, our experimental model of a 50% reduced liver showed NAC, promote to regeneration of the remnant as evidenced by the total number of hepatocytes and PCNA, as well as its function, even associated with I/R injury.

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