Ischemic and ozone oxidative preconditioning in the protection against hepatic ischemic-reperfusion injury

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Abstract

It has been demonstrated that ozone, probably by means of an oxidative preconditioning (OP) mechanism, protected the liver against the damage mediated by reactive oxygen species. Taking into account that ischemic preconditioning (IschP) is also a protective mechanism, a comparative study between both preconditioning effects, with the aim to study the effectiveness of both protective procedures, was performed. Rats were divided at random in: 1- control, sham operated (anesthesia and laparotomy plus surgical manipulation); 2- I/R (ischemia for 90 min followed by 90 min reperfusion); 3- IschP + I/R, as group 2 but submitted to a previous ischemic preconditioning (ischemia 10 min and reperfusion 10 min); 4- OzoneOP + I/R, as group 2 but submitted to a previous oxidative preconditioning with 15 sessions, daily, of ozone by rectal administration (dose = 1 mg/kg). The comparison between both preconditionings showed no biochemical differences for the parameters evaluated. Nevertheless, the histological study demonstrated that the protective effect produced by the OzoneOP is superior to that achieved with the IschP.

Introduction

Microscopic studies of tissues exposed to ischemia/reperfusion (I/R) have revealed an acute inflammatory response. Although the inflammatory response elicited by I/R has been extensively characterized, the mechanism underlaying this phenomenom remain poorly understood. Several factors, including reactive oxygen species (ROS) (1-5), leukotrienes (6), platelet-activation-factor (7) and nitric oxide (8) have been implicated.

Many studies (9-10) indicate that oxygen free-radical formation after reoxygenation of liver may initiate the cascade of hepatocellular injury. Acute ischemia apparently produces minor tissue damage, the major damage is apparently secondary to reperfusion (11). The major source of free radicals in post-ischemic tissue appears to be initiated by the formation of superoxide anion, formed by the action of xanthine oxidase. It catalyzes the conversion of

xanthine and hypoxanthine to uric acid and superoxide anion (12). It is frecuently implicated as a significant source of ROS (13-14). Two molecules of superoxide anion react simultaneously to form hydrogen peroxide (H_2O_2). In the presence of various transition metals, especially iron and copper, H_2O_2 is rapidly converted to the extremely reactive hydroxyl radical (15).

Low levels of ozone may protect the cells against subsequent ozone exposure. This protection may contribute to the adaptation after multiple ozone exposures (16). Moreover, not only ozone could induce tolerance to itself it could prepare the host to face physiopathological conditions mediated by ROS. It has been demonstrated (17-18) that ozone, probably by means of an oxidative preconditioning mechanism, protected the liver against the damage produced by a chemical challenge mediated by ROS. Also, ozone therapy applied in an experimental model of liver ischemia-reperfusion, protected the organ against the injury caused by this surgical procedure, decreasing transaminases and lactate figures, preserving the hepatocellular integrity and reducing the ROS by the stimulation and/or preservation of the endogenous antioxidant systems (19). In addition, ozone was able to induce an adaptation to oxidative stress with a preservation of the antioxidant endogenous systems in an animal model of renal ischemia-reperfusion (20).

The protection associated with preconditioning is one of the most powerful mechanisms of protection known. Ischemic preconditioning, short periods of ischemia with intermittent reperfusion, has been shown to protect the myocardium from a subsequent longer ischemic insult (21-23). Also, preconditioning has been demonstrated in intestine (24), brain (25) and liver (26). Although the mechanism of preconditioning is not yet known, it has been suggested that liver ischemic preconditioning is mediated by the inhibitory action of nitric oxide on endothelin and through the balance of adenosine and xanthine levels (24,27).

Taking into account both protective effects, ischemic and ozone oxidative preconditioning, against liver injury by I/R, the aim of this study is to stablish a comparison between them, in a liver I/R model.

Materials and Methods

Animals

Adult male Wistar rats weighing 250-300 g, were used in this study (n = 32). Rats were housed in plexiglass cages, maintained in an air-filtered and temperature-conditioned (20 - 22 ^{o}C) room with a relative humidity of 50 - 52 % and under an artificial light/dark cycle of 12 h. Animals were fed with standard laboratory chow and water *ad libitum*. All procedures were performed as approved by the International Animal Care Committees and in accordance with the European Union Guidelines for animal experimentation.

Treatment schedule

Ozone (O3) was generated by an OZOMED equipment manufactured by the Ozone Research Center (Cuba). Ozone obtained from medical grade oxygen was used inmediately and it represented only about 3% of the gas (O2 + O3) mixture. The ozone concentration was measured by using an UV spectrophotometer at 254 nm. The ozone dose is the product of the ozone concentration, expressed as mg/L, by the gas (O2 + O3) volume (L). By knowing the body weight of the rat, the ozone dose is calculated as 1 mg/kg, as in our previous papers (17-20, 28).

The protocol consisted of four experimental groups, with 8 animals each: 1- Control (sham operated), animals submitted to anesthesia and laparatomy plus surgical manipulation (including the isolation of the right hepatic artery and vein vs the left hepatic artery and vein without the induction of hepatic ischemia); 2- I/R, animals submitted to 90 min of right lobe hepatic ischemia, followed by 90 min reperfusion; 3- Ischemic Preconditioning (IschP), previous to the I/R period (as in group 2), animals were subjected to 10 min of ischemia and 10 min reperfusion and 4- Ozone Oxidative Preconditioning (OzoneOP), previous to the I/R period (as in group 2), animals received by rectal insufflation, 15 ozone treatments, one per day (4.5 - 5.0 ml of O3 at a concentration of 50 mg/L).

Surgical procedure

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 - 37 ^{o}C. To induce hepatic ischemia, laparatomy was performed and the blood supply to the right lobe of the liver was interrupted by placement of a buldog clamp at the level of the hepatic artery and portal vein. Reflow was initiated by removing the clamp (24).

Sample preparation

Blood samples were obtained from the abdominal aorta for biochemical determinations. Afterwards, some representative samples of different liver portions were taken for histopathological studies and tissue homogenates. Liver homogenates were obtained using a tissue homogenator Edmund Bulher LBMA at 4 ^{o}C. The homogenates were prepared by using a 50 mM KCl/histidine buffer pH 7.4, 1:10 (w/v) and were spun down with a Sigma Centrifuge 2K15, at 4 ^{o}C and 8500 x g for 20 min. The supernatans were taken for biochemical determinations. Preparation of enzyme fraction for evaluation of total enzyme (xanthine dehydrogenase: XDH + xanthine oxidase: XO) and XO activities was performed (29). Livers were removed quickly in freeze-clamped way, washed and homogenized in 50 mM phosphate buffer, pH 7.4 containing 1 mM EDTA (1:5 w/v). The conversion of XDH to XO during handling was minimized by adding 10 mM 2-mercaptoethanol, trypsin inhibitor (5 mg ml-1; type II-S Sigma Chemical Co., Poole, U.K.) and leupeptin (0.5 mg l-1) to the buffer before use. Instead of dithiothreitol (DTT), 2-mercaptoethanol was added to the buffer as a thiol group protector as it prevents XDH to XO transformation without promoting the

conversion of XO to XDH. The homogenates was centrifuged at 1500 g for 10 min and then at 105 000 g for 60 min at 4 ^{o}C. The supernatant was dialysed for at least 4 hours against the same homogenization buffer at 4 ^{o}C.

Biochemical determinations

Plasma alanine aminotransferase (ALAT) and aspartate aminotransferase (ASAT) were measured using commercial kit from Boehringer Mannheim (Munich, Germany).

The measurements of XDH and XO activities were carried out within the first hour after supernantant isolation. For the evaluation of total enzyme activity defined as XDH + XO, aliquots (0.2 ml) of dialyzed enzyme fraction were preincubated for 30 min at 37 ^{o}C in the presence of 10 mM dithiotreitol (DTT). Diluted aliquots (50 g protein) were then incubated after the addition of 60 M xanthine and 0.67 mM NAD+ for 10 min at 25 ^{o}C (total volume 0.1 ml). DTT preincubation was carried out to transform the XOrev into XDH. For the evaluation of total XO activity DTT activation was avoided. Incubation was stopped by the addition of ethanol (1 ml), the samples centrifuged at 1000 g for 5 min and the supernatants dried under nitrogen flow. The residues were resuspended in 0.1 mM NH4H2PO4, pH 7 (0.3 ml). Activities were determined by spectrophotometric method using an Ultrospec Plus Spectrophotometer from Pharmacia LKB on the basis of uric acid formation at 292 nm. Each activity was expressed as mol/min/mg protein (29).

Lipid peroxidation, in supernatant of liver homogenates, was assessed by measuring the concentration of malonaldehyde using the Bioxytech LPO-586 kit. The assay was conducted according to the manufacturer's instructions.

Uric acid concentration was measured according to a commercial kit obtained from Sigma Chemical Co. (St. Louis, MO, USA).

Inorganic phosphorus, as a measured of ATP depletion, was measured according to a commercial kit obtained from Boehringer Mannheim GmbH (Munich, Germany).

Total protein concentration in supernatant of liver homogenates was determined using a commercial kit from Bio-Rad (Munich, Germany)

Histopathological study

For the histological analysis, 6 animals of each group were studied and at least two samples of liver were taken. Samples were fixed in 10 % neutral buffered formalin, processed and embedded in paraffin and stained with haematoxilin-eosin. The histological evaluation considered the grades of liver congestion, presence of leucocytes, activated Kupffer cells and liver necrosis.

Statistical analysis

Results are presented as means ± standar error of mean (SEM). The statistical analysis was started by using the OUTLIERS preliminary tests for detection of error values. Afterward, data were analysed by one-way analysis of variance (ANOVA) followed by homogeneity variance test (Bartlett-Box). In addition, a multiple comparison test was used (Duncan test). Different letters indicate a statistical significance of at least $P < 0.05$.

RESULTS

ASAT and ALAT (Table I) activities in serum samples, obtained after 90 minutes of ischemia and 90 minutes of reperfusion, increased in the group subjected to I/R. These increases were significantly lower when ischemia was preceded by both preconditionings. Nevetheless, IschP attenuated transaminase activities while OzoneOP was able to maintain ASAT and ALAT at control level.

Table I. Transaminase (ASAT and ALAT) levels in the groups included in this study.

Parameter	Control	I/R	OzoneOP	IschP
ASAT(U/L)	63.95 ± 11.18^{a}	200.00 ± 22.00^{b}	$89.55 \pm 12.63^{a,c}$	130.24 ± 11.42^{c}
ALAT(U/L)	9.48 ± 3.25^{a}	117.05 ± 21.74^{b}	$43.77 \pm 10.05^{a,c}$	74.95 ± 5.74^{c}

Each value is the mean value ± SEM from eight rats. Statistical significance among different letters of at least $p<0.05$.

The histological study of the liver was in accordance with the biochemical findings. In Table II are shown the histopathological results.The morphology of the hepatic lobuli was normal in OzoneOP group, a protection against liver I/R injury was observed. Only 1 animal presented minimal lesions, consisted of incipient necrosis of scattered hepatocytes through the hepatic parenchyma. Neutrophils and activated Kuppfer cells were not presented. In I/R group, multiple and extensive areas of hepatocyte necrosis, with dilated and hyperemic sinusoids where hyperplastic Kupffer cells and migrated leucocyte were evident. The lesions were randomly distributed through the hepatic parenchyma. Ischemic Preconditioning showed focal necrosis, presence of neutrophils and minimum and moderate congestion.. Histological studies demonstrated differences between both protective mechanisms, being more effective the oxidative preconditioning.

Table II. Histological results of the liver in the groups included in this study.

Animals	Control	I/R	OzoneOP	IschP
1	0	A2, B2, C, D	0	A1, C
2	A1	A3, B2, C	A1, B2	0
3	0	0	0	A1, C
4	0	A1	0	A2, B2, C
5	0	0	0	A1
6	0	0	0	A1

A: Congestion: 0 none
A1 minimum
A2 moderate
A3 severe

B: Necrosis: 0 none
B1 single cells
B2 focal necrosis

C: Presence of Neutrophils

D: Activated Kuppfer cells

The generation of inorganic phosphorus (Pi), as a measure of ATP degradation during the ischemia, showed that both preconditionings produced a similar protection. OzoneOP and IschP were capable to avoid the lost of high energy phosphate, diminishing significantly ($p<0.01$) inorganic phosphorus concentrations with respect to I/R group, even lower with regards to the negative control group (Table III).

Table III. Inorganic phosphorus figures, in supernatant of liver homogenates, for the different groups studied

Experimental groups	Pi (nmol/L)
Control	3.59 ± 0.22^{a}
I/R	4.71 ± 0.33^{b}
OzoneOP	2.92 ± 0.51^{c}
IschP	2.96 ± 0.40^{c}

Each value is the mean value ± SEM from eight rats. Different letters indicate statistical significance of at least $p<0.05$.

Results of XDH + XO and XO are shown in Table IV. There was no change in XDH + XO activity in liver. Ischemic-reperfusion increased total XO activity. In animals, control XO represented 10 % of total enzymatic activity (XDH + XO). After I/R this proportion increased to 53 %. IschP and OzoneOP attenuated this conversion of XDH to XO, since the XO represented 37 and 38 % of total enzymatic activity, respectively, with regard to I/R group. Both preconditionings caused a reduction in total XO; however enzyme concentrations were still higher in comparison with the control group.

Table IV. XDH + XO and XO activities in liver ischemia/reperfusion study.

Experimental groups	XDH-XO (U/mg prot)[2]	Total XO (U/mg prot)[2]
Control	6.01 ± 0.15[a]	0.60 ± 0.12[a]
I/R	7.36 ± 0.51[a]	3.92 ± 0.43[b]
OzoneOP	6.02 ± 0.28[a]	2.81 ± 0.36[c]
IschP	6.02 ± 0.60[a]	2.74 ± 0.23[c]

Each value is the mean value ± SEM from eight rats. Different letters indicate a statistical significance of at least $p<0.05$.(2) The values of U/mg/protein correspond to µmol/min/mg protein.

Acid uric concentrations, as a measure of the transformation of hypoxanthine and xanthine to superoxide anions, are represented in Figure 1. Both preconditionings attenuated the uric acid formation, with a significant decrease ($p<0.01$) with respect to I/R group, but still remained higher figures in comparison with the control group. OzoneOP decrease uric acid production in 37 % and IschP in 25.8 % with regard to I/R group.

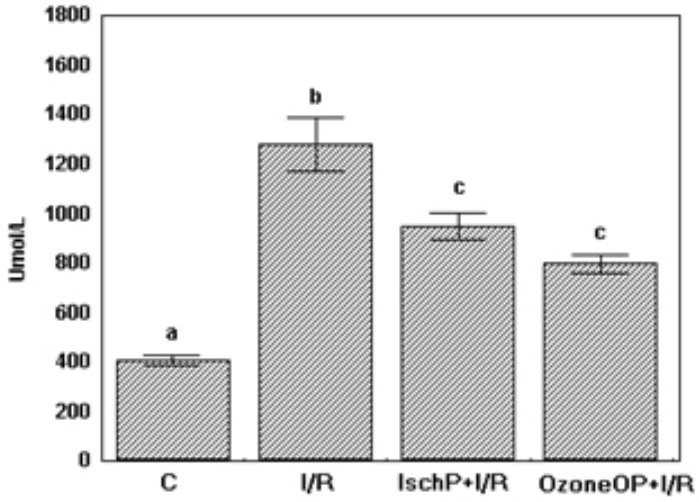

Figure 1. Acid uric concentration in the different experimental groups: Control, sham operated: animals subjected to anesthesia and laparotomy plus surgical manipulation; I/R: 90 minutes of ischemia followed by 90 minutes of reperfusion; IschP+I/R: Ischemic preconditioning + I/R; OzoneOP+I/R: Ozone oxidative preconditioning + I/R. Each value is the means ± SEM from eight rats. Different letters indicate a statistical significance of at least $p< 0.05$.

The results of MDA, as an index of lipid peroxidation are shown in Figure 2. Ischemic and ozone oxidative preconditionings maintained the concentrations of MDA at control level, avoiding lipid damage. In contrast, an increase of lipid peroxidation in I/R group was obtained.

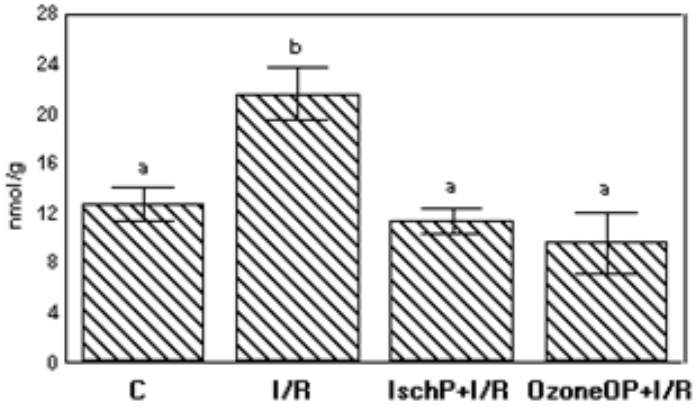

Figure 2. Malondialdehyde concentration in the different experimental groups: Control, sham operated: animals subjected to anesthesia and laparotomy plus surgical manipulation; I/R: 90 minutes of ischemia followed by 90 minutes of reperfusion; IschP+I/R: Ischemic preconditioning + I/R; OzoneOP+I/R: Ozone oxidative preconditioning + I/R. Each value is the means ± SEM from eight rats. Different letters indicate a statistical significance of at least $p< 0.05$.

Discussion

Oxidative preconditioning is analogous to other phenomena such as ischemic preconditioning (21), thermal preconditioning (30) and chemical preconditioning (31). All these processes have in common that a repeated and controled stress is able to protect against a prolonged and severe stress. Ozone could confer protection against the hepatic I/R injury by the adenosine accumulation and by blocking the xanthine/xanthine oxidase pathway, decreasing ROS generation after reperfusion (28). Potencials mediators of the protective effects of ischemia preconditioning include adenosine, prostanoids such as prostacyclin, nitric oxide and bradykinin (32), but its mechanism remains unknown and under intense investigation.

The biochemical parameters (Tables I, III, IV and Figures 1 and 2) and the microscopic study of liver ischemic-reperfusion injury indicated the protective effects of OzoneOP and IschP on preventing hepatic ischemia/reperfusion damage. ALAT and ASAT decrease significantly for both preconditionings with respect to I/R group. Nevertheless, OzoneOP decrease ALAT and ASAT in 62.6 and 55.4 %, in comparison with 36 and 35.2 % for IschP, respectively. OzoneOP was able to maintain ASAT and ALAT at control level, while IschP attenuated transaminase activities.

The anaerobic glycolisis, during the ischemia, have two important facts to be considered: ATP catabolism and lactate production. Lactate is the main source in the increase of hydrogen ions, found in the ischemia. It have been demonstrated a decrease in lactate figures during ischemia using ozone as an oxidative preconditioning (18,19). ATP catabolism is associated with ROS production. Both preconditionings prevented the release of inorganic phosphorus, of its original sources (ATP and adenosine), decreasing its figure with regards to I/R and control groups. Decrease levels of ATP, present in the cells of the hepatic parenchyma can produce a

lost in the electrolyte balance of the plasmatic membrane, obtaining a prolonging of the cell area and cytoskeletal alterations (38). In addition, the final product of ATP degradation is hypoxanthine, that constitutes the substrate of xanthine oxidase, important enzyme in the production of superoxide radicals, during the reperfusion.

Many studies have suggested that ROS, generated at the time of reperfusion, can cause a loss of organ function (34). In addition, after ischemia-reperfusion the liver release XDH + XO into the vasculature, damaging the vascular endothelium and activating oxidant-producing inflammatory cells (i.e., neutrophils), extending the oxidant-induced injury to tissues remote from the site of origin (35).

Previous findings (19,20) have demonstrated that ozone treatment promotes an increase in antioxidant endogenous systems reducing oxidative stress mediated by ischemia/reperfusion processes. Also, ozone treatment attenuated the conversion of XDH to XO during hepatic ischemia (28).

In this study in both preconditioning groups, total XO figures were lower with respect to I/R group. Xanthine oxidase, in the presence of molecular oxygen, converts xanthine to uric acid and superoxide radical. Therefore, a measure of XO activation can be the production of uric acid. In both preconditioning groups, the level of uric acid were significantly lower with respect to I/R group, but with figures higher with regards to control group (Figure 1). A controlled ischemia-reperfusion induced xanthine oxidase activation is achieved.

OzoneOP, similarly to IschP, maintained MDA concentrations at sham operated group levels (Figure 2), in contrast, in I/R group was increased.. Similar results were reported when it was studied MDA levels in rats treated previously with ozone and after that, subjected to liver ischemia/reperfusion (28). Repeated administration of ozone, via rectal administration, have induced a sort of cross-tolerance to free radicals released after the ischemia-reperfusion procedure, demonstrating that hepatocytes have become resistant to this damage. It have been demonstrated that low doses of ozone increased antioxidant endogenous systems as glutathione, superoxide dismutase and catalase (17-20, 36). Therefore, antioxidant-prooxidant balance is favored to preservation of cell redox state and toxic aldehydes are not formed. This have also been proved in the treatment of different diseases and beneficial effects have been observed with the use of ozone therapy (36-38).

Taking into account the results of previous studies (17,19,20,28) and those obtained in this paper, an integrated picture of the different events regulated by ozone in liver ischemiareperfusion is showed in Figure 3. All those ozone biological effects produced a liver protection, as has been proved histologically.

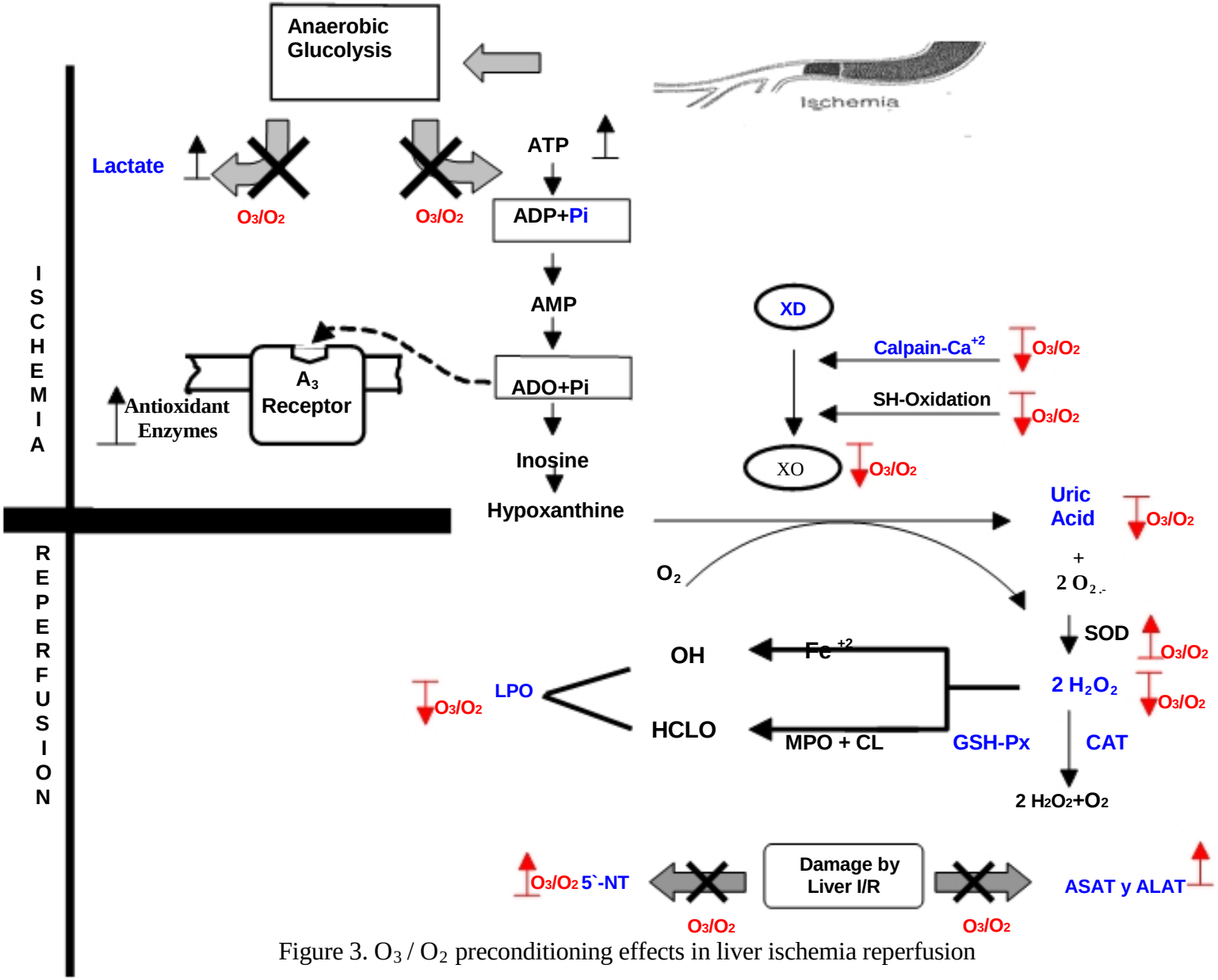

Figure 3. O_3 / O_2 preconditioning effects in liver ischemia reperfusion

Conclusions

The comparison between the ischemic and ozone oxidative preconditioning showed no biochemical differences for the parameters evaluated. A protection against liver ischemic-reperfusion injury was achieved. Nevertheless, the histological study demonstrated that the protective effect produced by the ozone oxidative preconditioning is superior to that achieved with the ischemic preconditioning. Therefore, ozonetherapy may become an important prophylactic treatment able to improve the success of organ transplantation and similar surgical procedures.

Keywords

Ozone; liver ischemia/reperfusion; oxidative stress; preconditioning; antioxidant defense system.

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