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Protective Effect of Ozone Treatment on the Injury Associated with Hepatic Ischemia-Reperfusion: Antioxidant–Prooxidant Balance

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The effects of ozone treatment on the injury associated to hepatic ischemia-reperfusion (I/R) was evaluated. Ozone treatment (1 mg/kg daily during 10 days by rectal insufflation) is shown to be protective as it attenuated the increases in transaminases (AST, ALT) and lactate levels observed after I/R. I/R leads to a decrease in endogenous antioxidant (SOD and glutathione) and an increase in reactive oxygen species (H_2O_2) with respect to the control group. However, ozone treatment results in a preservation (glutathione) or increase (SOD) in antioxidant defense and maintains H_2O_2 at levels comparable to those in the control group. The present study reports a protective effect of ozone treatment on the injury associated to hepatic I/R. The effectiveness of ozone could be related to its action on endogenous antioxidants and prooxidants balance in favour of antioxidants, thus attenuating oxidative stress.

Keywords: Ozone, ischemia-reperfusion, reactive oxygen species, superoxide dismutase, glutathione

Abbreviations: AST, aspartate aminotransferase; ALT, alanine aminotransferase; I/R, ischemia-reperfusion; SOD, superoxide dismutase; ROS, reactive oxygen species

INTRODUCTION

It is well known that ischemia-reperfusion (I/R) leads to an inflammatory response which results in tissue injury. A significant component of this injury is due to the generation of reactive oxygen species (ROS) when ischemic tissues are re-exposed to oxygenated blood.[1,2]

There is evidence on the efficacy of administration of antioxidant agents such as superoxide dismutase (SOD), catalase (CAT) or glutathione in restricting ischemic injury.[3,4] Recently, some authors have focused their attention on the induction of organ stress to elicit the enhancement of endogenous antioxidant mechanisms.[5,6]

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Treatment with ozone may be effective in clinical use in different disorders, including ischemic conditions.[7] Recently, the effectiveness of ozone treatment in an experimental model of hepatotoxicity induced by CCl_4 in rat[8] has been reported. Although the mechanisms which underlie its beneficial actions are unknown, a relation between ozone and antioxidant mechanisms has been suggested. It seems reasonable that ozone, being an oxidant, could upregulate the intracellular antioxidant mechanisms by induction of organ stress.[7–9]

In the present study, the effectiveness of ozone treatment in an experimental model of hepatic ischemia-reperfusion has been evaluated.

MATERIALS AND METHODS

Surgical Procedure

The study was performed with male Wistar rats (6 in each group) weighing between 250 and 300 g. All animals (including controls) were anesthetized with urethane (10 mg/kg, i.p.) and placed in a supine position on a heating pad in order to maintain body temperature between 36°C and 37°C. To induce hepatic ischemia, laparotomy was performed and the blood supply to the right lobe of the liver was interrupted by placement of a bulldog clamp at the level of the hepatic artery and portal vein. Reflow was initiated by removing the clamp.[10] The studies were performed in concordance with the European Union regulations for animal experiments.

Experimental Design

The following experimental groups were used:

Group 1. Control: Animals subjected to anesthesia and laparotomy.

Group 2. Ischemia-reperfusion (I/R): Animals subjected to 90 min of right-lobe hepatic ischemia followed by 90 min of reperfusion.

Group 3. Ischemia-reperfusion + ozone treatment ($I/R + O_3$): Same as group 2, but with previous ozone treatment before ischemic period. Ozone was administered by rectal insufflation using an equipment from Biozon, Germany. The O_3 concentration was measured by using a UV spectrophotometer at 254 nm. The O_3 dose is the product of the O_3 concentration by the gas volume. By knowing the body weight of the rat the O_3 dose is calculated as 1 mg/kg. Rats received 10 ozone treatments, one per day, of 5–5.5 ml at an O_3 concentration of 50 μg/ml.[8]

Control experiments were performed with the vehicle (O_2) used for the ozone administration.

Biochemical Determinations

Hepatic injury Plasma alanine aminotransferase (ALT) and aspartate aminotransferase (AST) were measured using a commercial kit from Boehringer Mannheim (München, Germany).

Lactate content Livers were freeze-clamped and the lactate extracted with H_2O/acetone (1/1.2). The lactate content was measured using a commercial kit from Boehringer Mannheim (München, Germany).

Glutathione measurement Liver samples were homogenized in KCl (1.1%). After protein precipitation in $HClO_4$ (1 N), the samples were neutralized with K_2CO_3 (10%). The amount of GSH was measured using glutathione transferase and 1-chloro-2,4-dinitrobenzene.[11]

Superoxide dismutase activity SOD activity was measured by the inhibition of pyrogallol autooxidation where a unit of activity was defined as the amount of enzyme required to inhibit the rate of pyrogallol autooxidation by 50%.

H_2O_2 measurement H_2O_2 was determined following the method used by Slezak *et al.*[12] in tissue samples.

Protein measurement Total protein content in liver homogenates was determined using a commercial kit from Bio-Rad (München, Germany).

Histopathologic Analyses

Liver samples were fixed in 10% neutral buffered formalin, embedded in paraplast and 5 mm sections were stained with hematoxylin and eosin, according to standard procedures. Sections were evaluated by light microscopy.

Statistics

Data are expressed as means ± SEM. Mean of different groups ($n = 6$, each group) were compared using a one-way analysis of variance. Student's t test was performed for evaluation of significant differences between groups. Significance was determined at the 5% level ($p < 0.05$).

RESULTS

As shown in Table I, I/R leads to significant increases in transaminase levels (AST, ALT) with respect to the control group. These increases were nonsignificant with ozone treatment. As with transaminase levels, ozone treatment also attenuated the significant increases in lactate content observed after I/R (see Table I). The histological study of the liver was in accordance with the biochemical findings. In this way no lesions were present in the control group (Figure 1A). Liver samples of the animals subjected to I/R (Figure 1B) showed multiple and extensive areas of hepatocyte necrosis and a mild sinusoidal

TABLE I Transaminase (AST, ALT), lactate, pro-oxidant (H_2O_2) and antioxidant (SOD, GSH) levels in the groups included in this study. Control; I/R: 90 min of ischemia followed by 90 min of reperfusion; I/R + O_3: same as I/R but with previous ozone treatment. Results expressed as mean ± SEM ($n = 6$)

	Control	I/R	I/R + Ozone
AST (U/l)	39.5 ± 2.7	151.7 ± 13.8*	57.0 ± 14.4†
ALT (U/l)	23.7 ± 2.0	255.7 ± 32.8*	54.5 ± 20.1†
Lactate (μmol/g)	5.2 ± 1.2	21.5 ± 1.8*	11.9 ± 3.8†
H_2O_2 (μmol/g)	31.8 ± 4.1	64.2 ± 3.8*	37.8 ± 4.7†
SOD (U/mg protein)	12.4 ± 1.4	6.6 ± 2.0*	16.0 ± 0.5†*
GSH (nmol/mg protein)	8.4 ± 1.4	4.3 ± 1.1*	7.9 ± 1.1†

*$p < 0.05$ vs. Control.
†$p < 0.05$ vs. I/R.

A

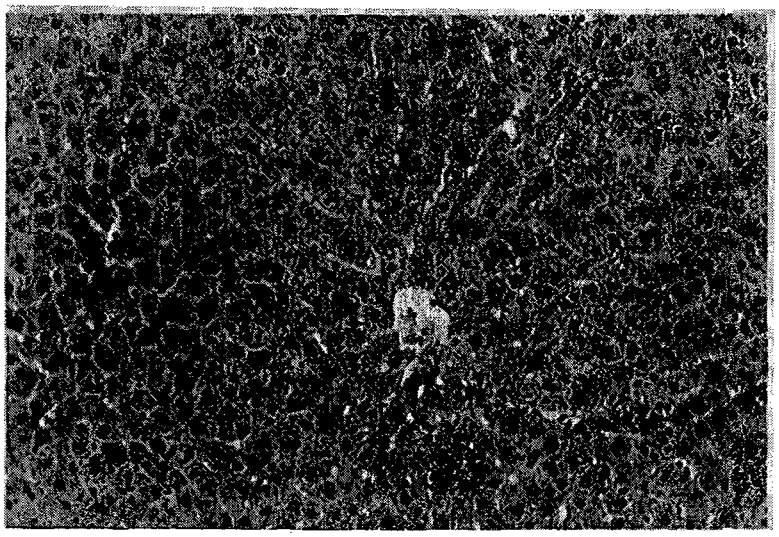

FIGURE 1A

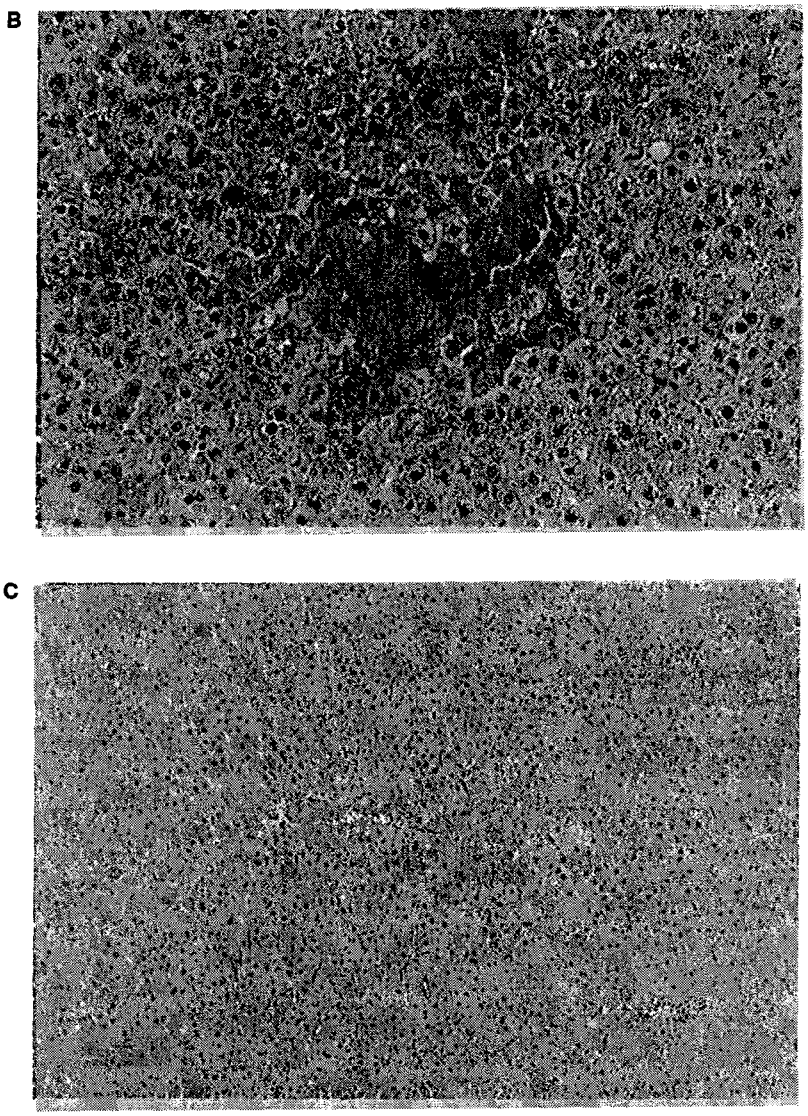

FIGURE 1B and C

FIGURE 1 Histological lesions. (A): Control group, no apparent hepatic lesions. (B): Ischemia-reperfusion (I/R), extended area of hepatic necrosis. (C): Ischemia-reperfusion + Ozone treatment (I/R + O_3), incipient necrosis of scattered hepatocytes. Hematoxylin and eosin (×215).

neutrophil infiltration. The lesions were randomly distributed through the hepatic parenchyma. In contrast, liver samples of the animals subjected to I/R with previous ozone treatment (Figure 1C) showed minimal lesions which consisted of incipient necrosis of scattered hepatocytes through the hepatic parenchyma. The alterations observed in these hepatocytes were cytoplasmatic eosinophilia and nuclear pycnosis.

The ozone effects on ROS and antioxidant defense mechanisms have been evaluated and the results obtained are shown in Table I. The increase in H_2O_2 levels observed in the animals subjected to I/R was prevented in the animals treated with ozone. With respect to antioxidant

mechanisms, I/R leads to a significant decrease in the SOD activity and GSH levels with respect to the control group. However, ozone treatment prior to I/R results in a significant increase in both SOD activity and GSH levels, becoming increased and similar, respectively, to those in the control group. The vehicle (O_2) used did not modify the biochemical and histological results obtained in I/R group (data not shown).

DISCUSSION

Ischemia-reperfusion is characterized in liver by circulatory and metabolic derangement, liver dysfunction and tissue damage.[13] Studies reported up to now focused on the use of the exogenous protective agents for attenuating the I/R injury.[3,4] Similar beneficial effects have also been reported after stimulation of endogenous protective systems by induction of organ stress.[5,6] It seems reasonable that ozone, being an oxidant, could promote organ stress inducing an enhancement of the endogenous protective mechanisms in order to attenuate the hepatic I/R injury.

In the present study we report on the protective effect of ozone treatment on the injury associated to I/R. The increases in transaminases (AST and ALT) observed after I/R were attenuated with previous ozone treatment (Table I). The ozone treatment was also able to lower the increase in lactate levels found in I/R group. It is well known that the lactate accumulation produced in I/R contributes to increased cellular injury and exacerbate ischemic damage.[14] Lactate accumulation during ischemia and reperfusion is associated with intracellular acidosis, an important cause of cellular injury,[15] resulting in direct structural damage to cells[16,17] and increased Ca^{2+} accumulation in tissue.[18] Elevated Ca^{2+} levels are believed to have a role in the activation of several destructive mechanisms responsible for cell damage in these settings.[19] The ozone treatment, by lowering the lactate accumulation (Table I), could probably attenuate the injurious consequences following intracellular acidosis.

In line with the biochemical results, the histopathological analyses showed that liver damage induced by I/R (Figure 1B) was partially prevented by ozone pretreatment (Figure 1C).

It has also been suggested that the production of ROS is the major reason for tissue damage after reperfusion.[20,21] In pathologic situations, the defence mechanisms could be overwhelmed allowing the ROS to exert their deleterious effect.[22] This disruption in pro-oxidant–antioxidant balance is known as oxidative stress.[23] H_2O_2 is an important cytotoxic ROS involved in some hepatic disorders, including the I/R process.[24] Thus, it has been reported that SOD and glutathione represent the main enzymatic[25] and nonenzymatic mechanisms,[26] respectively, involved in antioxidant defense.

The results found in hepatic I/R show a disturbance in the pro-oxidant–antioxidant balance in favour of the pro-oxidant species. In this sense, the decrease in SOD activity and glutathione levels was accompanied by an increase in H_2O_2 levels (see Table I). Several hypotheses have been suggested to explain the drop in antioxidant defense mechanisms in several disorders including I/R process.[22,27] The drop in GSH levels may result either from an arrest of synthesis or a higher consumption. The decrease in SOD activity levels, could be associated with their inhibition or inactivation related to I/R process or free radical attack itself.

It is known that the maintenance or increase of endogenous antioxidant mechanisms could attenuate the injurious effect of ROS in I/R process.[1,28,29] Preservation in antioxidant mechanisms such as SOD and glutathione by ozone treatment was accompanied by lowered H_2O_2 levels which became comparable to those of the control group (see Table I).

In summary, this work has shown the effectiveness of ozone treatment on the injury associated to hepatic I/R. Accordingly, the ozone action on endogenous antioxidants and pro-oxidants

attenuating oxidative stress could probably contribute to the restriction of postischemic injury.

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