

## ORIGINAL ARTICLE

# Role of protein synthesis in the protection conferred by ozone-oxidative-preconditioning in hepatic ischaemia/reperfusion

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## Keywords

liver ischaemia/reperfusion, ozone, protein synthesis.

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Received: 5 May 2004

Revised: 11 August 2004

Accepted: 23 December 2004

doi:10.1111/j.1432-2277.2005.00101.x

## Summary

The liver is damaged by sustained ischaemia during liver transplantation, and the reperfusion after ischaemia results in further functional impairment. Ozone oxidative preconditioning (OzoneOP) protected the liver against ischaemia/reperfusion (I/R) injury through different mechanisms. The aim of this study was to investigate the influence of the inhibition of protein synthesis on the protective actions conferred by OzoneOP in hepatic I/R. Rats were treated with cycloheximide (CHX) in order to promote protein synthesis inhibition after OzoneOP treatment. Plasma transaminases, malondialdehyde and 4-hydroxyalkenals and morphological characteristics were measured as an index of hepatocellular damage; Cu/Zn-superoxide dismutase (SOD), Mn-SOD, catalase, total hydroperoxides and glutathione levels as markers of endogenous antioxidant system. OzoneOP increased Mn-SOD isoform and ameliorated mitochondrial damage. CHX abrogated the protection conferred by OzoneOP and decreased Mn-SOD activity. Cellular redox balance disappeared when CHX was introduced. Protein synthesis is involved in the protective mechanisms mediated by OzoneOP. Ozone treatment preserved mitochondrial functions and cellular redox balance.

## Introduction

Liver transplantation is the therapy of choice for end-stage liver disease and the demand for donor organs has surpassed the supply resulting in the death of thousands of patients [1]. However, 30% of transplants still fail from acute or chronic rejection within 5 years [2]. An understanding of the mechanism involved in ischaemia/reperfusion (I/R) is essential for the design of therapeutic strategies to improve the outcome of liver transplantation.

The mechanism of acute liver damage following I/R are thought to involve a complex interaction of immediate

cellular damage caused by different mediators. In the setting of prolonged ischaemia, a major source of primary liver dysfunction in donor grafts results from the generation of reactive oxygen species (ROS) during the reperfusion phase [3] leading to inflammation, cell death and ultimate organ failure. A crucial role in the pathophysiology of liver reperfusion injury has been attributed to activated Kupffer cells which generate a spectrum of bioactive molecules including eicosanoids, tumour necrosis factor- $\alpha$  (TNF $\alpha$ ), nitric oxide (NO $\cdot$ ) and ROS [4]. In addition, it has been demonstrated the activation of nuclear factor kappaB (NF $\kappa$ B) and heat shock protein 70 (HSP 70) [5,6].

Superoxide is one of the most relevant radicals in biological regulation. Many regulatory effects are mediated by hydrogen peroxide and other ROS that are chemically derived from superoxide [7].

During the ischaemic period, excessive ATP consumption leads to the accumulation of the purine catabolites, hypoxanthine and xanthine, which upon subsequent reperfusion and influx of oxygen are metabolized by xanthine oxidase to yield massive amount of superoxide and hydrogen peroxide [8]. ROS formation after I/R can lead to oxidative damage of DNA, proteins and lipids which contribute to cellular dysfunction or can directly regulate signal transduction [9]. Therefore, therapeutic approaches aimed at reducing oxidative stress in transplanted organs have been considered rational strategies for decreasing the complications associated to I/R damage.

Superoxide dismutase (SOD) activities are recognized scavengers. SOD are the first and most important line of antioxidant enzyme defence systems against ROS and particularly superoxide anion radicals. At present, three distinct isoforms of SOD have been identified in mammals, and their genomic structure, cDNA and proteins have been described [10]. Two isoforms of SOD (cytoplasmatic CuZn-SOD or SOD1 and mitochondrial Mn-SOD or SOD2) which attenuated the liver damage by I/R have been characterized.

Although SOD could protect against liver I/R injury, the administration of SOD does not protect the liver against I/R damage [11]. The protein SOD degrades rapidly when administered parenterally. Gene delivery has been used to increase protein expression in the cell [12]. Adenoviral-mediated gene delivery to the liver is highly effective even under conditions of cold organ storage [13]. It was shown that overexpression of Cu/Zn-SOD by adenovirus reduced I/R injury and improved survival after liver transplantation in rats [13]. Moreover, lipid-derived free radical adducts were blunted by about 60% in rats infected with adenovirus containing the transgene for cytosolic Cu/Zn-SOD and mitochondrial Mn-SOD [14].

Other strategies against liver I/R injury have been used. Surgical and pharmacological strategies present approaches to enhance the survival and viability of the liver in various surgical procedures including liver transplantation [15]. Ischaemic preconditioning and intermittent clamping are in clinical use. Although the benefit of ischaemic preconditioning in the liver already has been suggested in clinical pilot study [16], knowledge of the molecular mechanism remains vague. Intermittent clamping currently is used in practice by many centres. Although the protective mechanism of intermittent clamping still remain elusive, a similar mechanism to those described in ischaemic preconditioning, mainly by reduction of apoptosis [17] is assumed. A large number of pharmacological

agents were shown to confer protection against ischaemic injury in the liver. These agents include antioxidant, adenosine agonists and nitric oxide (NO<sup>•</sup>) donors, pentoxifylline and others. Nevertheless, only a few drugs are currently at the point of clinical application [16].

Recently ozone has been identified as a pharmacological agent able to reduce liver I/R injury through its effects on adenosine production [16]. However, not only adenosine production but also other mechanisms are involved.

The ROS (superoxide, hydrogen peroxide, hydroxyl radicals) generated from brief I/R have been recognized as possible 'triggers' in the initiation of preconditioning. Other studies have showed that antioxidants abolished the induction of preconditioning [18]. Endothelial preconditioning by transient oxidative stress reduced inflammatory responses of cultured endothelial cell to TNF $\alpha$  [19].

Ozone has been used as a therapeutical agent for the treatment of different diseases and beneficial effects have been observed [20,21]. On the contrary, it has been demonstrated that low levels of ozone exposure have distinct effects within cells. They may also protect the cell against subsequent ozone exposure [22].

In our laboratory we demonstrated that a controlled number of treatment and dose of ozone conferred protection against different physiopathological processes mediated by ROS [23–25]. The same protective effects were found in renal and liver I/R [26–29]. We called this phenomenon ozone oxidative preconditioning (OzoneOP) which did not evidence any difference with ischaemic preconditioning from the biochemical point of view [29]. More recently it was demonstrated the role of NO<sup>•</sup> in the OzoneOP in hepatic I/R [30].

Taking into account that SOD promote a protection against liver I/R injury and OzoneOP was able to increase total SOD activity in different experimental models [23,25,26,30], the aim of this work was to investigate the influence of the inhibition of protein synthesis with cycloheximide (CHX) on the protective actions conferred by OzoneOP in hepatic I/R and the effects of the protein synthesis inhibition on SOD isoforms (CuZn-SOD, Mn-SOD). The morphological characteristics of the liver submitted to OzoneOP + I/R were evaluated by histopathological procedures.

## Materials and methods

The protocol was approved by the College of Pharmacy (Havana University) Animal Care Committee and the experimental procedures were carried out in accordance with the guidelines established by the Principles of Laboratory Animal care (NIH publication No. 86–23, revised 1985).

## Animals

Adult male Wistar rats (10 each group, 250–275 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*.

## Surgical procedure

All animals (including controls) were anaesthetized with urethane (1 g/kg, 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, 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 [31].

## Experimental design

To study the effects of inhibition of protein synthesis on the protection conferred by OzoneOP, the following experimental groups were performed:

*Group 1.* Sham operated ( $n = 10$ ): Animals subjected to anaesthesia and laparotomy plus surgical manipulation (including isolation of the right hepatic artery and vein versus the left hepatic artery and vein without the induction of hepatic ischaemia).

*Group 2.* I/R ( $n = 10$ ): Animals subjected to 90 min of right lobe hepatic ischaemia as it was described in surgical procedure, followed by 90 min of reperfusion.

*Group 3.* OzoneOP + I/R ( $n = 10$ ): Before the I/R procedure (as in group 2), animals were treated with ozone by rectal insufflation 1 mg/kg. Nelaton canule No. 8 was introduced 6 cm by rectal way. The possible damage generated by this procedure was evaluated. Histopathological studies have not shown any injury at macroscopic and microscopic levels. Rats received 15 ozone treatments, one per day of 5–5.5 ml at an ozone concentration of 50 µg/ml. Ozone was obtained from medical grade oxygen, was used immediately as generated and it represented only about 3% of the gas ( $O_2/O_3$ ) mixture. The ozone concentration is measured by using a build-in UV spectrophotometer at 254 nm (accuracy: 0.002 at 1 absorbance unit, repeatability: 0.001 absorbance unit and calibrated with internal standard). The ozone dose is the product of the ozone concentration [expressed as mg/ml by the gas ( $O_3/O_2$ ) volume]. By knowing the body weight of the rat the ozone dose was calculated as mg/kg as in our previous papers [23–28].

*Group 4.* CHX + I/R ( $n = 10$ ): Animals were treated with CHX (1 mg/kg intravenously) during 3 days previous to I/R procedure (as in group 2).

*Group 5.* OzoneOP + CHX + I/R ( $n = 10$ ): Animals were treated with ozone (as in group 3). Afterwards they received CHX (as in group 4) and finally they were submitted to I/R (as in group 2).

Control experiments were performed including two additional groups: sham-operated + CHX ( $n = 10$ ) and OzoneOP + CHX ( $n = 10$ ). There were not differences between these groups with regard to sham-operated (data not shown).

## Sample preparations

Blood samples were obtained from the abdominal aorta in order to evaluate the degree of hepatic injury. Afterwards, the hepatic right lobe of each animal was extracted and they were homogenized in 20 mM KCl/histidine buffer pH 7.4, 1:10 w/v using a tissue homogenator Edmund Bülher LBMA (Edmund Bülher Co., Bodelshausen, Germany) at 4 °C and were centrifuged for 10 min at  $12\,000 \times g$ . The supernatants were taken for biochemical determinations.

## Biochemical determinations

### Markers of hepatic injury

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

### Determination of Cu/Zn-SOD and Mn-SOD in supernatant of liver homogenates

Total SOD activity was measured using pyrogallol as substrate [32]. This method follows the superoxide driven auto-oxidation of pyrogallol at pH 8.2 in the presence of EDTA. The assay mixture contained 1 mM of EDTA in 50 mM Tris-HCl buffer (pH 8.2) with or without the sample. The reaction was started by the addition of pyrogallol (final concentration 0.124 mM) and the oxidation of pyrogallol was followed for 1 min at 420 nm. The inhibition percentage of the auto-oxidation of pyrogallol by SOD present in the tissue sample was determined, and standard curves using known amounts of purified SOD (Sigma Chemical Co., St Louis, MO, USA) under identical conditions were established. One unit (U) of SOD activity was defined as the amount that reduced the absorbance change by 50%, and results were normalized on the basis of total protein content (U/mg protein). Cu/Zn-SOD was differentiated from Mn-SOD by addition of 2 mM sodium cyanide to inhibit the activity of Cu/Zn-SOD from total SOD activity. Cu/Zn-SOD activity was calculated as the difference between total SOD and Mn-SOD activities as in a previous report [33].

### Markers of antioxidant–prooxidant balance in liver I/R

Catalase (CAT) activity was measured by following the decomposition of hydrogen peroxide at 240 nm at 10-s intervals for 1 min [34]. The quantification of total hydroperoxides (TH) was measured by Bioxytech H<sub>2</sub>O<sub>2</sub>-560 kit (Oxis International Inc., Portland, OR, USA) using xylenol orange to form a stable coloured complex, which can be measured at 560 nm. Reduced and oxidized glutathione (GSH and GSSG respectively) were measured enzymatically in 5-sulphosalicylic acid-deproteinized samples using a modification [35] of the procedure [36]. Lipid peroxidation was assessed by measuring the concentration of malondialdehyde (MDA) and 4-hydroxyalkenals (4-HDA). Concentrations of MDA + 4-HDA were analysed using the LPO-586 kit obtained from Calbiochem (La Jolla, CA, USA). In the assay, the production of a stable chromophore after 40 min of incubation at 45 °C was measured at a wavelength of 586 nm. For standards, freshly prepared solutions of MDA bis[*N,N*-dimethyl acetal] (Sigma Chemical Co.) and 4-hydroxynonenal diethyl-acetal (Cayman Chemical Ann Arbor, MI, USA) were employed and assayed under identical conditions. Total protein were determined using the method described by Bradford [37] and analytical grade bovine serum albumin was used to establish a standard curve.

Unless otherwise stated, all chemicals were obtained from Sigma Chemical Co.

### Light and electron microscopy

The samples of liver were submitted to 10% buffered formalin fixation for 12 h at 8 °C and the trimmed sections embedded in paraffin; the slides were stained with haematoxylin and eosin.

For the electron microscopy, the samples were immediately placed in 3.2% glutaraldehyde during 1 h, fixed in osmic acid for 1 h at 4 °C. Subsequently the samples were washed with PBS (0.1 M) at pH 7.4 after which they were dehydrated in graded ethanol (30, 50, 70 and 100%) during 10 min. One to 400–500 Å sections were obtained using an Ultratome NOVA LKB (Leica, Solms, Germany) which were contrast stained with uranyl acetate and lead citrate and visualized using an electron microscope JEOL JEM 2000 Ex (JEOL, Tokyo, Japan).

### Statistical analysis

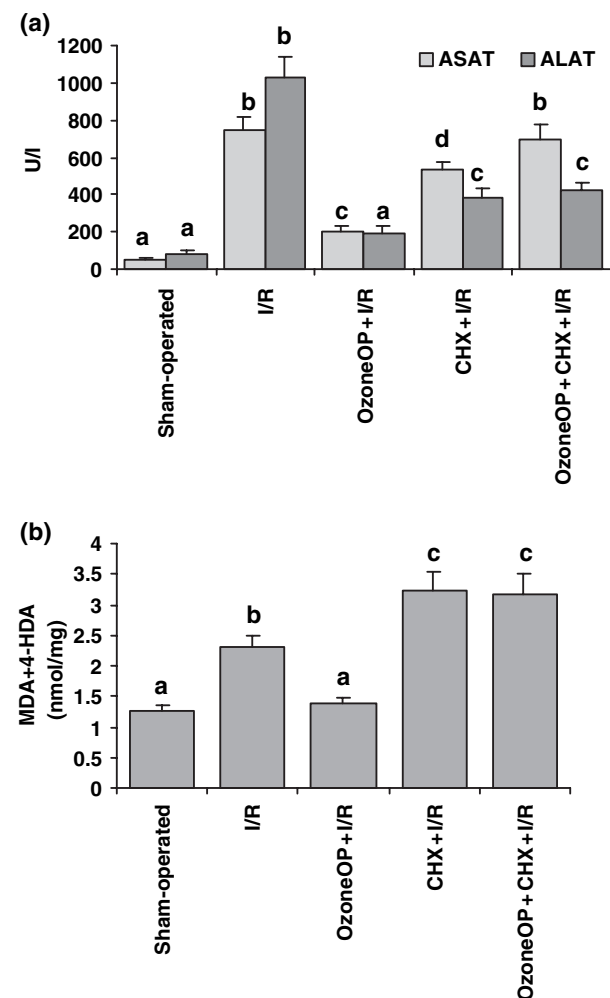
The statistical analysis was started by using the outliers preliminary tests for the detection of error values. Afterward, homogeneity variance test (Bartlett-Box) was used followed by the ANOVA method (one-way). In addition, a multiple comparison test was used (Duncan test); values were expressed by the mean ± standard error of mean

( $n = 10$  per group). The significance levels was set at  $P < 0.05$ .

## Results

### Effects of CHX on the protection conferred by OzoneOP in liver I/R

As shown in Fig. 1a, the degree of hepatic damage induced by 90 min of ischaemia and 90 min of reperfusion significantly increased ( $P < 0.05$ ) in the group subjected to I/R as evaluated by the plasma levels of AST and ALT. OzoneOP prevented and ameliorated the damage in



**Figure 1** (a) Plasma activities of aspartate aminotransferase (AST) and alanine aminotransferase (ALT); (b) hepatic tissue levels of malondialdehyde and 4-hydroxyalkenals. Ischaemia-reperfusion (I/R), 90 min of ischaemia followed by 90 min of reperfusion; OzoneOP, ozone oxidative preconditioning; CHX, cycloheximide. Cycloheximide was administered after OzoneOP during 3 days previous to I/R procedure. Each value is the mean ± SEM from 10 rats. Mean values having different superscript letters indicate significant difference ( $P < 0.05$ ) between groups.

accordance with ALT and AST activities respectively. CHX treatment reduced transaminases in CHX + I/R in comparison with I/R group but the activities were higher than OzoneOP + I/R ( $P < 0.05$ ). The inhibition of protein synthesis by CHX increased transaminase activities in OzoneOP + CHX + I/R with regard to OzoneOP + I/R and sham-operated groups.

The MDA + 4-HDA is an index of hepatic damage associated with lipid peroxidation. The results are shown in Fig. 1b. There was a significant increase ( $P < 0.05$ ) of lipid peroxidation in I/R. OzoneOP maintained MDA + 4-HDA generation to sham-operated levels in OzoneOP + I/R. CHX increased the hepatic damage mediated by I/R as the raise of MDA + 4HDA was higher in CHX + I/R. CHX treatment increased MDA + 4 HDA concentrations in OzoneOP + CHX + I/R without differences with CHX + I/R.

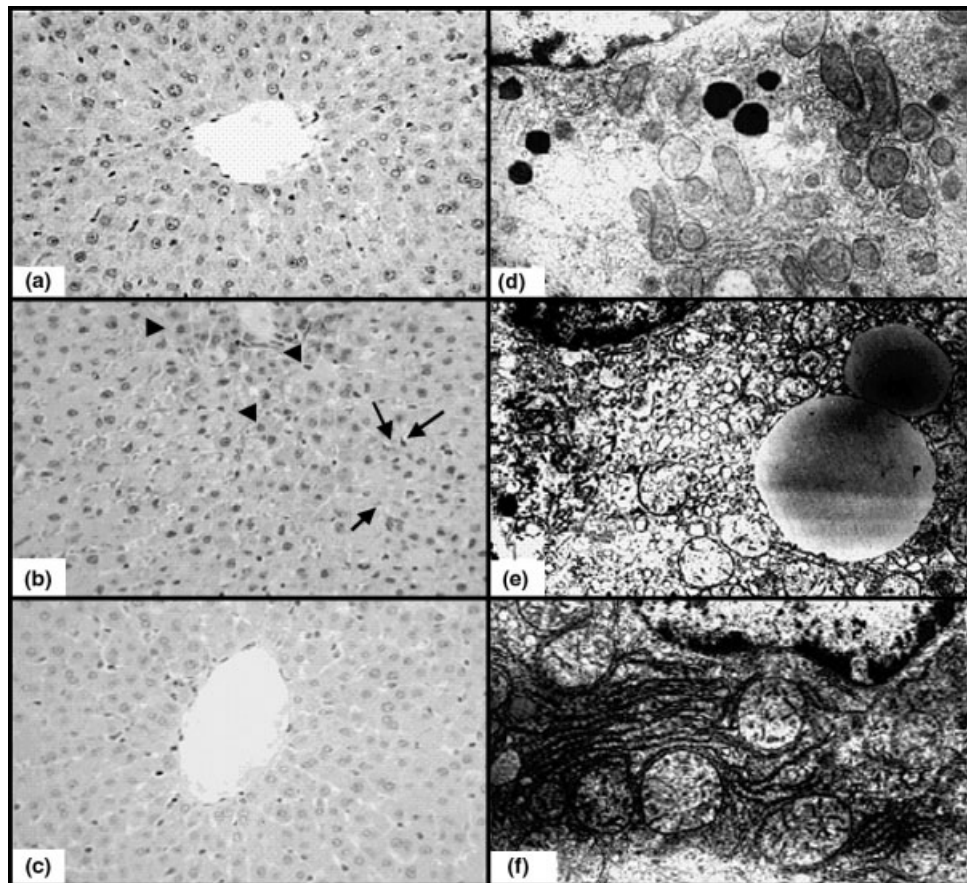
In contrast with the sham-operated group (Fig. 2a), the liver of the animals submitted to I/R showed microvascular steatosis and nuclear condensation of the hepatocytes at the zone 3 of the acinus (Fig. 2b).

The liver sections of the OzoneOP + I/R group showed a normal structure when compared with the sham-operated (Fig. 2c).

At the ultrastructural level great lipid droplets and moderated alteration of mitochondrial membranes and crests were found while these changes have been partially reverted in OzoneOP + I/R group (Fig. 2d–f).

#### OzoneOP and CHX actions on Cu/Zn-SOD, Mn-SOD, CAT activities and TH concentrations

The effects of OzoneOP on SOD activities are shown in Table 1. The activity of total SOD decreased in I/R (27%)



**Figure 2** Histological lesions: Optical Microscopic analysis: (a) sham-operated, zone 3 of hepatic acinus presents normal morphology. (b) I/R, nuclear condensation of hepatocytes at the zone 3 of the acinus (head arrow). Hyperaemic dilatation on sinusoids and microvascular steatosis (arrow). (c) OzoneOP + I/R, normal morphology of zone 3 of the acinus like sham-operated (HE; original magnifications 400 $\times$ ). Ultrastructural analysis: (d) normal appearance of mitochondrial, rough endoplasmatic reticulum and peroxisome, no alteration is observed on nucleus structure. (e) I/R, great lipid droplets and moderated alteration of mitochondrial membranes and crests were found. (f) Changes observed on I/R group have been partially reverted in the OzoneOP + I/R group.

**Table 1.** Superoxide dismutase, catalase activities and hydroperoxides concentrations in hepatic tissue.

Experimental groups	Total SOD (U/g tissue)	Mn-SOD (U/g tissue)	Cu/Zn-SOD (U/g tissue)	CAT (U/g tissue)	TH ( $\mu\text{mol/g}$ tissue)
Sham-operated	31 127 $\pm$ 5267 <sup>a</sup>	19 310 $\pm$ 5775 <sup>a</sup>	11 816 $\pm$ 5916 <sup>a</sup>	87.1 $\pm$ 10.4 <sup>a</sup>	10.8 $\pm$ 1.5 <sup>a</sup>
I/R	22 600 $\pm$ 4506 <sup>b</sup>	3560 $\pm$ 630 <sup>b</sup>	19 040 $\pm$ 4539 <sup>b</sup>	316.4 $\pm$ 23.0 <sup>b</sup>	62.5 $\pm$ 9.7 <sup>b</sup>
OzoneOP + I/R	37 026 $\pm$ 4390 <sup>c</sup>	27 903 $\pm$ 7357 <sup>c</sup>	9122 $\pm$ 4548 <sup>a</sup>	154.4 $\pm$ 8.2 <sup>a</sup>	17.8 $\pm$ 2.4 <sup>ac</sup>
CHX + I/R	17 534 $\pm$ 4289 <sup>b</sup>	6109 $\pm$ 1406 <sup>b</sup>	11 424 $\pm$ 3706 <sup>a</sup>	358.2 $\pm$ 32.8 <sup>b</sup>	11.1 $\pm$ 4.1 <sup>a</sup>
OzoneOP + CHX + I/R	19 916 $\pm$ 4766 <sup>b</sup>	3494 $\pm$ 1159 <sup>b</sup>	16 408 $\pm$ 4830 <sup>b</sup>	612.5 $\pm$ 65.9 <sup>c</sup>	21.9 $\pm$ 6.7 <sup>c</sup>

Ischaemia/reperfusion (I/R), 90 min of ischaemia followed by 90 min of reperfusion; OzoneOP, ozone oxidative preconditioning; CHX, cycloheximide administered after OzoneOP during 3 days previous to I/R procedure.

Each value is the mean  $\pm$  SEM from 10 rats. Means having different superscript letter indicate significant difference ( $P < 0.05$ ) between groups within the same set.

and CHX + I/R (43%) groups with regard to sham-operated animals. Ozone treatment was not only able to maintain total SOD but also to increase it in OzoneOP + I/R with regard to sham-operated groups (37 026  $\pm$  4390 U/g vs. 31 127  $\pm$  5267 U/g protein respectively). In contrast, when CHX was introduced (OzoneOP + CHX + I/R) total SOD activity decreased and it did not differ ( $P > 0.05$ ) from I/R group (19 916  $\pm$  4766 U/g vs.  $\pm$  4506 U/g protein respectively).

Mn-SOD was the isoform which contributed to the rise in total SOD in OzoneOP + I/R. Ozone promoted an increase of Mn-SOD (44%) in comparison with sham-operated animals while Mn-SOD activity decreased in the rest of the groups and there were no differences in I/R, CHX + I/R and OzoneOP + CHX + I/R groups.

The Cu/Zn-SOD activity was maintained at sham-operated levels in OzoneOP + I/R and CHX + I/R. An increase was observed in Cu/Zn-SOD in I/R and OzoneOP + CHX + I/R with regard to the rest of the groups.

OzoneOP maintained CAT activity and TH at sham-operated levels in OzoneOP + I/R. CAT activity was increased in I/R with a similar figure in TH. CHX did not modify CAT activity in CHX + I/R with regard to I/R group. OzoneOP + CHX + I/R showed the higher CAT activity in comparison with the rest of the groups. An increase of TH concentrations was observed in OzoneOP + CHX + I/R.

#### Influence of OzoneOP and CHX on glutathione (reduced and oxidized) generation

The results for total glutathione (GSH + GSSG) concentrations are shown in Table 2. A depletion of GSH and an increase of GSSG in I/R group were observed. OzoneOP (OzoneOP + I/R) prevented the GSH depletion and the GSSG increment. In contrast, CHX increased GSSG levels in CHX + I/R and OzoneOP + CHX + I/R groups. In line with these results GSH/GSSG ratio showed that glutathione existing in the oxidized form was significantly ( $P < 0.05$ ) higher in I/R, CHX + I/R and

**Table 2.** Glutathione concentrations in hepatic tissue in different experimental conditions.

Experimental groups	GSH + GSSG ( $\mu\text{g/g}$ tissue)	GSH ( $\mu\text{g/g}$ tissue)	GSSG ( $\mu\text{g/g}$ tissue)	GSH/GSSG
Sham-operated	49.64 $\pm$ 4.7 <sup>a</sup>	32.99 $\pm$ 6.50 <sup>a</sup>	13.64 $\pm$ 4.13 <sup>a</sup>	2.42
I/R	52.96 $\pm$ 4.91 <sup>ac</sup>	14.45 $\pm$ 2.90 <sup>b</sup>	38.52 $\pm$ 7.30 <sup>b</sup>	0.38
OzoneOP + I/R	37.86 $\pm$ 8.64 <sup>b</sup>	28.51 $\pm$ 72 <sup>a</sup>	9.35 $\pm$ 3.50 <sup>a</sup>	3.10
CHX + I/R	59.17 $\pm$ 12.92 <sup>c</sup>	29.56 $\pm$ 3.9 <sup>a</sup>	27.99 $\pm$ 8.80 <sup>c</sup>	1.10
OzoneOP + CHX + I/R	55.74 $\pm$ 5.11 <sup>a</sup>	35.53 $\pm$ 5.6 <sup>a</sup>	20.20 $\pm$ 4.60 <sup>d</sup>	1.80

Ischaemia/reperfusion (I/R), 90 min of ischaemia followed by 90 min of reperfusion; OzoneOP, ozone oxidative preconditioning; CHX, cycloheximide administered after OzoneOP during 3 days previous to I/R procedure.

Each value is the mean  $\pm$  SEM from 10 rats. Mean values having different superscript letters indicate significant difference ( $P < 0.05$ ) between groups within the same set.

OzoneOP + CHX + I/R groups in comparison with OzoneOP + I/R and sham-operated.

#### Discussion

OzoneOP may promote a moderate oxidative stress which, in turn, increases antioxidant endogenous systems protecting against liver damage [23,24]. Cells or tissues are in a stable state if the rates of ROS production and scavenging capacity are essentially constant and in balance. Redox signalling requires that this balance be disturbed, either by an increase in ROS concentrations or a decrease in the activity of one or more antioxidant systems. In higher organisms, such an oxidative event may be induced in a regulated way by the activation of endogenous RNS- or ROS-generating systems [7]. However similar responses may be induced by oxidative stress conditions like the hydrogen peroxide in human umbilical vein endothelial cells [19] and the ozone treatment in controlled doses [23–29]. The protective mechanism mediated by OzoneOP may involve protein synthesis. Elevated ROS concentrations induce in many cells the expression of genes whose products exhibit anti-

oxidative activity. A major mechanism of redox homeostasis is based on the ROS-mediated induction of redox sensitive signal cascades that lead to increase expression of antioxidative enzymes or an increase in the cystine transport system which, in turn, facilitates in certain cell types the increase in intracellular glutathione [7].

There was correspondence between transaminases and MDA + 4HDA concentrations as markers of liver damage (Fig. 1a and b). OzoneOP protected against I/R injury in OzoneOP + I/R. The reduction in transaminase activities observed in CHX + I/R with regard to I/R groups may reflect the inhibition of protein synthesis associated to liver injury such as interleukins, hydrolytic enzymes, etc. [4]. However, the damage mediated by lipid peroxidation was dramatically increased in CHX + I/R in comparison with I/R (Fig. 1b).

When CHX was introduced (OzoneOP + CHX + I/R) the protection conferred by ozone treatment was abrogated in accordance with the increase in transaminase activities and MDA + 4-HDA concentrations. These results suggested that the protection against liver I/R injury by ozone required protein synthesis.

Acute damage following I/R in the liver is in part caused by the generation of ROS, such as superoxide during the reperfusion event [38]. The superoxide anion is formed by the univalent reduction of triplet-state molecular oxygen ( $^3\text{O}_2$ ). This process is mediated by enzymes such as NADPH oxidases and xanthine oxidase or nonenzymatically by redox reactive compounds such as the semi-ubiquinone compound of the mitochondrial electron transport chain. SOD converts superoxide enzymatically into hydrogen peroxide [39]. Upon reperfusion, a burst of anion superoxide would be expected to occur because of the increased autoxidation rate of the intramitochondrial sources of the anion superoxide, mainly the semiquinone (QH $\cdot$ ) of complexes I and II.

OzoneOP increased total SOD activity in particular the isoform Mn-SOD in OzoneOP + I/R with regard to the rest of the experimental groups including sham-operated animals (Table 1). The role of Mn-SOD in the protection against liver I/R damage was evident when the protein synthesis was manipulated with CHX. The protein synthesis inhibitor reduced drastically Mn-SOD activity in OzoneOP + CHX + I/R (87% with regard to OzoneOP + I/R) and the protection conferred by OzoneOP disappeared, observing instead an increase in both MDA + 4 HDA and transaminase levels (Fig. 1a and b).

The contribution of OzoneOP to Mn-SOD activity may be a consequence of its actions on gene expression in a similar way to OzoneOP effects on NO $\cdot$  generation [30]. Ozone administration under our experimental conditions (15 days, low controlled doses administered by rectal insufflation) may prime and activate the genes associated

to Mn-SOD expression which decrease ROS formation in the required concentrations for protecting against liver I/R injury. Those results will be confirmed in future works through Western blot and RT-PCR. As it was mentioned before, the controlled exposure to ROS induced by OzoneOP may regulate various signal transduction cascades and increase the activities of several transcription factors. ROS and other oxidants were also found to induce Mn-SOD mRNA levels to a moderate extent in several cell types [40].

Large amounts of radicals interfere with mitochondrial function and the cell dies because of lack of energy. Transmission electronic microscopy evidenced OzoneOP attenuated mitochondrial damage. The availability of ATP produced by mitochondria favours Ca $^{2+}$ -ATPase activity which is responsible for calcium homeostasis. This enzyme was protected by OzoneOP against hepatocellular injury mediated by CCl $_4$  [23]. In line with these results OzoneOP avoided the increase of calcium in liver I/R [29]. Mitochondrial integrity and the expression of Mn-SOD activity may be an explanation of how OzoneOP protects the hepatocyte against ROS. On the contrary, if any ROS escape from the mitochondria to cytosol Cu/Zn-SOD may detoxify them because of Cu/Zn-SOD activity did not differ ( $P > 0.05$ ) from the sham-operated group (Table 1). Cu/Zn-SOD activity increased in I/R and OzoneOP + CHX + I/R groups. These results may correspond to the activation of antioxidant defence systems before an oxidative challenge.

The effects of OzoneOP on Mn-SOD isoform trend has particular importance. Mn-SOD was the first protein transfected in mice by adenoviral gene therapy [41] and the gene therapy is considered a great promise in reducing acute hepatocellular damage in liver I/R in spite of the potential negative side-effects which are currently ethically unacceptable [15,38]. Other therapeutic systems include modified enzymes (e.g. mutated forms of SOD) and synthetic low molecular weight SOD mimics. In line with these therapeutic proposals OzoneOP effects on Mn-SOD isoform has special importance. OzoneOP is able to promote an increase of endogenous Mn-SOD activity devoid of typical undesirable reactions which are common to those newly developed free radical scavenger.

The protective effects of OzoneOP through activation of Mn-SOD isoform reduce oxidative stress. Nevertheless, not only Mn-SOD activity but also other protective antioxidant mechanisms against liver I/R injury are mediated by OzoneOP. These mechanism include the regulation of xanthine oxidase, calpain, total sulphhydryl groups [23,29] and NO $\cdot$  generation [30].

The antioxidant effects mediated by OzoneOP may play an important role against activation of AP-1 and NF- $\kappa$ B as it has been suggested to be a common mitochondrial

redox-sensitive pathway for activation of both transcriptional factors which take part in inflammatory and apoptotic processes [41].

The maintenance of antioxidant–prooxidant balance by OzoneOP was evident. CAT activity and TH concentrations were maintained to sham-operated levels (Table 1), suggesting the regulation of cellular redox balance. The intracellular oxidation of GSH to GSSG (Table 2) protects the enzyme sulphhydryl groups and vital membrane components. OzoneOP avoid GSH depletion as a result of the prevention of oxidative stress mediated by I/R injury. These results were in line with the reduction of lipid peroxidation (Fig. 1b) which suggest the preservation of membrane integrity by ozone treatment.

In summary, protein synthesis is involved in the protective mechanisms mediated by OzoneOP. Ozone treatment preserved mitochondrial functions and cellular redox balance. The increase of endogenous Mn-SOD activity contributed to reduce hepatic damage in liver I/R. Therefore, OzoneOP represents a potential therapeutic strategy in liver transplantation.

### Acknowledgements

We gratefully acknowledge the support from the Humboldt Foundation (Germany), the Department of Chemistry and Medical Biochemistry, University of Milan (Italy) and Laboratory of Pharmacological Biotechnology, University of Ancona (Italy).

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