

# Effects of ozone oxidative preconditioning on nitric oxide generation and cellular redox balance in a rat model of hepatic ischaemia–reperfusion

Ajamieh HH, Menéndez S, Martínez-Sánchez G, Candelario-Jalil E, Re L, Giuliani A, Fernández OS. Effects of ozone oxidative preconditioning on nitric oxide generation and cellular redox balance in a rat model of hepatic ischaemia–reperfusion.

Liver International 2004; 24: 55–62. © Blackwell Munksgaard, 2004

**Abstract:** *Background:* 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 and protecting against liver ischaemia–reperfusion (I/R) injury. *Aims:* In the present study, the effects of ozone oxidative preconditioning (OzoneOP) on nitric oxide (NO) generation and the cellular redox balance have been studied. *Methods:* Six groups of rats were classified as follows: (1) sham-operated; (2) sham-operated+L-NAME (*N*<sup>ω</sup>-nitro-L-arginine methyl ester); (3) I/R (ischaemia 90 min–reperfusion 90 min); (4) OzoneOP+I/R; (5) OzoneOP+L-NAME+I/R; and (6) L-NAME+I/R. The following parameters were measured: plasma transaminases (aspartate aminotransferase, alanine aminotransferase) as an index of hepatocellular injury; in homogenates of hepatic tissue: nitrate/nitrite as an index of NO production; superoxide dismutase (SOD), catalase (CAT) and glutathione levels as markers of endogenous antioxidant system; and finally malondialdehyde+4-hydroxyalkenals (MDA+4-HDA) and total hydroperoxides (TH) as indicators of oxidative stress. *Results:* A correspondence between liver damage and the increase of NO, CAT, TH, glutathione and MDA+4-HDA concentrations were observed just as a decrease of SOD activity. OzoneOP prevented and attenuated hepatic damage in I/R and OzoneOP+L-NAME+I/R, respectively, in close relation with the above-mentioned parameters. *Conclusions:* These results show that OzoneOP protected against liver I/R injury through mechanisms that promote a regulation of endogenous NO concentrations and maintenance of cellular redox balance. Ozone treatment may have important clinical implications, particularly in view of the increasing hepatic transplantation programs.

**H. H. Ajamieh<sup>1</sup>, S. Menéndez<sup>2</sup>, G. Martínez-Sánchez<sup>1</sup>, E. Candelario-Jalil<sup>1</sup>, L. Re<sup>3</sup>, A. Giuliani<sup>4</sup> and Olga Sonia León Fernández<sup>1</sup>**

<sup>1</sup>Center of Studies for Research and Biological Evaluation (CEIEB-IFAL-UH), University of Havana, Havana City, Cuba, <sup>2</sup>Ozone Research Center, Havana City, Cuba, <sup>3</sup>University of Ancona, Ancona, Italy and <sup>4</sup>Department of Chemistry and Medical Biochemistry, University of Milan, Milan, Italy

**Key words:** ischaemia–reperfusion – liver damage – nitric oxide – ozone – ozone preconditioning

Olga Sonia León Fernández, PhD, Center of Studies for Research and Biological Evaluation (CEIEB-IFAL), University of Havana, Havana City 10400, Cuba.

Tel: +53 7 271 9531.

Fax: +53 7 336 811.

e-mail: olga@infomed.sld.cu

Received 18 April 2003,

accepted 25 September 2003

**Abbreviations:** 4-HDA, 4-hydroxyalkenals; ALT, alanine aminotransferase; AST, aspartate aminotransferase; CAT, Catalase; GSH, reduced glutathione; GSSG, oxidized glutathione; I/R, ischaemia–reperfusion; L-NAME, *N*<sup>ω</sup>-nitro-L-arginine methyl ester; MDA, malondialdehyde; NO, nitric oxide; NOS, nitric oxide synthetase; tNOS, total nitric oxide synthetase; iNOS, inducible nitric oxide synthetase; eNOS, endothelial nitric oxide synthetase; OzoneOP, ozone oxidative preconditioning; ROS, reactive oxygen species; SOD, superoxide dismutase; TH, total hydroperoxides.

Liver transplantation is now accepted as the best treatment for end-stage liver disease. Nevertheless, hepatic ischaemia–reperfusion (I/R) injury associated with liver transplantation and hepatic resection are an unresolved problem in clinical practice (1, 2).

Although the inflammatory response elicited by I/R has been extensively characterized, the mechanisms underlying this phenomenon remain poorly understood. Several bioactive molecules,

including reactive oxygen species (ROS) (3), some cytokines, hydrolytic enzymes and nitric oxide (NO) are generated in response to soluble and particulate stimuli (3, 4).

NO, a hydrophobic gaseous molecule, is synthesized from L-arginine by different isoforms of nitric oxide synthetase (NOS). The properties of NO appear to depend on which isoform has contributed to its formation. Two principal forms of NOS have been described: a constitutive endothelial NOS (eNOS), which is dependent on intracellular calcium levels for its activity, and an inducible form (iNOS) expressed by a number of tissues and cells, usually in response to inflammatory mediators (5).

Ischaemic preconditioning is an inducible and potent endogenous mechanism by which repeated episodes of brief ischaemia and reperfusion confer a state of protection against subsequent sustained I/R injury (6). Although the mechanisms of preconditioning are not yet completely known, some hypotheses have been tested. The results indicate that organ protection depends on the release of endothelial substances such as NO. It has been demonstrated that the mechanism of hepatic preconditioning is mediated by the inhibitory action of NO on endothelin levels (7). A close relation between NO and adenosine in the protection of the liver by ischaemic preconditioning has been shown. The inhibition of NO abolished the preconditioning effect despite adenosine administration, whereas adenosine deaminase infusion plus NO administration failed to abolish the beneficial effect of preconditioning. These results suggest that the mechanism leading to preconditioning in the ischaemic liver involves the release of adenosine, which induces the generation of NO (8).

Ozone has been used as a therapeutic agent for the treatment of different diseases, and beneficial effects have been observed (9–11). It has been demonstrated that controlled ozone administration may promote an oxidative preconditioning or adaptation to oxidative stress that, in turn, increases antioxidant endogenous systems protecting against liver and pancreas damage (12–14).

We had demonstrated that ozone treatment was able to protect the liver against I/R damage by the accumulation of adenosine and by blocking the xanthine/xanthine oxidase pathway for ROS generation (15, 16). More recently, a similar protective effect of ischaemic and ozone oxidative preconditionings (OzoneOPs) in liver I/R injury was demonstrated, providing evidences that both preconditioning settings shared similar biochemical mechanisms of protection. However, the histological results showed a more effective

protection of OzoneOP than ischaemic preconditioning (17).

Taking into account the role of NO in liver I/R injury and the protection conferred by ischaemic and OzoneOPs, the aim of this study was to investigate the effects of OzoneOP on NO molecule generation and the relation of this with the antioxidant–pro-oxidant balance in a model of liver I/R in rats.

## Methods

The protocol was approved by the Havana University Faculty of Pharmacy Animal Care Committee and the experimental procedures were carried out in accordance with the guidelines established by the Canadian Council on Animal Care.

### Animals

Adult male Wistar rats (10 animals per 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*.

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 °C 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 (7).

### Experimental design

To study the effects of OzoneOP on NO generation and cellular redox balance, the following experimental groups were prepared:

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

*Group 2. Sham-operated+L-NAME (N<sup>ω</sup>-nitro-L-arginine methyl ester) (n = 10):* Animals subjected to anaesthesia and laparotomy plus surgical manipulation (as group 1) were treated with L-NAME (10 mg/kg, i.v.) 10 min before laparotomy.

*Group 3. I/R (n = 10):* Animals subjected to 90 min of right lobe hepatic ischaemia, followed by 90 min of reperfusion.

**Group 4. OzoneOP+I/R ( $n = 10$ ):** Before the I/R procedure (as in group 3), animals were treated with ozone by rectal insufflation 1 mg/kg. 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 built-in UV spectrophotometer at 254 nm (accuracy, 0.002 A at 1 A, repeatability 0.001 A and calibrated with internal standard). The ozone dose is the product of the ozone concentration (expressed as mg/l by the gas ( $O_3/O_2$ ) volume (l)). By knowing the body weight of the rat, the ozone dose was calculated as mg/kg as in our previous papers (12–17).

**Group 5. OzoneOP+L-NAME+I/R ( $n = 10$ ):** Animals treated with ozone (as in group 4) were treated with L-NAME (10 mg/kg, i.v.) 10 min before the I/R procedure.

**Group 6. L-NAME+I/R ( $n = 10$ ):** Animals treated with L-NAME (10 mg/kg, i.v.) 10 min before the I/R procedure.

#### 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 homogenizer (Edmund Bühler LBMA, Germany) at 4 °C and centrifuged for 10 min at 12 000g. 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 kits from Boehringer Mannheim (Munich, Germany).

##### *Markers of antioxidant–pro-oxidant balance in supernatants of liver homogenates*

Nitrite/nitrate levels as a measure of NO generation were determined by the Griess reaction by first converting nitrates to nitrites using nitrate reductase (Boehringer Mannheim Italy SpA, Milan, Italy). Then the Griess reagent (1% sulphanilamide, 0.1% *N*-(1-naphthyl)-ethylenediamine dihydrochloride in 0.25% phosphoric acid) was added (18). Samples were incubated at room temperature for 10 min and absorbance was measured at 540 nm using a microplate reader. Superoxide dismutase (SOD) was measured using

a kit supplied by Randox Laboratories Ltd, Ireland (Cat. No. SD125). Catalase (CAT) activity was measured by following the decomposition of hydrogen peroxide at 240 nm at 10 s intervals for 1 min (19). The quantification of total hydroperoxides (TH) was measured by Bioxytech  $H_2O_2$ -560 kit (Oxis International Inc., Portland, OR) 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 (20) of the procedure of Tietze (21). 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). 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 malondialdehyde bis[*dimethyl acetal*] (Sigma Chemical Co., St Louis, MO) and 4-hydroxynonenal diethyl-acetal (Cayman Chemical, Ann Arbor, MI) were employed and assayed under identical conditions. Total protein was determined using the method described by Bradford (22), and analytical grade bovine serum albumin was used to establish a standard curve.

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

#### 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 are expressed by the mean  $\pm$  standard error of mean ( $n = 10$  per group). The significance level was set at  $P < 0.05$ .

## Results

### Effects of OzoneOP on hepatic injury

As shown in Fig. 1A, the degree of hepatic damage induced by 90 min of ischaemia and 90 min of reperfusion significantly ( $P < 0.05$ ) increased in the group subjected to I/R as evaluated by the plasma levels of AST and ALT. OzoneOP ameliorated the damage in both treatments OzoneOP+I/R and OzoneOP+L-NAME+I/R. Nevertheless, the ozone-protective effects were

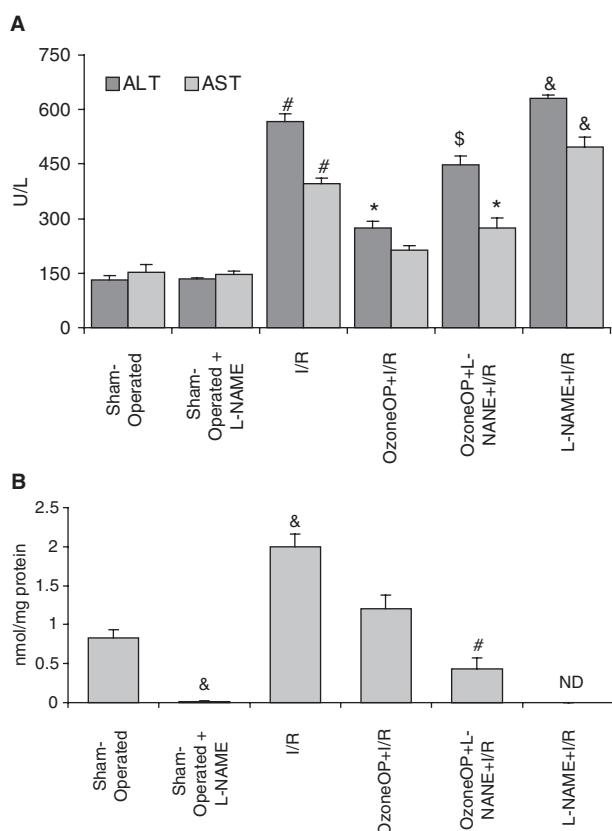


Fig. 1. (A) Plasma activities of aspartate aminotransferase (AST) and alanine aminotransferase (ALT); (B) hepatic tissue levels of nitrite/nitrate ( $\text{NO}_2^-/\text{NO}_3^-$ ). Ischaemia–reperfusion (I/R): 90 min of ischaemia followed by 90 min of reperfusion; OzoneOP: ozone oxidative preconditioning; L-NAME:  $N^G$ -nitro-L-arginine methyl ester. Each value is the mean  $\pm$  SEM from 10 rats. ND, not detectable levels. &, #, \* <sup>§</sup>Statistical significance of at least  $P < 0.05$  compared with the rest of the groups.

lesser in the group treated with OzoneOP+L-NAME+I/R than the OzoneOP+I/R group. Transaminase activities were not different in the sham-operated group+L-NAME with regard to the sham-operated animals.

#### OzoneOP actions on $\text{NO}_2^-/\text{NO}_3^-$ generations

Figure 1B shows the effects of ischaemia and treatments on NO generation. NO increased in I/R compared with all treatments. In the group only subjected to I/R, the NO levels reached the maximal values as compared with all other treatments. OzoneOP (OzoneOP+I/R) afforded complete protection against the marked increased in  $\text{NO}_2^-/\text{NO}_3^-$  concentrations induced by the I/R episode, as there were no statistical differences between the OzoneOP+I/R and sham-operated control group. The inhibition of NO synthesis by L-NAME decreased  $\text{NO}_2^-/\text{NO}_3^-$  levels in the presence of OzoneOP (OzoneOP+L-NAME+I/R). When animals were not subjected to preconditioning with ozone (OzoneOP), the L-NAME

treatment (L-NAME+I/R) completely abolished (undetectable levels) NO production induced by I/R. In the group treated only with L-NAME (sham-operated+L-NAME), the NO production was not different from the sham-operated control group as shown in Fig. 1B.

#### Effects of OzoneOP on the antioxidant–prooxidant balance in liver I/R

The effects of OzoneOP on SOD and CAT activities and TH concentrations are shown in Table 1. The activity of SOD decreased in I/R (42%) and L-NAME+I/R (38%) groups with regard to sham-operated animals, while CAT concentrations increased in the same groups. The activity of SOD was not different in OzoneOP+I/R and sham-operated groups. Ozone treatment ameliorated the decrease in SOD activity in OzoneOP+L-NAME+I/R (13% with regard to the sham-operated group). The enzyme levels in this group increased compared with L-NAME+I/R ( $6936 \pm 343$  vs.  $4928 \pm 205$  U/mg protein, respectively).

TH was maintained at sham-operated levels in OzoneOP+I/R, OzoneOP+L-NAME+I/R and sham-operated+L-NAME groups. However, there was a significant increase of this ROS in I/R and L-NAME+I/R. 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 and L-NAME+I/R groups were observed. OzoneOP prevented (OzoneOP+I/R) or attenuated (OzoneOP+L-NAME+I/R) the GSH depletion and the GSSG increment, respectively. GSH/GSSG ratio showed that glutathione existing in the oxidized form was significantly ( $P < 0.05$ ) higher in I/R and L-NAME+I/R groups than in the remaining groups. MDA+4-HDA is an index of lipid oxidation. The results of these parameters are shown in Fig. 2. There was a significant increase ( $P < 0.05$ ) in lipid peroxidation in I/R. The rise of MDA+4-HDA was higher in L-NAME+I/R, which was different from all experimental groups including I/R.

In a similar way to parameters as transaminases,  $\text{NO}_2^-/\text{NO}_3^-$  levels, SOD and CAT activities, TH and glutathione concentrations, OzoneOP maintained lipid peroxidation levels to sham-operated in OzoneOP+I/R and ameliorated MDA+4-HDA concentrations in OzoneOP+L-NAME+I/R group.

#### Discussion

The mechanisms underlying preconditioning remain unknown and are currently under intense

Table 1. SOD and CAT activities and TH concentrations in hepatic tissue

Experimental groups	SOD activity (U/g protein)	CAT activity (U/g protein)	TH ( $\mu\text{mol/g}$ protein)
Sham-operated	7952 $\pm$ 296	215 $\pm$ 45	8.28 $\pm$ 0.80
Sham-operated + L-NAME	7485 $\pm$ 150 <sup>#</sup>	174 $\pm$ 40	5.70 $\pm$ 0.35
I/R	4566 $\pm$ 374 <sup>&amp;</sup>	764 $\pm$ 53 <sup>&amp;</sup>	42.73 $\pm$ 2.31 <sup>&amp;</sup>
OzoneOP + I/R	8013 $\pm$ 123	282 $\pm$ 27	11.40 $\pm$ 1.46
OzoneOP + L-NAME + I/R	6936 $\pm$ 343 <sup>#</sup>	238 $\pm$ 33	12.21 $\pm$ 1.24
L-NAME + I/R	4928 $\pm$ 205 <sup>&amp;</sup>	988 $\pm$ 78 <sup>#</sup>	42.20 $\pm$ 2.03 <sup>#</sup>

SOD, superoxide dismutase; CAT, catalase; TH, total hydroperoxide. Sham-operated, rats subjected to anaesthesia and laparotomy plus surgical manipulation; I/R, 90 min of ischaemia followed by 90 min of reperfusion; OzoneOP, ozone oxidative preconditioning; L-NAME, *N*<sup>ω</sup>-nitro-L-arginine methyl ester. Each value is the means  $\pm$  SEM from 10 rats. <sup>&</sup>, <sup>#</sup>Statistical difference of at least  $P < 0.05$  compared to the rest of the groups between the same column. \*No different from sham-operated.

Table 2. Glutathion concentrations in hepatic tissue in different experienced conditions

Experimental groups	GSH+GSSG ( $\mu\text{g/g}$ tissue)	GSH ( $\mu\text{g/g}$ tissue)	GSSG ( $\mu\text{g/g}$ tissue)	Ratio GSH/GSSG
Sham-operated	115.9 $\pm$ 14.2	76.8 $\pm$ 14.1	39.1 $\pm$ 14.5	1.96
Sham-operated + L-NAME	127.7 $\pm$ 12.7	77.8 $\pm$ 14.1	49.9 $\pm$ 11.4	1.55
I/R	170.7 $\pm$ 14.2 <sup>&amp;</sup>	32.3 $\pm$ 11.2 <sup>&amp;</sup>	138.4 $\pm$ 17.2 <sup>&amp;</sup>	0.23
OzoneOP + I/R	97.5 $\pm$ 18.2 <sup>#</sup>	60.5 $\pm$ 17.3 <sup>#</sup>	37.0 $\pm$ 19.1	1.63
OzoneOP + L-NAME + I/R	124.5 $\pm$ 9.5	45.9 $\pm$ 5.5 <sup>S</sup>	78.6 $\pm$ 13.5 <sup>#</sup>	0.58
L-NAME + I/R	156.9 $\pm$ 5.9 <sup>&amp;</sup>	12.4 $\pm$ 4.7 <sup>*</sup>	144.5 $\pm$ 7.1	0.086

GSH, reduced glutathione; GSSG, oxidized glutathione; sham-operated, rats subjected to anaesthesia and laparotomy plus surgical manipulation; I/R, 90 min of ischaemia followed by 90 min of reperfusion; OzoneOP, ozone oxidative preconditioning; L-NAME: *N*<sup>ω</sup>-nitro-L-arginine methyl ester. Each value is the means  $\pm$  SEM from 10 rats. <sup>&</sup>, <sup>#</sup>, <sup>S</sup>, <sup>\*</sup>Statistical difference of at least  $P < 0.05$  compared to the rest of the groups between the same column.

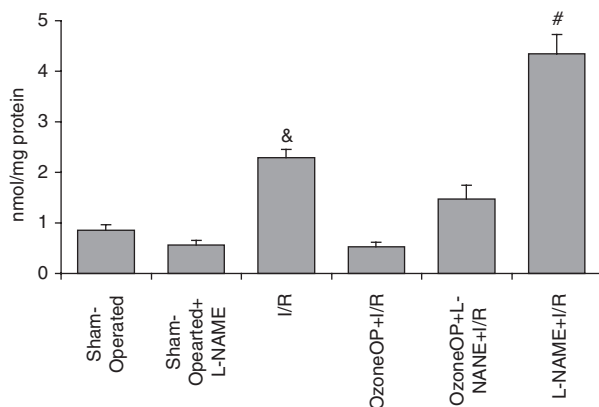


Fig 2. Hepatic tissue levels of malondialdehyde+4-hydroxyalkenals. Each value represents the mean  $\pm$  SEM from 10 rats. <sup>&</sup>, <sup>#</sup>Statistical significance of at least  $P < 0.05$  compared with the rest of the groups.

investigation. It has been suggested that protection depends on the release of substances by the organ helping to protect it against injury. NO is one of these mediators (7, 23).

The role of NO in liver I/R injury remains controversial (24). It has been suggested that the decrease of NO levels in liver may be caused by peroxynitrite formation by the reaction of NO with superoxide. Peroxynitrite is considered as a putative cytotoxin, which has been implicated in

the pathophysiology of a variety of processes including I/R injury (25). However, it has been reported that the inhibition of NOS activity reduces peroxynitrite formation, but aggravates liver injury and increases neutrophil accumulation, which suggests that the anti-inflammatory function of NO is more important than the cytotoxic potential of peroxynitrite in acute inflammation (26). Another crucial point is which isoform of NOS is activated. It has been suggested that systemic as well as local sources of iNOS regulate reperfusion, and local iNOS contributes to hepatic injury, while eNOS is protective in warm hepatic I/R (27). On the contrary, other studies have demonstrated the importance of both isoforms in liver protection. iNOS deficiency produces unanticipated genetic alterations that renders mice subjected to liver I/R injury more sensitive to liver I/R damage (28). The ability of eNOS and iNOS to protect the post-ischaemic liver in a murine model of hepatic I/R has been demonstrated; however, their mechanisms of action may be very different (29). The role of NO in rat hepatic I/R injury has been studied. I/R increased the activity of total NOS (tNOS) and iNOS, but not the eNOS activity. It was suggested that Kupffer cells might be the major source of the induction of iNOS

activity. An iNOS-specific inhibitor increased the lipid peroxidation and tissue damage in hepatic I/R injury but a NO donor increased the activity of iNOS and decreased the hepatic injury, thus NO production has a beneficial role in hepatic I/R injury (30).

There are different experimental results and opinions around NO generation and its function in liver I/R injury just as its participation in the protective effects. Nevertheless, the role of NO as a regulator of important processes in liver I/R is unquestionable.

There was a correspondence between transaminases, as markers of liver damage, and NO generation (Fig. 1A, B). OzoneOP regulated NO formation in the OzoneOP+I/R group and decreased the liver damage (increases in AST were prevented and those in ALT were attenuated). L-NAME is an inhibitor of NO synthesis. It was able to reduce NO generation in sham-operated+L-NAME, and NO levels were not detectable in the L-NAME+I/R group (Fig. 1B). Nevertheless, OzoneOP promoted NO formation in OzoneOP+L-NAME+I/R in spite of L-NAME's presence, but lesser than OzoneOP+I/R. There was a concomitant increase in transaminase activities in this group (OzoneOP+L-NAME+I/R). These results suggest that the protection conferred by OzoneOP against the damage in liver I/R seems to be mediated, at least in part, by NO generation.

The contribution of OzoneOP to NO generation may be a consequence of its actions on gene expression. Punjabi et al. (31) and Pendino et al. (32) have shown that exposure to ozone causes NO production in macrophages and type II cells of rat, whereas Haddad et al. (33) demonstrated iNOS induction in rats. More recently, it has been found that ozone-induced lung hyperpermeability is associated to iNOS and that iNOS mRNA levels are mediated through Tlr-4, which has been identified as the gene that determines susceptibility to endotoxin. There was a correlative pattern of gene expression in two strains (ozone-susceptible and ozone-resistant, respectively), which support a role of Tlr4 in the regulation of iNOS during ozone exposure in the mouse (34).

Ozone administration under our experimental conditions (15 days, low controlled doses administered by rectal insufflation) may prime and activate the genes associated to NOS expression, which promotes NO formation in the required concentrations for protecting against liver I/R injury.

Adenosine production is another mechanism that may explain OzoneOP contribution to NO formation. We had demonstrated that ozone

treatment was able to reduce ATP depletion after ischaemia. Adenosine was preserved and hypoxanthine and xanthine concentrations were reduced in comparison with the ischaemic group (ischaemia without any treatment). On the other hand, adenosine deaminase activity was maintained at the control level by OzoneOP (16).

Adenosine is a major component of vascular homeostasis playing an important role in regulating smooth muscle tone acting via cAMP-mediated cascades to induce vascular smooth muscle relaxation (35). It has been suggested that the protective effect of adenosine in hepatic I/R is a result of the prevention of eNOS downregulation within the hepatic sinusoidal cells, so adenosine may act as a potent preconditioning agent (36). Therefore, if OzoneOP increases adenosine levels, the available nucleoside may prevent the downregulation of eNOS and increase in NO generation. All these events are associated with protection against liver I/R injury. Also, the increase of adenosine by OzoneOP may prevent the processes resulting from activation of pro-inflammatory nuclear transcription factors, thereby exerting its protective effect. Recent experimental work has shown that adenosine prevents the activation of a potent pro-inflammatory nuclear transcription factor when it was administered prior to cardiac I/R (37). Adenosine has also been linked with mechanisms of activation of antioxidant enzymes. Ramkumar et al. (38) have proposed that an ischaemic insult increases the generation of adenosine derived from the utilization of ATP. Adenosine activates an adenosine receptor (possibly A<sub>3</sub> receptor subtype), which generates second messengers and activates kinases. It has been proposed that protein kinase C directly phosphorylates (and activates) antioxidant enzymes or phosphorylates a substrate that promotes activation of antioxidant enzymes. The net result of this process is a more efficient scavenging of ROS and a reduction in peroxidation of membrane lipids.

OzoneOP favoured antioxidant-pro-oxidant balance. It preserved the increase and ameliorated the rise of lipid peroxidation in OzoneOP+I/R and OzoneOP+L-NAME+I/R, respectively, in line with transaminase activities. These results indicate that the presence of lipid oxidative processes that promote liver damage are avoided or attenuated by OzoneOP. Inhibition of NO production (levels not detectable) in L-NAME+I/R correlated with the rise of lipid peroxidation, which was higher than that found in the I/R group, underlying the importance of NO when liver I/R damage has been induced. Glutathione

is a ubiquitous intracellular antioxidant that plays a key role in the defence against oxygen free radicals. The intracellular oxidation of GSH to GSSG is protective of enzyme sulphhydryl groups and vital membrane components (39). OzoneOP avoided 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, which suggest the preservation of membrane integrity by ozone treatment.

Injury in the I/R group may be explained when we analysed SOD, CAT and TH levels. A decrease in SOD activity was observed. It suggests a superoxide accumulation, which, in the presence of high levels of NO may promote the peroxynitrite formation. It should be pointed out that NO is the only known biological molecule generated in high enough concentrations under pathological conditions to compete and overcome the effects of endogenous SOD for superoxide (40). CAT activity increased in the above-mentioned group, but this increase was not enough to overcome TH concentrations. Our results are in accordance with those reported by Susuki et al. (41). They have demonstrated an increase of hydroperoxides in I/R injury of rat liver, and hydroperoxides levels were correlated with transaminases and ATP depletion. These results indicated that prolonged hepatic ischaemia with reperfusion produced bursts of oxygen-derived free radicals, which overwhelmed the defence mechanism of the cells, with a resultant decrease in energy charge associated with an increase in lipid peroxidation (40).

In summary, OzoneOP protected against liver I/R injury through mechanisms that promote a regulation of endogenous NO concentrations and the maintenance of an adequate cellular redox balance. Ozone treatment may have important clinical implications, particularly in view of the increasing hepatic transplantation programmes.

#### Acknowledgements

These studies were supported in part by Randox Laboratories (Antrim, UK) and the Department of Chemistry and Medical Biochemistry (University of Milan, Italy).

#### References

1. BILZER M, GERBER A L. Preservation injury of the liver: mechanisms and novel therapeutic strategies. *J Hepatol* 2000; 32: 508–15.
2. PERALTA C, BARTRONS R, SERAFÍN A, et al. Adenosine monophosphate-activated protein kinase mediates the protective effects of ischaemic preconditioning on hepatic ischaemia–reperfusion injury in rat. *Hepatology* 2001; 34: 1164–73.
3. CUTRÍN J C, LLESUG S, BOVERIS A. Primary role of Kupffer cell–hepatocyte communication in the expression of oxidative stress in the post-ischaemic liver. *Cell Biochem Funct* 1998; 16: 65–72.
4. DECKER K. Biologically active products of stimulated liver macrophages (Kupffer cells). *Eur J Biochem* 1990; 192: 245–61.
5. STEWART A G, BARKER J E, HICKEY M J, et al. Nitric oxide in ischaemia–reperfusion injury. In: *Ischaemia–Reperfusion Injury*. Oxford: Blackwell Science, 1999; 180–95.
6. MURRY C E, JENNINGS R B, REIMER K A. Preconditioning with ischaemia: a delay of lethal cell injury in ischaemic myocardium. *Circulation* 1986; 74: 1124–36.
7. PERALTA C, CLOSA D, HOTTER G, et al. Liver ischaemia preconditioning is mediated by the inhibitory actions of nitric oxide on endothelin. *Biochem Biophys Res Commun* 1996; 229: 264–70.
8. PERALTA C, HOTTER G, CLOSA D, et al. Protective effect of preconditioning on the injury associated to hepatic ischaemia–reperfusion in the rat: role of nitric oxide and adenosine. *Hepatology* 1997; 25: 934–7.
9. ROMERO A, MENÉNDEZ S, GÓMEZ M, et al. Ozone therapy in the advanced stages of arteriosclerosis obliterates. *Angiología* 1993; 45: 146–8.
10. MENÉNDEZ S, IGLESIAS O, BIDOT C, et al. Application of ozone in children with humoral immunity deficiency. In: *Proceedings of the 12th Ozone World Congress Ozone in Medicine*. Lille: International Ozone Association 1995; 271–74.
11. HERNÁNDEZ F, MENÉNDEZ S, WONG R, et al. Decrease of blood cholesterol and stimulation of antioxidative response in cardiopathy patients treated with endovenous ozone therapy. *Free Rad Biol Med* 1995; 19: 115–9.
12. LEÓN O S, MENÉNDEZ S, MERINO N, et al. Ozone oxidative preconditioning: a protection against cellular damage by free radicals. *Mediat Inflamm* 1998; 7: 289–94.
13. CANDELARIO-JALIL E, AL-DALAIN S M, LEÓN O S, et al. Oxidative preconditioning affords protection against carbon tetrachloride-induced glycogen depletion and oxidative stress in rats. *J Appl Toxicol* 2001; 21: 297–301.
14. AL-DALAIN S M, MARTÍNEZ G, CANDELARIO-JALIL E, et al. Ozone treatment reduces markers of oxidative and endothelial damage in an experimental diabetes model in rats. *Pharmacol Res* 2001; 44: 391–6.
15. PERALTA C, LEÓN O S, XAUS C, et al. Protective effect of ozone treatment on the injury associated with hepatic ischaemia–reperfusion: antioxidant–prooxidant balance. *Free Radical Res* 1999; 3: 191–6.
16. PERALTA C, XAUS C, BARTRONS R, et al. Effect of ozone treatment on reactive oxygen species and adenosine production during hepatic ischaemia–reperfusion. *Free Radical Res* 2000; 33: 595–605.
17. AJAMIEH H, MERINO N, CANDELARIO-JALIL E, et al. Similar protective effect of ischaemia and oxidative preconditioning in liver ischaemic/reperfusion injury. *Pharmacol Res* 2002; 45: 333–9.
18. GRANGER D L, TAINTOR R R, BOOCKVAR K S, et al. Determination of nitrate and nitrite in biological samples using bacterial nitrate reductase coupled with the Griess reaction. *Methods Companion. Meth Enzymol* 1995; 7: 78–83.
19. BOEHRINGER MANNHEIM Biochemica Information. A Revised Biochemical Reference Source. *Enzymes for Routine*, 1. Germany: Boehringer Mannheim, 1987; 15–16.
20. ANDERSON M E. Determination of glutathione and glutathione disulfide in biological samples. *Meth Enzymol* 1985; 113: 548–55.
21. TIETZE F. Enzymatic method for quantitative determination of nanogram amounts of total and oxidized glutathione:

- applications to mammalian blood and other tissues. *Anal Biochem* 1969; 27: 502–22.
22. BRADFORD M M. A rapid and sensitive method for the quantitation of micrograms quantities of protein utilizing the principle of protein–dye binding. *Anal Biochem* 1976; 72: 248–54.
  23. PERALTA C, RULL R, RIMOLA A, et al. Endogenous nitric oxide and exogenous nitric oxide supplementation in hepatic ischaemia–reperfusion injury in the rat. *Transplantation* 2001; 71: 529–36.
  24. RIVERA-CHÁVEZ F A, TOLEDO-PEREYRA L H, DEAN R E, et al. Exogenous and endogenous nitric oxide but not iNOS inhibition improves function and survival of ischaemically injured livers. *J Invest Surg* 2001; 14: 267–73.
  25. TUNKAY A D, CAKICI I, KANZIK I. Peroxynitrite a putative cytotoxin. *Pharmacol Toxicol* 1998; 82: 113–7.
  26. LIU P, YIN K, NAGELE A R, et al. Inhibition of nitric oxide synthase attenuates peroxynitrite generation but augments neutrophil accumulation in hepatic ischaemia–reperfusion in rats. *J Pharmacol Exp Ther* 1998; 284: 1139–46.
  27. LEE V G, JOHNSON M L, BAUST J, et al. The roles of iNOS in liver ischaemia–reperfusion injury. *Shock* 2001; 16: 355–60.
  28. HINES I N, HARADA H, BHARWANI S, et al. Enhanced post-ischaemic liver injury in iNOS-deficient mice: a cautionary note. *Biochem Biophys Res Commun* 2001; 284: 972–6.
  29. HINES I N, KAWACHI S, HARADA H, et al. Role of nitric oxide in liver ischaemia and reperfusion injury. *Mol Cell Biochem* 2002; 235: 229–37.
  30. HSU C M, WANG J S, LIU C H, et al. Kupffer cells protect liver from ischaemia–reperfusion injury by an inducible nitric oxide synthase-dependent mechanism. *Shock* 2002; 17: 280–5.
  31. PUNJABI C J, LASKIN J D, PENDINO K J, et al. Production of nitric oxide by rat type II pneumocytes: increased expression of inducible nitric oxide synthase following inhalation of a pulmonary irritant. *Am J Respir Cell Mol Biol* 1994; 11: 165–72.
  32. PENDINO K J, GARDNER C R, SHULER R L, et al. Inhibition of ozone-induced nitric oxide synthase expression in the lung by endotoxin. *Am J Respir Cell Mol Biol* 1996; 14: 516–25.
  33. HADDAD E B, LIU S F, SALMON M, et al. Expression of inducible nitric oxide synthase mRNA in Brown Norway rats exposed to ozone: effect of dexamethasone. *Eur J Pharmacol* 1995; 293: 287–90.
  34. KLEEBERGER S R, SEKHAR P M, ZHANG L Y, et al. Toll-like receptor 4 mediates ozone-induced murine lung hyperpermeability via inducible nitric synthase. *Am J Physiol Lung Cell Mol Physiol* 2001; 280: L326–33.
  35. STILES G L. Adenosine receptors and beyond: molecular mechanism of physiological regulation. *Clin Res* 1990; 38: 10–18.
  36. SERRACINO-INGLOTT F, IOANNIS T V, NAGY A H, et al. Adenosine preconditioning attenuates hepatic reperfusion injury in the rat preventing the downregulation of endothelial nitric oxide synthase. *BMC Gastroenterol* 2002; 2: 22–6.
  37. LI C, HA T, LIU L, et al. Adenosine prevents activation of transcriptional factor NF-κB and enhances activator protein-1 binding activity in ischaemic rat heart. *Surgery* 2000; 127: 161–9.
  38. RAMKUMAR V, NIE A, RYBAK L P, et al. Adenosine antioxidant enzymes and cytoprotection. *Trends Pharmacol Sci* 1995; 16: 283–5.
  39. MEISTER A. Glutathione. In: ARIAS I M, JAKOBY W B, POPPER H, et al. *The Liver: Biology and Pathology*, 2nd edn, Chapter 23. New York: Raven Press Ltd, 1988; 401–17.
  40. BECKMAN J S, KAPPENOL W H. Nitric oxide, superoxide and peroxynitrite: the good, the bad and the ugly. *Am J Physiol* 1996; 271: C1424–37.
  41. SUSUKI M, FUKUHARA K, UNNO M, et al. Correlation between plasma and hepatic phosphatidylcholine hydroperoxide, energy charge and total glutathione content in ischaemia–reperfusion injury of rat liver. *Hepatogastroenterology* 2000; 47: 1082–9.