SIMILAR PROTECTIVE EFFECT OF ISCHAEMIC AND OZONE OXIDATIVE PRECONDITIONINGS IN LIVER ISCHAEMIA/REPERFUSION INJURY

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Many studies indicate that oxygen free-radical formation after reoxygenation of liver may initiate the cascade of hepatocellular injury. It has been demonstrated that controlled ozone administration may promote an oxidative preconditioning or adaptation to oxidative stress, preventing the damage induced by reactive oxygen species (ROS) and protecting against liver ischaemia-reperfusion (I/R) injury. On the basis of those results we postulated that ozone treatment in our experimental conditions has biochemical parameters similar to the ischaemic preconditioning (IscheP) mechanism. Four groups of rats were classified as follows: (1) sham-operated animals subjected to anaesthesia and laparotomy, plus surgical manipulation; (2) I/R animals were subjected to 90 min of right-lobe hepatic ischaemia, followed by 90 min of reperfusion; (3) IscheP, previous to the I/R period (as in group 2): animals were subjected to 10 min of ischaemia and 10 min of reperfusion; (4) ozone oxidative preconditioning (OzoneOP), previous to the I/R period (as in group 2): animals were treated with ozone by rectal insufflation 1 mg kg\(^{-1}\). The rats received 15 ozone treatments, one per day, of 5–5.5 ml at the ozone concentration of 50 µg ml\(^{-1}\). The following parameters were measured: serum transaminases (AST, ALT) and 5′-nucleotidase (5′-NT), with morphological determinations, as indicators or hepatocellular injury; total sulphydryl groups, calcium levels and calpain activity as mediators which take part in xanthine deshydrogenase (XDH) conversion to xanthine oxidase (XO) (reversible and irreversible forms, respectively); XO activities and malondialdehyde + 4-hydroxyalkenals as indicators of increased oxidative stress. AST, ALT levels were attenuated in the IscheP (130 ± 11.4 and 75 ± 5.7 U l\(^{-1}\)) with regard to the I/R group (200 ± 22 and 117 ± 21.7 U l\(^{-1}\)) while the OzoneOP maintained both of the enzyme activities (89.5 ± 12.6 and 43.7 ± 10 U l\(^{-1}\)) without statistical differences (P < 0.05) in comparison with the sham-operated (63.95 ± 11 and 19.48 ± 3.2 U l\(^{-1}\)). Protective effects of both the preconditioning settings on the preservation of total sulphydryl groups (IscheP: 6.28 ± 0.07, OzoneOP: 6.34 ± 0.07 µmol mg prot\(^{-1}\)), calcium concentrations (IscheP: 0.18 ± 0.09, OzoneOP: 0.20 ± 0.06 µmol mg prot\(^{-1}\)), and calpain activity (IscheP: 1.04 ± 0.58, OzoneOP: 1.41 ± 0.79 U mg prot\(^{-1}\)) were observed. Both of the preconditionings attenuated the increase of total XO associated to I/R injury. Generation of malondialdehyde + 4 hydroxyalkenals was prevented by IscheP and OzoneOP without statistical differences between the two protective procedures. These results provide evidence that both of the preconditioning settings share similar biochemical mechanisms of protection in the parameters which were measured. Although there were no differences from a biochemical point of view between Ischaemic and OzoneOPs, the histological results showed a more effective protection of OzoneOP than IscheP in our experimental conditions.

KEY WORDS: ozone, liver ischaemia/reperfusion, oxidative stress.
INTRODUCTION

The liver is damaged by sustained ischaemia in liver transplantation or in liver surgical procedures, and the reperfusion after ischaemia results in further functional impairment [1]. After the first report describing protection against myocardial necrosis [2], preconditioning has been demonstrated in intestine [3], brain [4] and liver [5] indicating that it is not a mechanism restricted to the myocardium. In spite of the fact that the ischaemic preconditioning (IscheP) has been extensively studied on heart, only a few studies have been performed on the model of ischaemic-reperfusion (I/R) injury in the liver.

Many studies indicate that, after reoxygenation of the liver, oxygen free-radical formation may initiate the cascade of hepatocellular injury, necrosis/apoptosis, and subsequent infiltration of inflammatory cells [6, 7]. Although reactive oxygen species (ROS) can arise from a number of sources, xanthine oxidase (XO) is frequently implicated as a significant source of these toxic oxygen species [8, 9]. Superoxide radicals are generated by XO. Two molecules of superoxide anion react simultaneously to form hydrogen peroxide, and then, by the addition of one electron, the highly reactive hydroxyl radical is formed [10].

The IscheP is an inducible and potent endogenous mechanism by which repeated episodes of brief I/R confer a state of protection against subsequent sustained I/R injury [2]. It has been suggested that the hepatic protective effects of the IscheP against the postischaemic injury could be partly attributed to a decrease in activity of the ping-pong mechanism between the generation of ROS and the neutrophil infiltration. Furthermore, the IscheP preventing the increase of neutral fatty acids seems to reduce the substrate of lipid peroxidation [2, 11]. Although the mechanism of preconditioning is not yet known, some hypotheses have recently been tested. It has been suggested that the liver IscheP is mediated by the inhibitory action of nitric oxide on endothelin [12] and through the balance of adenosine and xanthine levels [13].

Ozone has been used as a therapeutic agent for the treatment of different diseases and beneficial effects have been observed [14–16]. It has been demonstrated that low levels of ozone exposure have distinct effects within cells, they may also protect the cells against subsequent ozone exposure. This protection may contribute to the adaptation after multiple ozone exposures [17]. Moreover, ozone could not only induce tolerance to itself, it could also prepare the host to face physiopathological events mediated by ROS. It has recently been discovered that ozone is able to induce an adaptation to oxidative stress or promote an oxidative preconditioning through the increase and preservation of antioxidant endogenous systems in animal models of hepatotoxicity, induced by carbon tetrachloride and hepatic and renal I/R [18–21].

Taking into account the protective effects conferred by the IscheP and the ozone oxidative preconditioning (OzoneOP) against liver injury by I/R, the aim of this work is to establish a comparison between both of the preconditioning settings. Biochemical and histological parameters were taken into consideration in order to find out whether there were differences and/or similarities between these protecting procedures.

MATERIALS AND METHODS

Animals

Adult male Wistar rats (250–300 g) were used for these studies. Rats were maintained in an air filtered and temperature conditioned (20–22°C) room with a relative humidity of 50–52%. Rats were fed with standard commercial pellets and water ad libitum.

All procedures were performed as approved by the Institutional Animal Care Committees (ARCA No. 015) and in accordance with the European Union Guidelines for animal experimentation.

Chemicals

Ozone (O₃) was generated by an OZOMED equipment manufactured by the Ozone Research Centre (Cuba) and was administered by rectal insufflation. Ozone was obtained from medical grade oxygen, was used immediately as generated and it represented only about 3% of the gas (O₂ + O₃) mixture. The ozone concentration was measured by using a built-in UV spectrophotometer set at 254 nm (accuracy, 0.002 A at 1A, repeatability 0.001 A and calibrated with internal standard). The ozone dose was the product of the ozone concentration [expressed as mg l⁻¹ by the gas (O₂ + O₃) volume (l)]. By knowing the body weight of the rat, the ozone dose was calculated as mg kg⁻¹ as in our previous papers [18–22]. Unless otherwise stated, all chemicals were obtained from Sigma Chemical Company (St Louis, MO, USA).

Treatment schedule

The protocol consisted of five experimental groups (n = 32). Group 1: Sham-operated (n = 8): animals subjected to anaesthesia 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 ischaemia). Group 2: I/R (n = 8): animals were subjected to 90 min of reperfusion. Group 3: IscheP (n = 8): previous to the I/R period (as in group 2), animals were subjected to 10 min of ischaemia and 10 min of reperfusion as in the previous experiments [13]. Group 4: OzoneOP (n = 8): previous to the I/R period (as in group 2), animals were treated with ozone by rectal insufflation 1 mg kg⁻¹. The rats received 15 ozone treatments, one per day, of 5–5.5 ml at an ozone concentration of 50 µg ml⁻¹.

Control experiments were performed with the vehicle (O₂) used for the ozone administration. The sham-
operated animals (as in group 1) were subjected to the previous ozone treatment.

**Surgical procedure**

The study was performed as in our previous papers [20, 21]. In brief, all animals (including the sham-operated) were anaesthetized with urethane (10 mg kg$^{-1}$, i.p.) and placed in a supine position on a heating pad in order to maintain body temperature between 36 and 37°C. To induce hepatic ischaemia, laparatomy 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 [20].

**Sample preparation**

Blood samples (4 ml) were obtained from the abdominal aorta for biochemical determinations of hepatic injury. Afterwards, some representative samples of different liver portions from the right ischaemic lobe were taken for histopathological studies and tissue homogenates. Liver homogenates were obtained using a tissue homogenator Edmund Bülher LBMA at 4°C. The homogenates for calcium, calpain activity, total sulfhydryl groups and 4-hydroxyalkenals determinations were prepared by using a 50 mM KCl/histidine buffer pH 7.4, 1 : 10 (w/v) and were centrifuged with a Sigma centrifuge 2K15, at 4°C and 8500 × g for 20 min. The supernatants were taken for biochemical determinations. Preparation of the enzyme fraction for evaluation of total enzyme activity defined as XDH + XO, aliquots (0.2 ml) of dialyzed enzyme fraction were preincubated for 30 min at 37°C in the presence of 10 mM DTT, diluted aliquots (50 µg protein) were then incubated after the addition of 60 µM xanthine and 0.67 mM NAD$^+$ for 10 min at 25°C (total volume 0.1 ml). DTT preincubation was carried out to transform the XO reversible (XO$r$) into XDH. XO irreversible (XO$i$) and total XO (XO$r$ + XO$i$) activities were determined in the absence of NAD$^+$; DTT activation was avoided in evaluating the total XO activity. Values of XO$i$ activity were obtained by subtracting the XO$r$ value from that of total XO. Incubation was stopped by the addition of ethanol (1 ml), the samples were centrifuged at 1000 × g for 5 min and the supernatants dried under nitrogen flow; the residues were resuspended in 0.1 mM NH$_4$H$_2$PO$_4$, pH 7 (0.3 ml). Activities were determined by using the HPLC method on the basis of uric acid formation at 292 nm. Each activity was expressed as µmol min$^{-1}$ mg protein$^{-1}$ [23].

**Calcium levels, total sulphhydryl groups, calpain activity and lipid peroxidation in supernatants of liver homogenates.** These parameters were determined by spectrophotometric methods using an Ultrospec Plus Spectrophotometer from Pharmacia LKB. Calcium levels were measured using standard commercial kit produced by Sigma (St Louis, MO, USA). Determination of the total sulphhydryl groups was performed according to the method of Sedlak and Lindsay [24] with Ellman’s reagent. Calpain activity was measured as the release of trichloroaetic acid-soluble peptides from azocasein (Sigma, St Louis, MO, USA) in an assay mixture (1.5 ml) containing 20 mM tris-HCl buffer pH 7.4, 4 mg ml$^{-1}$ azocasein, 20 mM β-mercaptoethanol, 5 mM Ca$^{2+}$, 1% triton X-100 and 0.3 ml samples at 25°C as previously reported [25]. Lipid peroxidation was assessed by measuring the concentration of malonaldehyde (MDA) and 4-hydroxyalkenals using the Bioxytech LPO-586 kit. The assay was conducted according to the manufacturer’s instruction.

**Protein measurement**

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

**Histopathological procedure**

Two samples of liver of each animal were taken and fixed in 10% neutral formaline, 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**

The statistical analysis was started by using the OUTLIERS preliminary tests for detection of error values. The homogeneity variance test (Bartlett-Box)
Enzymatic serum markers of liver damage. AST, ALT (a) and 5′-NT (b) activities (U l−1) in the following experimental groups. Sham-operated: animals subjected to anaesthesia and laparotomy, plus surgical manipulation; I/R: 90 min of ischaemia followed by 90 min of reperfusion; IscheP + I/R: ischaemia preconditioning + I/R; OzoneOP + I/R: OzoneOP + I/R. Different letters indicate a statistical significance of at least $P < 0.05$. Results are expressed as the mean ± SEM from eight rats.

was used afterwards, followed by the ANOVA method (one way). In addition, a multiple comparison test was used (Duncan test); values are expressed by the mean ± standard error of mean ($n = 8$ per group). Different letters indicate a statistical significance of at least $P < 0.05$.

RESULTS

Effects of ischaemic and ozone oxidative preconditionings on hepatic injury

As shown in Fig. 1(a, b) the degree of hepatic damage evaluated by the serum levels of AST, ALT and 5′-NT induced after 90 min of ischaemia and 90 min of reperfusion increased in the group subjected to I/R. IscheP attenuated transaminase activities while OzoneOP was able to maintain AST and ALT at the level of the sham-operated. The ischaemic and OzoneOPs prevented 5′-NT increase observed in I/R group.

Figure 2(a–d) shows the histopathological results in different experimental groups. It was observed that OzoneOP protected against liver I/R injury since the morphology of hepatic lobuli was normal. The I/R group showed dilated and hyperaemic sinusoids where hyperplastic Kupffer cells and migrated leucocytes were evident, HE 250×, (c) IscheP + I/R, slight proliferation of Kupffer cells, HE 100×, (d) OzoneOP + I/R, normal morphology of hepatic lobuli, HE 100×.
Role of IscheP and OzoneOP on XDH and XO activities

The effects of both the preconditioning settings on calcium concentrations, calpain activity and total sulfhydryl groups were evaluated in liver homogenates (Table 1).  
I/R increased total XO activity. In correspondence, a significant increase ($P < 0.05$) was found in calcium levels, calpain activity and decreased total sulfhydryl groups. In the sham-operated animals, XO represented 10% of the total enzymatic activity (XDH + XO). After I/R, this proportion was increased to 53%. Both the ischaemic and OzoneOPs attenuated this conversion since XO represented 45.5 and 46.6% of total enzymatic activity, respectively, compared to XDH + XO. Both of the preconditionings caused a reduction in total XO; however enzyme concentrations were still raised above that seen on the sham-operated rats. Total XO was only ameliorated by the ischaemic and OzoneOPs but calcium, calpain activity and total sulfhydryl groups were maintained to the concentrations of the sham-operated. In order to know the contribution of reversible and irreversible XO activities to total XO, the XO forms were evaluated. The results are shown in Table 1.

XO$_{o}$ was increased compared to the sham-operated group. XO$_{o}$ was unaffected by I/R when rats were previously subjected to the ischaemic and OzoneOPs.

Effects of the ischaemic and ozone oxidative preconditionings on MDA and 4-hydroxyalkenals generation. MDA plus 4-hydroxyalkenals is an index of lipid injury. The results of these parameters are shown in Fig. 3. There was a significant increase ($P < 0.05$) in lipid peroxidation in the I/R group. The ischaemic and OzoneOPs avoided lipid damage and they maintained the concentrations of MDA + 4-hydroxyalkenals at the level of the sham-operated group.

The results of biochemical and histopathological analysis reached in the sham-operated group were no different from the control group (oxygen).

DISCUSSION

The mechanism underlying preconditioning remains unknown and is currently under intense investigation. It has been suggested that protection depends on the release of substances by the organ, helping to protect it against injury. Potential mediators include adenosine [26], nitric oxide [12, 27] and others.

Ozone protected against I/R injury attenuating the increase of adenosine deaminase activity in liver. This process favoured adenosine accumulation and decreased hypoxanthine and xanthine formation, thus decreasing the ROS generation after reperfusion [21].

The biochemical markers and microscopic study of liver I/R injury indicate the protective effects of the ischaemic and OzoneOPs on preventing hepatic I/R damage (Figs 1 and 2). Nevertheless, there was a difference between the two preconditioning settings from a histological point of view. OzoneOP prevented Kupffer cell activation. Kupffer cells appear to be implicated in the regulation of the hepatic oxygen consumption. It has been postulated that in response to the oxygen stimulus, the Kupffer cell produces specific signal molecules (eicosanoids and/or cytokines) that regulate the oxygen uptake by liver parenchymal cells [28]. It has recently been reported that Kupffer cells are activated during the early phase after reperfusion in the ischaemic areas with the generation of ROS and production of proinflammatory cytokines [29].

Ozone treatment might avoid the Kupffer cell activation through its preconditioning mechanism which, during ischaemia, decreases or prevents the rise of precursors of ROS such as calcium levels, XO activity and xanthine generation [21].

Many studies have suggested that ROS, generated at the time of reperfusion, can cause a loss of organ function [30]. ROS production in the revascularized liver involves several mechanisms: (1) ischaemic conversion of XDH to XO, in addition to hypoxanthine generation from adenosine triphosphate (ATP), resulting in ROS production within the hepatocyte [31]; (2) activation of Kupffer cells, resulting in massive ROS production within the sinusoidal lumen which enhances endothelial cell injury and leads to polymorphonuclear cell accumulation and subsequent capillary plugging [32].

Liver, intestine and endothelial cells contain the highest specific activity of XDH + XO [33]. The liver releases XDH + XO into the vasculature after I/R. Circulating XDH + XO could result in direct damage to the vascular endothelium, activate oxidant-producing inflammatory cells (i.e. neutrophils), and then, via the direct production of ROS in the plasma or by activated neutrophils, extend oxidant-induced injury to tissues remote from the site of origin [34].

XDH is a homodimer with a subunit molecular mass of approximately 150 kDa [35]. Reversible conversion into XO may occur through sulfhydryl group oxidation. Irreversible conversion takes place by calcium-dependent
proteolytic processing of XDH during tissue ischaemia through cleavage of an approximately 15–20 kDa fragment and is believed to occur subsequent to the reversible conversion [35, 36].

OzoneOP maintained the reversible form of XO at the level of the sham-operated. It was in correspondence with preservation of total sulphhydryl group concentrations. It is in line with our previous findings that ozone treatment promotes an increase in antioxidant endogenous systems reducing oxidative stress mediated by liver I/R [20].

In both the preconditioning settings, XO accounts for almost the entire total XO activity present in liver samples (Table I).

The inability to maintain cellular calcium homeostasis appears to be an important event during ischaemia. Loss of calcium-pumping activity during ischaemia allows intracellular calcium concentrations to rise with associated increases in calcium dependent metabolic activities and eventual cell death. The activation of calcium-dependent enzymes, such as phospholipases, leads to membrane damage due to phospholipid degradation [3, 4]. Cellular calcium overload may also trigger the release of ROS and potentiate oxygen radical membrane injury through the increased conversion of XDH to XO.

There were no differences in calcium concentrations between the two preconditionings with regard to the sham-operated group (Table I). The protective effect of OzoneOP on cellular calcium homeostasis may be due to ozone’s capacity to protect Ca^{2+}-ATPase activities against inactivation of oxidative challenge [18]. Ca^{2+}-ATPase plays an important role in the transport of intracellular calcium in the liver.

Many lines of evidence suggest that calpain, a Ca^{2+}-dependent neutral protease, is implicated in injury during ischaemia or reperfusion. It has been demonstrated that reperfusion with EGTA [ethylene glycol bis (β-aminoethyl-ether)-N,N,N',N'-tetracetic acid] suppressed the ischaemia-induced increase in calpain activity which is the evidence that Ca^{2+} influx is involved in calpain activation [25]. Nevertheless, the role of calpain activity in conversion of XDH into XO is not yet a clear event. It has been shown that the cytoplasmic XDH of human liver is irreversibly cleaved to XO by mitochondrial protease and apparently not through a calcium-dependent protease (calpain). Intracellular calcium influx and subsequent mitochondrial Ca^{2+} overload during and after ischaemia have shown to promote the mitochondrial permeability transition. The role of excess intracellular calcium on XDH-XO conversion is thus likely to be indirect, rather than directly coupled to the activation mechanism of the protease [36].

In line with these reports, the effects of both preconditionings on XO conversion suggest that calpain is not the major protease which catalyses the irreversible conversion of XDH into XO.

Aldehyde production, as a result of ozone inhalation, could be an important mediator of ozone toxicity [37]. Although a large number of aldehydes can be formed during polyunsaturated fatty acid peroxidation, three aldehydes have been extensively studied as physiologically relevant lipid peroxidation products (4-hydroxynonenal, 4-hydroxyhexenal and MDA) [38].

The contribution of 4-hydroxynonenal to ozone injury was studied on a human exposed to 0.4 ppm ozone for 1 h with exercise. It was reported that 4-hydroxynonenal protein adducts after ozone exposure were consistent with a potential role for 4-hydroxynonenal in the cellular toxic effects of acute ozone exposure [39].

OzoneOP, similar to IscheP, maintained 4-hydroxyalkenals + MDA concentrations at the sham-operated group levels (Fig. 3). Similar results were reported when the MDA levels were studied on rats previously treated with ozone and, after that, subjected to liver I/R [20].

Low doses of ozone increased antioxidant endogenous systems such as glutathione, superoxide dismutase and catalase [16, 18, 20]. Therefore, an antioxidant-prooxidant balance is favoured for the preservation of the cell redox state and to ensure toxic aldehydes are not formed.

In summary, ischaemic and OzoneOP protected against liver I/R injury. This study provides evidence that both of the preconditioning settings share similar biochemical mechanisms of protection. It is noteworthy that histological results showed a more effective protection of OzoneOP than IscheP in our experimental conditions. Therefore, ozonetherapy may be considered as a potential medical approach against liver I/R damage.

### Table I

<table>
<thead>
<tr>
<th>Experimental groups</th>
<th>Ca^{2+} μmol mg prot.(^{-1})</th>
<th>Total-SH groups</th>
<th>Calpain(1) U mg prot.(^{-1})</th>
<th>XDH-XO(2) U mg prot.(^{-1})</th>
<th>Total XO(2) U mg prot.(^{-1})</th>
<th>Reversible XO(2) U mg prot.(^{-1})</th>
<th>Irreversible XO(2) U mg prot.(^{-1})</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sham-operated</td>
<td>0.18 ± 0.08(a)</td>
<td>6.21 ± 0.04(a)</td>
<td>1.65 ± 1.10(a)</td>
<td>6.01 ± 0.15(a)</td>
<td>0.60 ± 0.12(a)</td>
<td>0.11 ± 0.04(a)</td>
<td>0.49 ± 0.06(a)</td>
</tr>
<tr>
<td>I/R</td>
<td>0.52 ± 0.01(b)</td>
<td>6.06 ± 0.02(b)</td>
<td>7.48 ± 2.18(b)</td>
<td>7.36 ± 0.51(a)</td>
<td>3.92 ± 0.43(b)</td>
<td>0.50 ± 0.03(b)</td>
<td>3.42 ± 0.17(b)</td>
</tr>
<tr>
<td>IscheP + I/R</td>
<td>0.18 ± 0.09(a)</td>
<td>6.28 ± 0.07(a,c)</td>
<td>1.04 ± 0.58(a)</td>
<td>6.02 ± 0.60(a)</td>
<td>2.74 ± 0.23(c)</td>
<td>0.13 ± 0.03(a)</td>
<td>2.61 ± 0.23(c)</td>
</tr>
<tr>
<td>OzoneOP + I/R</td>
<td>0.20 ± 0.06(a)</td>
<td>6.34 ± 0.07(c,d)</td>
<td>1.41 ± 0.79(a)</td>
<td>6.02 ± 0.28(a)</td>
<td>2.81 ± 0.36(c)</td>
<td>0.12 ± 0.08(a)</td>
<td>2.69 ± 0.14(c)</td>
</tr>
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</table>

*Note: Each value is the mean ± SEM from eight rats. Different letters indicate a statistical significance of at least P < 0.05 within the same set.

(1) One unit activity was defined as an increase in A\(_{440}\) of 1.0 per ml sample per hour.

(2) The values of U mg protein\(^{-1}\) correspond to µmol min\(^{-1}\)mg protein\(^{-1}\).*
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