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 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\textsuperscript{o}-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 formation; 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.

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

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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, laparatomy was performed, and the blood supply to the right lobe of the liver was interrupted by placement of a bulldog clamp at the level of the hepatic artery and portal vein. Reflow was initiated by removing the clamp (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 laparatomy 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°-nitro-L-arginine methyl ester)** (n = 10): Animals subjected to anaesthesia and laparatomy 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 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 (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 (O₃/O₂) 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 (Munchen, 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% 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 H₂O₂-560 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 ± 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
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 NO2-/NO3- 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 NO2-/NO3- 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 NO2-/NO3- 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 ± 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
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Table 1. SOD and CAT activities and TH concentrations in hepatic tissue

<table>
<thead>
<tr>
<th>Experimental groups</th>
<th>SOD activity (U/g protein)</th>
<th>CAT activity (U/g protein)</th>
<th>TH (µmol/g protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sham-operated</td>
<td>7952 ± 296</td>
<td>215 ± 45</td>
<td>8.28 ± 0.80</td>
</tr>
<tr>
<td>Sham-operated + L-NAME</td>
<td>7485 ± 150</td>
<td>174 ± 40</td>
<td>5.70 ± 0.35</td>
</tr>
<tr>
<td>I/R</td>
<td>4566 ± 374</td>
<td>764 ± 53†</td>
<td>42.73 ± 2.31†</td>
</tr>
<tr>
<td>OzoneOP + I/R</td>
<td>8013 ± 123</td>
<td>282 ± 27</td>
<td>11.40 ± 1.46</td>
</tr>
<tr>
<td>OzoneOP + L-NAME + I/R</td>
<td>6936 ± 343</td>
<td>238 ± 33</td>
<td>12.21 ± 1.24</td>
</tr>
<tr>
<td>L-NAME + I/R</td>
<td>4928 ± 205†</td>
<td>988 ± 78†</td>
<td>42.20 ± 2.03†</td>
</tr>
</tbody>
</table>

SOD, superoxide dismutase; CAT, catalase; TH, total hydroperoxide. Sham-operated, rats subjected to anaesthesia and laparatomy plus surgical manipulation; I/R, 90 min of ischaemia followed by 90 min of reperfusion; OzoneOP, ozone oxidative preconditioning; L-NAME, Nω-nitro-l-arginine methyl ester. Each value is the means ± SEM from 10 rats. ‡, †, 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

<table>
<thead>
<tr>
<th>Experimental groups</th>
<th>GSH + GSSG (µg/g tissue)</th>
<th>GSH (µg/g tissue)</th>
<th>GSSG (µg/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>39.1 ± 14.5</td>
<td>1.96</td>
</tr>
<tr>
<td>Sham-operated + L-NAME</td>
<td>127.7 ± 12.7</td>
<td>77.8 ± 14.1</td>
<td>49.9 ± 11.4</td>
<td>1.55</td>
</tr>
<tr>
<td>I/R</td>
<td>170.7 ± 14.2§</td>
<td>32.3 ± 11.2§</td>
<td>138.4 ± 17.2§</td>
<td>0.23</td>
</tr>
<tr>
<td>OzoneOP + I/R</td>
<td>97.5 ± 18.2§</td>
<td>60.5 ± 17.3§</td>
<td>37.0 ± 19.1</td>
<td>1.63</td>
</tr>
<tr>
<td>OzoneOP + L-NAME + I/R</td>
<td>124.5 ± 9.5</td>
<td>45.9 ± 5.5§</td>
<td>78.6 ± 13.5§</td>
<td>0.58</td>
</tr>
<tr>
<td>L-NAME + I/R</td>
<td>156.9 ± 5.9‡</td>
<td>12.4 ± 4.7*</td>
<td>144.5 ± 7.1</td>
<td>0.086</td>
</tr>
</tbody>
</table>

GSH, reduced glutathione; GSSG, oxidized glutathione; sham-operated, rats subjected to anaesthesia and laparatomy plus surgical manipulation; I/R, 90 min of ischaemia followed by 90 min of reperfusion; OzoneOP, ozone oxidative preconditioning; L-NAME, Nω-nitro-l-arginine methyl ester. Each value is the means ± SEM from 10 rats. ‡, §, *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-hydroxynonenals. Each value represents the mean ± SEM from 10 rats. ‡, #Statistical significance of at least P<0.05 compared with the rest of the groups.](image)

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.
L-NAME is an inhibitor of NO synthesis. It was rated able to reduce NO generation in sham-operated and L-NAME’s presence, but lesser than OzoneOP+I/R. 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 correla-
tive 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 A3 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 ital membrane components (39). OzonoOP 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, OzonoOP 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

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