Effects of ozone oxidative preconditioning on nitric oxide generation and cellular redox balance in a rat model of hepatic ischemia/reperfusión

Olga Sonia León Fernández[1], Hussan H Ajamieh[1], Gregorio Martínez-Sanchez[1], Eduardo Candelario-Jalil[1], Anna M Carmona[2], Silvia Menéndez[3], Lamberto Re[4], Attilia Giuliani[5]

[1]Center for Research & Biological Evaluation (CIEB-IFAL). University of Havana, Havana City 10 400, Cuba.
[2]Faculty of Pharmacy, Univ. of Barcelona, Barcelona, Spain.
[3]Ozone Research Center. PO Box 6412, Havana City, Cuba.
[4]Institute of Experimental and Clinical Medicine, Laboratory of Pharmacology. University of Ancona. Via Ranieri 2, 60131, Ancona, Italy.
[5]Department of Chemistry and Medical Biochemistry, University of Milan, Via Saldini, 50-20133 Milan, Italy.

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Abstract

Background: Liver transplantation is now accepted as the best treatment for end-stage liver disease. Nevertheless, hepatic ischemia-reperfusion (I/R) injury associated with liver transplantation and hepatic resection are an unresolved problem in clinical practice. Many studies indicate that oxygen free-radical formation after reoxygenation of liver may initiate the cascade of hepatocellular injury.

Aim: In the present study, the effects of Ozone Oxidative Preconditioning (OzoneOP) on nitric oxide ($^{\cdot}$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^{ω}-nitro-L-arginine methyl ester); (3) I/R (ischemia 90 minutes + reperfusion 90 minutes); (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 levels and inducible Nitric Oxide Sintase (iNOS) by immunohistochemistry as an index of $^{\cdot}$NO production; superoxide dismutasa (SOD), catalase (CAT) and glutathione levels as markers of endogenous antioxidant system, and finally malondialdehyde + 4-hydroxyalkenals (MDA+4-HDA), total hydroperoxides (TH) and Tumor Necrosis Factor (TNF-α) as indicators of oxidative stress.

Results: A correspondence between liver damage and the increase of $^{\cdot}$NO, CAT, TH, glutathione and MDA + 4HDA concentrations were observed just as a decrease of SOD activity. OzoneOP prevented and attenuated hepatic damage in OzoneOP +I/R and OzoneOP+L-NAME+I/R, respectively, in close relation with the above-mentioned parameters. Immunohistochemistry of iNOS showed that OzoneOP regulated enzymatic activity while TNF-α levels were not detected in OzoneOP + I/R group.

Conclusions: These results show that OzoneOP protected against liver I/R injury through mechanisms that promote a regulation of endogenous $^{\cdot}$NO concentrations and maintenance of cellular redox balance. Ozone treatment may have important clinical implications, particularly in view of the increasing hepatic transplantation programs.

Introduction

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 the 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 [3-5].

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 ischaemia-reperfusion injury [6]. Although the mechanism of preconditioning is not yet known, some hypotheses have been tested. The results have 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 therapeutical 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 which, in turn, increases antioxidant endogenous systems protecting against liver and pancreas damage [12-14].

Taking into account the role of NO in liver I/R injury and the protection conferred by ischaemic and ozone oxidative preconditionings, the aim of this study was to assay the effects of OzoneOP on NO molecule generation and the relation of this with the antioxidant-prooxidant balance in a model of liver I/R in rats.

Material and 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 guidelines established by the Canadian Council on Animal Care.

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 ^{o}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 between 36-37 ^{o}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 anesthesia 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 (Nω-nitro-L-arginine methyl ester), (n=10): Animals subjected to anesthesia and laparatomy plus surgical manipulation (as group 1) were treated with L-NAME (10 mg/kg intravenously) 10 min before laparatomy. 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 build-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 is 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 intravenously) 10 min before the I/R procedure. Group 6. L-NAME + I/R (n=10): Animals treated with L-NAME (10 mg/kg intravenously) 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 at 4 ^{o}C and centrifuged for 10 min at 12 000 x 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 kits from Boehringer Mannheim (Munchen, Germany).

Markers of antioxidant-prooxidant 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 Mannheimm Italy SpA, Milan, Italy) [18]. 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 sec intervals for one min [19]. Quantification of total hydroperoxides (TH) was measured by Bioxytech H_2O_2-560 kit (Oxis International Inc., Portland, OR, USA) using xylenol orange to form a stable colored complex, which can be measured at 560 nm. Reduced and oxidized glutathione (GSH and GSSG respectively) were measured enzymatically in 5- sulphosalycilic 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 analyzed using the LPO-586 kit obtained from Calbiochem (La Jolla, CA). Total protein were determined using the method described by Bradford [22] and analytical grade bovine serum albumin was used to establish a standard curve.

Statistical analysis

The statistical analysis was started by using the OUTLIERS preliminary tests for 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 $p < 0.05$.

Result and Discussion

Effects of OzoneOP on hepatic injury:As shown in Figure 1 (A), the degree of hepatic damage induced by 90 min of ischaemia and 90 min of reperfusion significantly ($p<0.05$) increase 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.

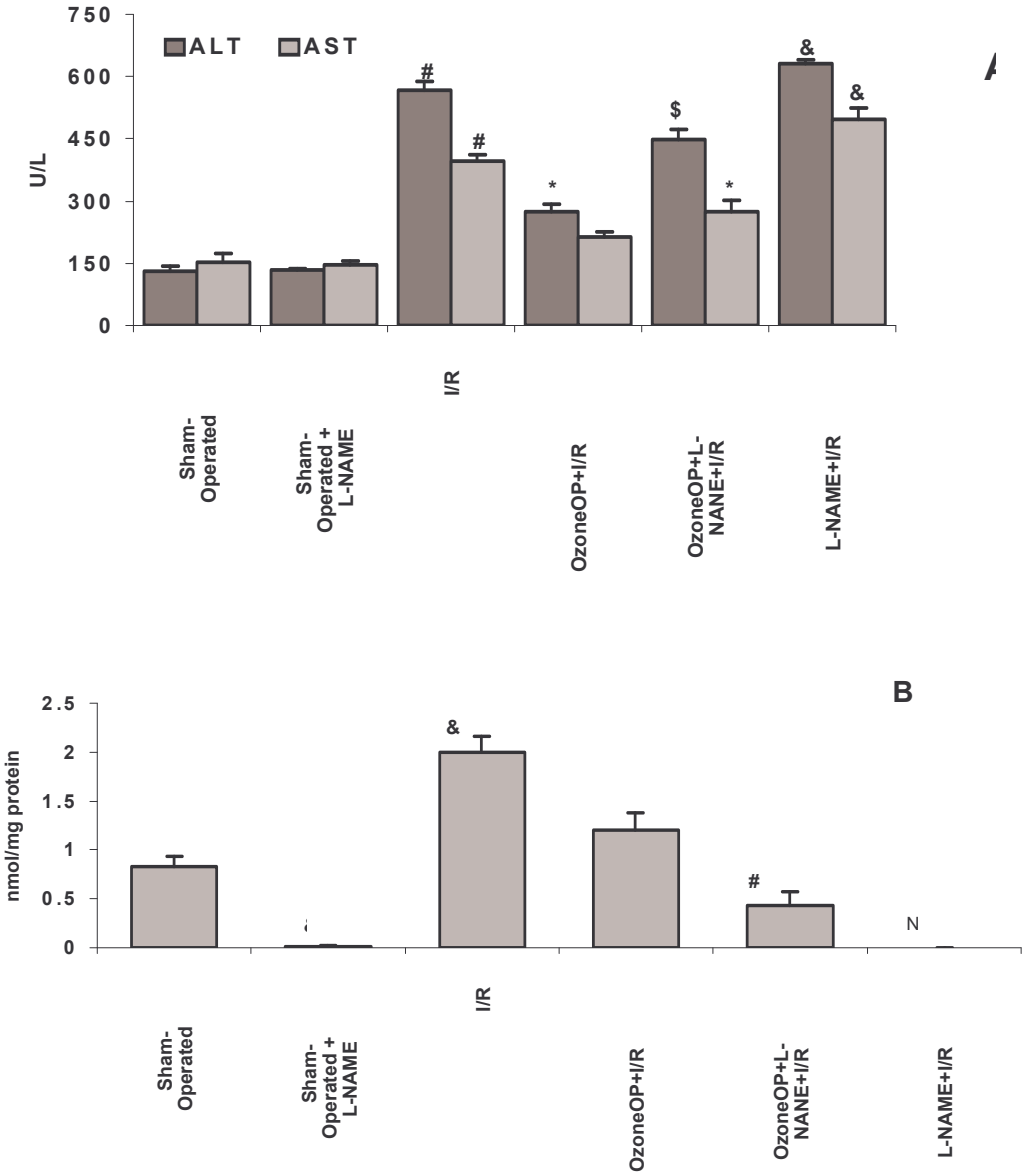

Fig. 1 (A) Plasma activities of aspartate aminotransferase (AST) and alanine aminotransferase (ALT); (B) Hepatic tissue levels of nitrite/nitrate (NO^-_2/NO^-_3). I/R: 90 minute of ischaemia followed by 90 min of reperfusion; OzoneOP: Ozone Oxidative Preconditioning; L-NAME: Nω-nitro-L-arginine methyl ester. Each value is the mean ± SEM from 10 rats. ND, not detectable levels. &,#,,$ Statistical significance of at least p < 0.05 compared with the rest of the groups.*

OzoneOP actions on NO^-_2/NO^-_3 generation: Figure 1 (B) shows the effects of ischaemia and treatments on NO generation. NO increased in I/R compared to all treatments. In the group only subjected to I/R, the NO levels reached the maximal values as compared to all other treatment. OzoneOP (OzoneOP + I/R) afforded complete protection against the marked increased in NO^-_2/NO^-_3 concentrations induced by the I/R episode as these were no statistical differences between

OzoneOP + I/R and sham-operated control group. The inhibition of NO synthesis by L-NAME decreased NO^-_2/NO^-_3 levels in the presence of OzoneOP (OzoneOP + NAME + I/R). When animals were not preconditioning with ozone (OzonoOP) the L-NAME treatment (L-NAME + I/R) completely abolish (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. 1(B).

OzoneOP on the antioxidant-prooxidant balance in liver ischaemia-reperfusion: The effects of OzoneOP on SOD and CAT activities and TH concentrations are shown in Table 1. 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. 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 sham-operated). The enzyme levels in this group increased compared to L-NAME + I/R (6936 ± 343 vs. 4928 ± 205 U/mg protein respectively).

Table 1. Superoxido dismutase (SOD) and Catalase (CAT) activities and Total Hydroperoxides concentrations in hepatic tissue

Experimental Groups	SOD Activity (U/g protein)	CAT Activity (U/g protein)	Total hydroperoxides (μmol/g protein)
Sham-Operated	7952±296	215±45	8.28 ±0.80
Sham-Operated+L-NAME	7485±150.05$^{\#*}$	174±40	5.70±0.35
I/R	4566±374$^{\&}$	764±53$^{\&}$	42.73±2.31$^{\&}$
OzoneOP+I/R	8013±123	282±27	11.40±1.46
OzoneOP+L-NAME+I/R	6936±343$^{\#}$	238±33	12.21±1.24
L-NAME+I/R	4928±205$^{\&}$	988±78$^{\#}$	42.20±2.03$^{\#}$

SOD, superoxide dismutase; CAT, catalase; TH, Total hydroperoxide. Sham Operated: rats subjected to anesthesia 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 group between the same column. * No different from Sham operated.

TH were maintained at sham-operated levels in OzoneOP + I/R, OzoneOP + L-NAME + I/R and sham-operated + L-NAME groups. However, there was an increase of this ROS in I/R and L-NAME + I/R. Results for total glutathione (GSH+GSSG) concentrations are shown in Table 2. It was observed a depletion of reduced glutathione (GSH) and an increase of oxidized glutathione

(GSSG) in I/R and L-NAME + I/R groups. OzoneOP prevented (OzoneOP + I/R) or attenuated (OzoneOP + L-NAME + I/R) the GSH depletion and the GSSG increment, respectively.

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

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

Note: GSH, reduced glutathione; GSSG, oxidized glutathione. Sham Operated: rats subjected to anesthesia 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 group between the same column.

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 than the remainder groups. MDA + 4-HDA is an index of lipid oxidation. The results of these parameters are shown in Figure 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 to all experimental groups including I/R.

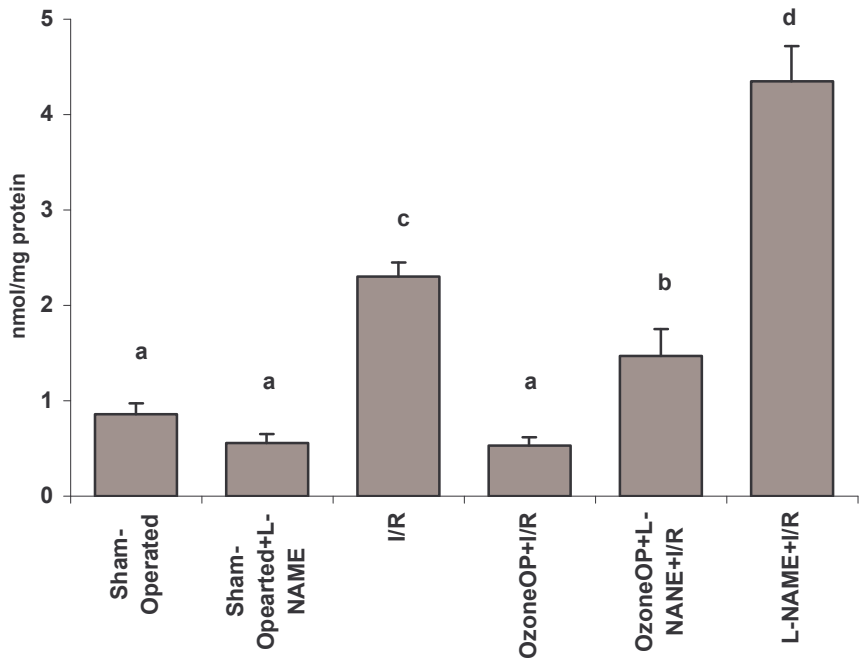

Fig 2. Hepatic tissue levels of Malondialdehyde + 4 Hydroxyalkenals. Each value represent the mean ± SEM from 10 rats. Different letters indicate a statistical significance of at least $p < 0.05$.

In a similar way to parameters as transaminases, NO^-_2/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. The mechanisms underlying preconditioning remains unknown and is currently under intense investigation. It has been suggested that protection depends on the release of substances by the organ helping to protect it against injury. NO is one of these mediators [7,23].

There was a correspondence between transaminases, as markers of liver damage, and NO generation (Fig. 1 A,B). OzoneOP regulated NO formation in OzoneOP + I/R group and decreased the liver damage (increases in AST was prevented and those in ALT was 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 L-NAME + I/R group (Fig. 1B). Nevertheless, OzoneOP promoted NO formation in OzoneOP + L-NAME + I/R in spite of L-NAME 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 at*. and Pendino *et at*. have shown that exposure to ozone causes NO production in macrophages [24] and type II cells [25] of rat, whereas Haddad *et al.* [26] demonstrated iNOS induction in rats. More recently it has been found that ozone-induced lung hyperpermeability is associated to iNOS and that NOS_2mRNA levels are mediated through Tlr-4 which has been identified as the gene that determines susceptibility to endotoxin. There was a correlative patterns of gene expression in two strains (ozone-susceptible and ozone-resistant respectively) which support a role of Tlr4 in the regulation of NOS_2 during ozone exposure in the mouse [27].

Ozone administration in 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 which 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 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 [28] 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 [29] Therefore, if OzoneOP rise adenosine, the available nucleoside may prevent the downregulation of eNOS and increase NO generation. All these events are associated to protection against liver I/R injury. Also, the increase of adenosine by OzoneOP may avoid the processes resulting from activation of proinflammatory nuclear transcription factors, thereby exerting its protective effect. Recent experimental work has shown that adenosine prevents the activation of a potent proinflammatory nuclear transcription factor when it was administered prior to cardiac I/R [30] Adenosine has been also linked with mechanisms of activation of antioxidant enzymes. Ramkumar *et al*. have proposed that an ischaemic insult increases the generation of adenosine derived from utilization of ATP. Adenosine activates an adenosine receptor (possibly A_3 receptor subtype) which generate second messengers and activates kinases. It has been proposed that protein kinase C directly phosphorylates (and activates) antioxidant enzymes or phosphorylate a substrate which promote 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 [31].

OzoneOP favored antioxidant-prooxidant 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 which promote liver damage are avoided or attenuated by OzoneOP. NO inhibition (levels not detectable) in L-NAME + I/R correlated with the rise of lipid peroxidation which was higher than found in the I/R group, underlying the importance of NO when liver I/R damage has been induced. Glutathione is an ubiquitous intracellular antioxidant that has a key role in the defense against oxygen free radicals. The intracellular oxidation of GSH to GSSG is protective of enzyme sulphydryl groups and vital membrane components [32]. 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.

Conclusion

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

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