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


Abstract: Background: Many studies indicate that oxygen free-radical formation after re-oxygenation of liver may initiate the cascade of hepatic cell 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 injury. Aim: 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 + t-NAME (N³-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 hepatic cellular injury; nitrite-nitrate levels as an index of NO production; superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GSH-Px) 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 was 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 a cellular redox balance. Ozone treatment may have important clinical implications, particularly in view of the increasing hepatic transplantation programs.

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,
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including reactive oxygen species (ROS) (3), some cytochrome P450 isoforms 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 synthase (NOS). The properties of NO appear to depend on which isofrom 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).

Ischemic preconditioning is an inducible and potent endogenous mechanism by which repeated episodes of brief ischemia 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 adenocine in the protection of the liver by ischemic preconditioning has been shown. The inhibition of NO abolishes the beneficial effect despite adenocine administration, whereas adenosine deaminase infusion plus NO administration failed to abolish the beneficial effect of preconditioning. These results suggest that the mechanism learned in the ischemic liver involves the release of adenocine, which induces the formation 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 adenocine and by blocking the xanthine/xanthine oxidase pathway for ROS generation (15, 16). More recently, a similar protective effect of ischemic 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 ischemic preconditioning (17).

Taking into account the role of NO in liver I/R injury and the protection conferred by ischemic and OzoneOPs, the aim of this study was to investigate the effects of OzoneOP on NO molecule generation and the antioxidant-prooxidant 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 anesthetized with urethane (1 g.kg. i.p.) and placed in a supine position on a heating pad in order to maintain body temperature of 36 °C and 37 °C. To induce hepatic ischemia, laparotomy was performed, and the blood supply to the right lobe of the liver was interrupted by placement of a bulldog clamp at the level of the hepatic artery and portal vein. Reperfusion 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 anesthesia and laparotomy plus surgical manipulation (excluding isolation of the right hepatic artery and vein vs. the left hepatic artery and vein without the induction of hepatic ischemia).

**Group 2. Sham-operated + L-NAME (N’’’-monomethyl-L-arginine methly ester) (n = 10):** Animals subjected to anesthesia and laparotomy plus surgical manipulation (as group 1) were treated with L-NAME (10mg.kg. i.v.) 10 min before laparotomy.

**Group 3. I/R (n = 10):** Animals subjected to 90 min of right lobe hepatic ischemia, 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 (O₂/O₃) 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 (O₂/O₃) 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+t-NAME+I/R (n = 10): Animals treated with ozone (as in group 4) were treated with t-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 preparation
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 12000g. 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 (München, Germany).

Markers of antioxidants/pro-oxidants balance in supernatant of liver homogenates
Nitrite/nitrate levels as a measure of NO generation were determined by the Griess reaction by first converting nitrites to nitrites using nitrate reductase (Boehringer Mannheim Italy SpA, Milan, Italy). Then the Griess reagent (1% sulfanilamide, 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 Hi-O₂/500 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-sulfosalicylic 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-886 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-hydroxyalkenal 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 ± 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 ischemia 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+t-NAME+I/R. Nevertheless, the ozone-protective effects were
Treatment of 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 respect 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 respect to the sham-operated group). The enzyme levels in this group increased compared with L-NAME+I/R (6936 ± 343 vs. 4928 ± 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, NO2/NO3 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 investigation.
Effects of ozone in ischemia-reperfusion

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

<table>
<thead>
<tr>
<th>Experimental groups</th>
<th>SDH activity (μg/g protein)</th>
<th>CAT activity (μg/g protein)</th>
<th>TH (μmol/g protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sham-operated</td>
<td>202 ± 1.26</td>
<td>2.6 ± 0.45</td>
<td>8.28 ± 0.03</td>
</tr>
<tr>
<td>Sham-operated + NAME</td>
<td>950 ± 130*</td>
<td>114 ± 40</td>
<td>9.79 ± 0.35</td>
</tr>
<tr>
<td>I/R</td>
<td>456 ± 324</td>
<td>142 ± 82*</td>
<td>11.37 ± 2.31*</td>
</tr>
<tr>
<td>OzoneN³ + I/R</td>
<td>830 ± 123*</td>
<td>66 ± 23*</td>
<td>10.43 ± 1.45*</td>
</tr>
<tr>
<td>OzoneN³ + NAME + I/R</td>
<td>6055 ± 345*</td>
<td>230 ± 23*</td>
<td>12.21 ± 1.24*</td>
</tr>
<tr>
<td>t-NAMe + I/R</td>
<td>462 ± 0.36*</td>
<td>88 ± 7.6*</td>
<td>12.83 ± 2.03*</td>
</tr>
</tbody>
</table>

SDH: superoxide dismutase; CAT, catalase; TH, total hydroperoxides. Sham-operated, rats subjected to anesthesia and laparotomy plus surgical manipulation; I/R, 90 min of ischemia followed by 90 min of reperfusion. OzoneN³, ozone oxidative preconditioning; t-NAMe, Acetyl-L-carnitine methyl ester. Each value is the mean ± SEM from 10 rats. *Significant difference at least P < 0.05 compared to the rest of the groups between the same columns. No different from sham-operated.

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

<table>
<thead>
<tr>
<th>Experimental groups</th>
<th>GSH+GSSG (μmol/g tissue)</th>
<th>GSH (μmol/g tissue)</th>
<th>GSSG (μmol/g tissue)</th>
<th>Ratio GSH/GSSG</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sham-operated</td>
<td>115.9 ± 14.2</td>
<td>76.8 ± 14.1</td>
<td>24.0 ± 14.3</td>
<td>1.55</td>
</tr>
<tr>
<td>Sham-operated + NAME</td>
<td>127.9 ± 12.7</td>
<td>85.3 ± 11.3*</td>
<td>47.3 ± 11.4</td>
<td>1.60</td>
</tr>
<tr>
<td>I/R</td>
<td>137.9 ± 14.2*</td>
<td>103.9 ± 14.2*</td>
<td>33.2 ± 11.4</td>
<td>1.55</td>
</tr>
<tr>
<td>OzoneN³ + I/R</td>
<td>80.5 ± 18.2*</td>
<td>60.5 ± 17.2*</td>
<td>25.4 ± 18.1</td>
<td>1.84</td>
</tr>
<tr>
<td>OzoneN³ + NAME + I/R</td>
<td>124.9 ± 5.9</td>
<td>45.9 ± 4.3*</td>
<td>78.5 ± 13.5*</td>
<td>0.58</td>
</tr>
<tr>
<td>t-NAMe + I/R</td>
<td>196.9 ± 6.8*</td>
<td>124 ± 4.7*</td>
<td>72.5 ± 7.1</td>
<td>0.86</td>
</tr>
</tbody>
</table>

GSH, reduced glutathione; GSSG, oxidized glutathione; sham-operated, rats subjected to anesthesia and laparotomy plus surgical manipulation; I/R, 90 min of ischemia followed by 90 min of reperfusion. OzoneN³, ozone oxidative preconditioning; t-NAMe, Acetyl-L-carnitine methyl ester. Each value is the mean ± SEM from 10 rats. *Significant difference at least P < 0.05 compared to the rest of the groups between the same columns.

Fig. 2. Hepatic tissue levels of malondialdehyde + 4-hydroxyalkenals. Each value represents the mean ± SEM from 10 rats.

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 systems 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 unexpected 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-ischemic 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 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 differences between experiments and opinions about 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+1/R group and decreased the liver damage (increase 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+1/R group and NO levels were undetectable in the L-NAME+1/R group (Fig. 1B). Nevertheless, OzoneOP promoted NO formation in OzoneOP+L-NAME+1/R in spite of L-NAME’s presence, but lower than OzoneOP+1/R. These results are consistent with the transaminase activities in this group (OzoneOP+L-NAME+1/R). These results suggest 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. Punjani 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 Hashid 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 Th1, which has been identified as the gene that determines susceptibility to endotoxin. There was a correlation pattern of gene expression in two strains (ozone-susceptible and ozone-resistant, respectively), which support a role of Th1 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 instillation) 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 ischemia. Adenosine deaminase, hypoxanthine and xanthine concentrations were reduced in comparison with the ischemic group (ischemia without any treatment). On the other hand, adenosine deaminase activity was maintained at the control level by OzoneOP (16). 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 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. Recently, we have proved that adenosine prevents the activation of a potent pro-inflammatory nuclear transcription factor when it was administered to cardiac I/R (37). Adenosine has also been linked with the mechanisms of activation of antioxidant enzymes. Ramakumar et al. (38) have proposed that an ischemic insult increases the generation of adenosine derived from the utilization of ATP. Adenosine activates an adenosine receptor (possibly A2 receptor subtypes), 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+1/R and OzoneOP+L-NAME+1/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+1/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
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