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Research Paper

Ozone oxidative post-conditioning in acute renal failure

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Abstract

Objectives The ischaemia–reperfusion process is largely mediated by reactive oxygen species. Taking into account that a transient and controlled administration of ozone is able to upregulate cellular antioxidant enzymes, a morphological, biochemical and functional renal study was performed in rats undergoing warm renal ischaemia.

Methods Rats were divided into four groups. All except the negative controls underwent 60 min' bilateral renal ischaemia followed by 10 days' reperfusion. The positive control group received no further treatment. The ozone group received an ozone/oxygen mixture (ozone dose 0.5 mg/kg) immediately after the ischaemia and daily for the 10 days' reperfusion; the oxygen group were given the same concentration of oxygen alone (13 mg/kg). Biochemical parameters fructosamine, phospholipase A_2, catalase, superoxide dismutase and thiobarbituric acid reactive substances were measured, as well as renal plasma flow and glomerular filtration rate.

Key findings Renal plasma flow and glomerular filtration rate decreased significantly in the positive controls and the oxygen group whereas values in the ozone group were similar to those in the negative control group. With respect to the biochemical parameters, ozone maintained a homeostasis redox, with significant increases in catalase and superoxide dismutase activities and similar values for phospholipase A_2 and fructosamine compared with the negative control group. Fewer morphological alterations were seen in kidneys from the ozone group. No advantages were obtained in the positive control and oxygen groups.

Conclusions The protective effect of ozone may be explained by upregulation of the antioxidant defence system and beneficial effects on blood circulation and in oxygen metabolism. Ozone treatment may represent a therapeutic approach for minimising renal damage after transplantation.

Keywords ozone therapy; reactive oxygen species; reperfusion; superoxide dismutase; warm ischaemia

Introduction

Changes that occur during ischaemia affect cells and provide the basis for increased damage during reperfusion. One of the characteristics of cells submitted to ischaemia–reperfusion is that they can recover and have normal function if the damage is not lethal (no lesions in the tubular basal membrane) or where attempts are made to stop the routes of this damage.[1]

Renal vasoconstriction and tubular dysfunction have been clearly defined as the most important mechanisms that reduce the glomerular filtration rate (GFR) after ischaemia–reperfusion.[1] Decreases in renal blood flow of 40–50% have been reported after renal ischaemia, in experimental models and in humans. This decrease provokes an increase in shearing force in the endothelium, as well as of neutrophils and reactive oxygen species (ROS). All these effects increase endothelial damage, favouring the maintenance of vasoconstriction and therefore decreasing GFR.[2,3]

At present, a protagonist role has been attributed to free radicals, in that increasing pro-oxidant substances produces an imbalance with antioxidant substances, creating oxidative stress.[4,5] The main oxidative products in biological systems are the ROS which include superoxide anions, hydrogen peroxide, hydroxyl radicals, singlet oxygen and hypochlorous

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acid.[6,7] ROS are implicated in renal damage caused by ischaemia–reperfusion.[3–5] When ROS are increased and not neutralised by antioxidant substances, they are capable of inducing lipid peroxidation in membranes. They can react with unsaturated fatty acids, provoking structural and functional modifications. In addition, ROS oxidise the lateral chains of amino acid residues and stimulate the formation of protein–protein cross bonds, as well as the oxidation of the protein skeleton, resulting in fragmentation.

Some researchers have tried to neutralise ROS damage using antioxidant enzymes such as catalase, superoxide dismutase (SOD) and glutathione peroxidase, or allopurinol (xanthine oxidase inhibitor) and deferroxamine (inhibitor of the Fenton reaction). They obtained reductions in cell damage and organ dysfunction caused by ischaemia–reperfusion, proving the role of oxidative stress in the ischaemia–reperfusion process.[7–9] In a rat model of hepatic ischaemia–reperfusion, ozone corrected the chronic oxidative stress by upregulating the antioxidant system, achieving a homeostasis redox.[10–12]

Repeated non-lethal cell stress has been demonstrated to give protection to a later severe stress, as occurs in ischaemic, thermal and chemical preconditioning,[13–15] as well as in ozone oxidative preconditioning.[10–12,16–20]

A comparison between ischaemic and ozone oxidative preconditioning showed no biochemical difference among the antioxidant/pro-oxidant enzymes measured. Nevertheless, the histological study demonstrated that the protective effect produced by the ozone oxidative preconditioning was superior to that achieved with ischaemic preconditioning.[17]

Recent studies have also demonstrated that ischaemic post-conditioning during reperfusion inhibits myocardial injury (highlighting the myocardial reperfusion phase as a target for cardioprotection).[21,22] Clinical trials using ozone oxidative post-conditioning in cardiopathy and in diabetic patients have demonstrated control of oxidative stress, improvements in the antioxidant defence system and satisfactory clinical responses.[23,24]

Taking into account that ischaemia–reperfusion is a process largely mediated by ROS generation and that a controlled and judicious administration of ozone is able to stimulate the endogenous antioxidant systems,[10–12,16–20] and also to enhance blood circulation and oxygen metabolism,[6] among other beneficial effects, the aim of this study is to evaluate the efficacy of ozone oxidative post-conditioning (applied after the ischaemia) on renal morphology, function and biochemical parameters in rats undergoing a warm renal ischaemia.

Materials and Methods

Animals and sample preparation

Forty adult male Wistar rats (250–260 g) were maintained in an air-filtered and temperature-conditioned room (20–22°C), relative humidity 50–52%. Rats were fed with a standard commercial diet and water *ad libitum.*

Experiments were conducted in accordance with the ethical guidelines established by the Principles of Laboratory Animal Care (NIH publication No. 86-23, revised 1985) and were approved by the Ethical Committee for Animal Experimentation of the National Center for Scientific Research, Havana, Cuba.

Ozone was generated using OZOMED equipment (Ozone Research Center), from medical-grade oxygen by means of a silent electric discharge, representing about 3% of the gas mixture (ozone + oxygen). The schedule and ozone dosing used have been demonstrated to be optimal in previous studies.[10–12,16–20]

Under constant sodium pentobarbital anaesthesia, after a renal ischaemia of 60 min, we allowed a reperfusion period of 10 days. Heparin (50 U) was administered subcutaneously before induction of ischaemia. Within 10 min of the end of the reperfusion period we measured renal plasma flow (RPF) and GFR by means of plasma clearance of p-amino-hippurate (PAH) and inulin, respectively. A constant plasma concentration of both substances was used (2 mg PAH and 20 mg inulin in 100 ml saline), given by continuous perfusion through the left femoral vein at a rate of 0.15 ml/min, after a loading dose of 0.8 ml PAH (12 mg/mJ) and 0.8 ml inulin (2 mg/mJ). For these analyses, blood was withdrawn by intracardiac puncture and urine was collected from the bladder. Rats were then euthanised under deep anaesthesia.

Representative samples of different kidney portions were taken for histopathological studies and preparation of tissue homogenates. Kidney homogenates were obtained using a Edmund Bühler LBMA tissue homogenator at 4°C. The homogenates were prepared in 50 mM KCI/histidine buffer pH 7.4, 1 : 10 (w/v) and were centrifuged at 8500*g* at 4°C for 20 min. The supernatants were used for biochemical determinations.

Treatment schedule and renal ischemia

Animals were divided into four groups of 10 animals. All animals were anaesthetised with sodium pentobarbital (30 mg/kg i.p.) and were given 50 IU heparin by subcutaneous injection. A laparotomy was then performed. The negative control group underwent sham exposure of the kidneys, followed by resuturing of the abdominal wall, allowing normal renal function for the next 10 days. The other three groups of rats were submitted to bilateral renal ischaemia by cross-clamping of both renal arteries for 60 min. Reperfusion thus occurred over the next 10 days, after which the morphological, functional and biochemical renal study was done. The positive control (ischaemia) group received no further treatment. The ozone group were treated with the ozone/oxygen gas mixture, administered by rectal insufflation performed with a polyethylene cannula in a volume of 2.5–2.6 ml; the ozone concentration was 50 μg/ml (representing a dose of 0.5 mg/kg weight). Administration was started immediately after the 60 min' renal ischaemia and was repeated once daily during the 10-day reperfusion period. The oxygen group were treated in the same way as the ozone group, but insufflated with oxygen alone (13 mg/kg). This group was included to determine the effect of the oxygen in the oxygen/ozone gas mixture.

Biochemical determinations

Levels of PAH and inulin were determined in plasma and urine samples deproteinated by cadmium sulfate. PAH was measured

using a photocolorimetric[25] technique as modified by Smith and Tinkelstein.[26] Inulin was measured by the direct method of resorcinol without alkaline treatment.[27] Plasma creatinine concentrations were measured in deproteinated filtrates using the sodium tungstate method[28] and evaluated by the method of Brot *et al.*[29] Absorption was measured at 160 nm.

Kidney homogenates were assayed for total SOD (Cu/Zn and Mn SOD) activity by determining the capacity of the enzyme to inhibit the autoxidation of pyrogallol by 50%.[30]

Catalase activity was determined according to the method of Rice and Diplock[31] and the absorbance was measured at 240 nm for 30 s in the spectrophotometer.

A lipid peroxidation assay was used to estimate thiobarbituric acid-reactive substances (TBARS) levels, as described by Ohkawa *et al.*,[32] where the absorbance of 3 ml of the coloured layer was measured spectrophotometrically at 532 nm, using 1,1,3,3-tetraethoxypropane as the standard.

Phospholipase A_2 activity was determined according to a standard procedure;[33] enzymatic activities are expressed as U/g protein.

Protein concentrations were determined by the method of Lowry *et al.*[34] using bovine serum albumin as standard.

Fructosamine was determined by means of a colorimetric procedure.[35] Values represent the difference in units of optical density per g renal tissue.

Histological study

Samples of kidneys from the different groups were fixed in neutral 10% formalin, processed and embedded in paraffin. The histological sections, stained with haematoxylin and eosin, were examined by a pathologist who was unaware of the treatment schedule.

Statistical analysis

First, the outliers preliminary test for detection of error values was performed. One-way analysis of variance was then used, followed by the Bartlett–Box homogeneity variance test. In addition, a multiple comparison test was used (Duncan test). Comparisons between two groups were done using the Student's *t*-test. Differences between groups in the histological study were evaluated with a non-parametric test (Fisher's test).

Results are presented as means ± SD. $P < 0.05$ was considered significant.

Results

Renal function

Table 1 shows the effects of treatments on renal function. RPF showed significant differences between the negative control group and the other groups. Values were lower in the positive control and oxygen groups than in the negative control and ozone groups, with significant differences between them. Values were lower in the ozone group than in the negative control group. Results for GFR showed a similar pattern.

With respect to plasma creatinine concentration, values were higher in the positive control and oxygen groups than in the negative control and ozone groups. Differences between the positive control and oxygen groups, and between the negative control and ozone groups, were not significant.

These results demonstrated a deterioration in renal function and that no protection occurs in the positive control and oxygen groups. Thus, oxygen does not favour recovery of renal function. However, ozone oxidative post-conditioning preserved renal function, maintaining values for RPF, GFR and creatinine concentration almost at levels in the negative control group.

Antioxidant–pro-oxidant balance and protein oxidation

Table 2 shows the behaviour of the different parameters relating to redox balance in renal tissue. With respect to TBARS, an indicator of lipid peroxidation, the values were significantly higher in the oxygen group than in the other groups. Values in the ozone and positive control groups were similar, and were significantly higher than in the negative control group. With regard to SOD, a scavenger of superoxide anions, enzymatic activity was significantly lower in the positive control group than in the other groups. A highly significant increase in SOD was found in the ozone group. Catalase showed similar behaviour to SOD. With respect to fructosamine (an indicator of advanced glycosylation protein products) and the enzyme activity of phospholipase A_2, significantly higher values were found in the positive control and oxygen groups than in the negative control and ozone groups. Differences between the negative control and the ozone groups were not significant.

Ozone oxidative post-conditioning was able to preserve the antioxidant–pro-oxidant balance. Also, protein oxidation was controlled, showing similar behaviour to the negative control group.

Histological study

The results of the histological study in renal tissue are shown in Table 3 and Figure 1. The different kind of lesions found were tubular tumefaction, tubular disorganisation and osmotic nephrosis (lesions that precede acute tubular necrosis). In the negative control group, only 5% moderate tubular tumefaction was found. The positive control and oxygen groups had similar results, with the presence of

Table 1 Plasma clearance of p-amino-hippurate (PAH), inulin and creatinine

	Negative control	Positive control	Ozone group	Oxygen group
PAH (ml/min)	1.93 ± 0.006^a	0.13 ± 0.003^b	0.50 ± 0.11^d	0.035 ± 0.0004^c
Inulin (ml/min)	0.78 ± 0.08^a	0.044 ± 0.010^b	0.24 ± 0.07^d	0.013 ± 0.002^c
Creatinine (μmol/l)	83.4 ± 24^a	177.8 ± 58^b	90.4 ± 32^a	181.9 ± 62^b

Values are means ± SD ($n = 10$ per group).
Different letters represent significant differences ($P < 0.05$) for each biochemical parameter.

Table 2 Biochemical parameters related to redox balance in renal tissue

	Negative control	Positive control	Ozone group	Oxygen group
Catalase (k_{15}/g wet tissue)	5.70 ± 0.30[a]	2.45 ± 0.40[b]	12.15 ± 3.55[c]	4.80 ± 0.42[a]
SOD (SOD units/mg protein)	7.73 ± 1.25[a]	5.73 ± 0.32[b]	18.50 ± 0.54[c]	6.85 ± 2.30[a]
Phospholipase A_2 (U/L · min)	52.5 ± 14.26[a]	205.7 ± 62.8[b]	63.4 ± 20.8[a]	175.6 ± 52.5[b]
Fructosamine (OD)	0.025 ± 0.003[a]	0.055 ± 0.007[b]	0.033 ± 0.008[a]	0.63 ± 0.008[b]
TBARS (nmol/mg protein)	0.18 ± 0.022[a]	0.30 ± 0.041[b]	0.33 ± 0.042[b]	0.53 ± 0.082[c]

Values are mean ± SEM (n = 10 animals per group).
Different letters represent significant differences ($P < 0.05$) for each biochemical parameter. Catalase activity is expressed as the first-order constant that describes the decomposition of hydrogen peroxide at room temperature. One unit of SOD enzymatic activity is equal to the amount of enzyme that diminishes the initial absorbance of nitro blue tetrazolium by 50%.
OD, optical density; SOD, superoxide dismutase; TBARS, thiobarbituric acid-reactive substances.

Table 3 Results of the histological study

	Negative control	Positive control	Ozone group	Oxygen group
Moderate tubular tumefaction (%)	3	–	65	25
Intense tubular tumefaction (%)	–	50	25	45
Osmotic nephrosis (%)	–	40	10	20
Tubular disorganisation (%)	–	10	–	5

intense tubular tumefaction, osmotic nephrosis and tubular disorganisation. However, in the ozone group, the higher percentage of lesions corresponded to a moderate tubular tumefaction, with a lower percentage of osmotic nephrosis and without tubular disorganisation. These results corroborate that ozone oxidative post-conditioning is able to diminish the damage caused by the ischaemia–reperfusion process.

Discussion

Oxidative stress is part of the oxygen paradox, in which the reoxygenation of ischaemic kidneys generates a degree of renal injury that greatly exceeds the injury induced by ischaemia alone.[3,5]

It has been clearly demonstrated that ozone oxidative preconditioning has a cytoprotective effect in liver and kidneys submitted to ischaemia–reperfusion challenge, activation of the endogenous antioxidant defence system produced by the ozone treatment protecting the tissue from oxidative damage.[10–12,16–20] Beneficial effects of using ozone oxidative post-conditioning have also been reported in different animal models such as diabetes or using adriamicin or cisplatin as

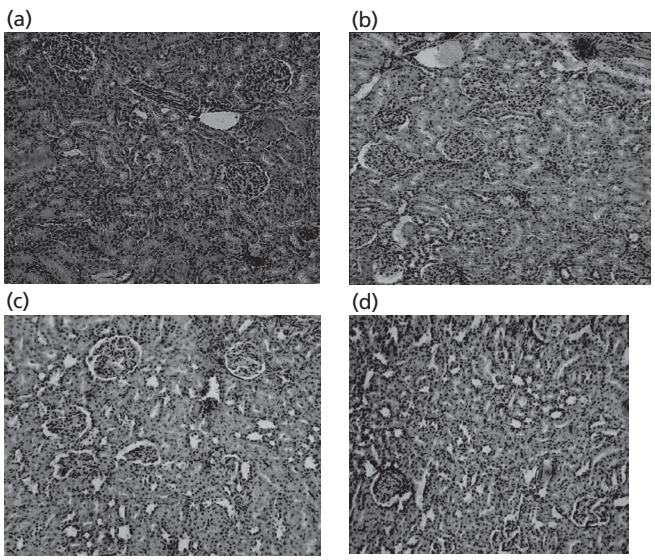

Figure 1 Histology of the renal cortex using haematoxylin and eosin stain (×200). (a) Positive control group – 50% intense tubular tumefaction. (b) Oxygen group – 45% intense tubular tumefaction and 25% moderate tubular tumefaction. (c) Ozone group – the higher percentage of lesions corresponds to moderate tubular tumefaction and a minimum of intense tubular tumefaction. (d) Negative control group – moderate tubular tumefaction.

nephrotoxic drugs.[36–38] Rectal ozone treatment after cisplatin challenge (ozone oxidative post-conditioning) effectively prevented the decrease in renal antioxidant defence system and certainly avoided the deleterious effect of the drug.[38]

Our study shows for the first time a protective effect of ozone oxidative post-conditioning in renal ischaemia–reperfusion phenomena. Repeated administration of ozone via rectal application induced a sort of cross-tolerance to free radicals released after the ischaemia–reperfusion procedure, demonstrating that renal cells have become resistant to this damage.

The mechanisms of protection induced by ozone oxidative post-conditioning as well as ischaemic post-conditioning[21,22,39] (which reduces myocardial infarct size by up to 50%), are not fully understood, but the procedure has been shown to target the important mediators of lethal reperfusion injury by reducing oxidative stress, as demonstrated in this study. Other factors can also be considered, such as decreasing calcium overload, improving endothelial function, increasing adenosine levels, and attenuating the apoptosis of renal cells, among others.[21,22,39] There is evidence that ozone is able to achieve calcium homeostasis in a rat model using carbon tetrachloride as a challenge.[40] Positive effects of ozone on nitric oxide and adenosine levels and in the preservation of mitochondria in hepatic ischaemia–reperfusion processes have also been demonstrated,[41,42] as well as the control of Bax protein in renal tissue in a cisplatin model.[43]

At the time of reperfusion, an increase in oxygen concentration occurs in dysfunctional mitochondria, which do not make the oxygen tetravalent reduction.[6] Also, because of neutrophil activation, there is a great release of ROS, explaining the TBARS increase (used as a measure of lipid peroxidation) in renal tissue submitted to ischaemia–reperfusion.[44] Non-significant differences were achieved between the positive control and ozone groups with respect to TBARS levels. This can be explained because, in its mechanism of action, ozone produces a small and controlled oxidative stress which does not saturate the antioxidant protective mechanism, but rather triggers activation of antioxidant enzymes.[10–12,16–20,23,24,38] An increase in TBARS was also demonstrated in the oxygen group. This group suffered the same lesions as the positive control group because when oxygen levels reaching the damaged tissue increase, more ROS are released and more lipid peroxidation products are formed, but with a lower antioxidant defence system to counteract the increase in ROS. In the ozone group, increases in SOD and catalase activities were demonstrated, achieving the homeostasis redox relating to protection of renal tissue after ischaemia. Levels of superoxide anions will be reduced by the activation of SOD in renal tissue, diminishing the damage to cell membranes and nephron components caused by ROS. During ischaemia, superoxide anions can react with nitric oxide to form peroxynitrite, a very toxic species.[41] By reducing levels of superoxide anions, less peroxynitrite will be formed and so more nitric oxide will be free to produce relaxation of the renal vascular smooth muscle, contributing to the recovery of RBF and GFR, as occurred in the ozone group.[2,3,45] With regard to catalase (which converts hydrogen peroxide to water and oxygen), a significant decrease occurred in the positive control group compared with all the other groups. An increase in hydrogen peroxide, which occupies the active sites of the enzyme, has been demonstrated during ischaemia–reperfusion. Saturation of catalase therefore occurs and the enzyme cannot respond to the excess of substrate (hydrogen peroxide), being essentially inactivated. This enzymatic inactivation by excess substrate[28,29] may explain the reduced catalase activity in the positive control group.

Phospholipase A_2 is an enzyme that splits double bonds in lipids present in membranes. It has been correlated with membrane damage by means of the release of lysophospholipids and toxic metabolites related to cell lysis in inflammatory tissues and in tissues submitted to ischaemia.[18,19] The increase in phospholipase A_2 activity in the positive control and oxygen groups suggests that this enzyme is responsible, in combination with other factors, for the tissue damage observed in these two groups, demonstrated by histological studies. However, the enzyme activity was similar in the negative control and ozone groups.

Fructosamine is an advanced glycosylation protein product highly related with protein damage and with blockade of the nitric oxide vasodilator response. Fructosamine levels were significantly higher in the positive control and oxygen groups, demonstrating the non-protection found in both these groups. However, fructosamine levels were similar in the ozone and negative control groups.

Analysing the results of renal function, significant decreases in RPF and GFR occurred in the oxygen and positive control groups compared with the ozone and negative control groups. Ischaemia damages the endothelial cells and when the blood flow decreases, shearing forces increase, producing more ROS release, damaging even more of the endothelium.[41] Ozone oxidative post-conditioning also influences tissue oxygenation via its haemorheological effects, increasing erythrocyte pliability, possibly diminishing blood viscosity and erythrocyte aggregation, allowing a higher renal blood flow during reperfusion in an organ previously submitted to a lack of blood flow.[18,19]

Creatinine is not reabsorbed and is poorly secreted and so depends mainly on glomerular filtration for its excretion.[1,2] When the glomerular filtration index diminishes, creatinine excretion also decreases, leading to an increase in the plasma concentration. Creatinine levels were lower in the ozone group than in the positive control and oxygen groups, demonstrating an improvement in renal function.

Histological studies showed that lesions were most severe in the positive control and oxygen groups (intense tubular tumefaction, osmotic nephrosis, accompanied by tubular disorganisation), correlating well with the groups that did not show a cytoprotective effect. Lesions were moderate in the ozone group, proving that the renal damage is minor compared with the positive control and oxygen groups. From a structural point of view, ozone treatment favours kidney recovery.

Conclusions

Ozone oxidative post-conditioning favours the functional and structural recovery of renal tissue submitted to 60 min' ischaemia and 10 days' reperfusion, through mechanisms that promote the maintenance of adequate cellular redox

balance and improvements in blood circulation and oxygen metabolism. This is the first demonstration of a protective effect of ozone oxidative post-conditioning in renal ischaemia–reperfusion. Therefore, ozone therapy may be considered as a potential medical approach against kidney ischaemia–reperfusion damage, improving kidney transplantation. These results allow us to consider other potential medical applications for ozone therapy, mainly in the cardiovascular system and central nervous system.

Conflict of interest

The Author(s) declare(s) that they have no conflicts of interest to disclose.

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