Ozone Biological Response in Kidneys of Rats Submitted to Warm Ischemia

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Abstract

A biochemical and morphofunctional renal study, applying different sessions of rectal ozone before a warm ischemia, was performed. Rats were divided in:1-control, a medial abdominal incision was performed for the exposure of the kidneys; 2-ischemia, animals with a bilateral renal ischemia (30 min), with subsequent reperfusion (3 h); groups 3, 4 and 5-ozone + ischemia, as group 2, but with previously treatment of 5, 10 and 15 sessions of rectal ozone, respectively; groups 6, 7 and 8-oxygen + ischemia, as groups 3, 4 and 5, respectively, but using rectal oxygen. A significant decrease of the flow and renal filtration, with high values of fructasamine and phospholipase A, in the ischemia and oxygen groups, with respect to control and ozone groups was obtained. Control and ozone groups, without any statistical difference among them. Morphological alterations were significantly less in the groups pretreated with ozone, with better results for 10 and 15 sessions.

Introduction

It have been demonstrated that reactive oxygen species (ROS) play a key intermediary role in the pathophysiologic processes of clinical and experimental renal diseases (1,2). They can promote DNA scission and base modification, inactivate plasma proteins, crosslink membrane proteins and induce lipid peroxidation in the polyunsaturated fatty acids of membrane lipids (3).

Tissue injury following renal ischemia represents the injurious effects of ROS and anoxia/hypoxia in varying degrees.(4) The major damage.is apparently secondary to reperfusion or reoxygenation. This reoxigenation leads to a massive production of toxic free radical species generated through several cytoplasmatic or mitochondrial mechanisms, producing an oxidative stress, which contributes to tissue injury. (3-7). Tissue ischemia, followed by reperfusion with oxygenated blood, occurs in a number of clinical situations, as

surgical revascularisation of the renal artery and after kidney transplantation. In order to improve the success rate of renal transplantation, the timing of warm and cold ischemia is determinant for kidney's viability (8) After reimplantation, the kidney may develop an acute tubular necrosis, and the recipient must required to undergo dialysis. Any prophylactic approach aiming at preserving the kidney is of great clinical importance.

In normal conditions, cells contain endogenous defense systems, as superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx) or non enzymatic components such as glutathione (GSH) and vitamins E, A, C (6). The SOD is the enzyme which detoxify the primary product of ROS generation, the superoxide anion radical (formed by the action of xanthine oxidase), leading to the production of hydrogen peroxide. This ROS is then converted to water, through the action of glutathione peroxidase or directly to water and oxygen by means of catalase (9). In several pathologic situations, an unbalance between oxidants and antioxidants occurs, and these defense mechanism can be overwhelmed, allowing the ROS to exert their deleterious effect (6).

Taking into account that ozone (O_3), by means of an oxidative preconditioning mechanism, is able to afford protection against cellular damage mediated by free radicals, stimulating the cellular antioxidant enzymes (8, 10-14), we decided to study, in rats, the influence of different sessions of rectal ozone before a warm ischemia, in the renal morphology and function, and in some biochemical parameters.

Materials and Methods

Animals

Eighty adult male Wistar rats (250-260 g) were maintained in an air filtered and temperature conditioned room (20-22 °C) with a relative humidity of 50-52 %. Rats were fed with standard laboratory chow and water *ad libitum* and were kept under an artificial light/dark cycle of 12 h.

Treatment Schedule and Renal Ischemia

O_3 was generated by an OZOMED equipment (Ozone Research Center, Cuba), from medical grade oxygen by means of a silent electric discharge, representing about 3 % of the gas mixture (O_3+O_2). The ozone concentration was measured by using an UV spectrophotometer at 254 nm. The ozone dose is the product of the ozone concentration, expressed as mg/L, by the gas (O_2 + O_3) volume (L). By knowing the body weight of the rat, the ozone dose is calculated as 1 mg/kg, as in our previous papers (8, 10-13).

Animals were allocated randomly to 8 experimental groups, of 10 animals each: 1-control, animals were anesthetized, using sodium pentobarbital at doses of 30 mg/Kg of weight,

receiving 50 I.U. of heparin by intraperitoneal injection. Afterwards, a laparotomy was performed for the sham exposure of the kidneys with successive laparorrhaphy; 2- positive control group (ischemia): animals were processed in the same way as group 1, but after the kidney exposition they were submitted to a bilateral renal ischemia. Both renal arteries were cross-clamped for 30 min, with subsequent reperfusion during 3 h, before the morphological, functional and biochemical renal study; groups 3, 4 and 5-ozone groups (O_2 + O_3 and ischemia), as group 2, but the animals were previously treated with 5, 10 and 15 sessions of a gas mixture composed of O_2 + O_3 (2.5-2.6 ml with O_3 concentration of 50 μg/ml, representing a dose of 0.5 mg/kg weight), by rectal insufflation, once per day, respectively; groups 6, 7 and 8-oxygen groups (O_2 + ischemia), as groups 3, 4 and 5, respectively, but using rectal oxygen (13 mg/kg weight) instead of the gas mixture composed of O_2 + O_3.

Sample Preparation

Under constant sodium pentobarbital anesthesia, after a renal ischemia of 30 min, we allowed a reperfusion of 3 h. Heparin (50 UI) was administered by intraperitoneal injection. Immediately, within the following 10 min were collected urine samples in the bladder for the renal function determinations: renal plasma flow (RPF) and the glomerular filtration rate (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 of PAH and 20 mg of inulin in 100 ml of saline solution) by a continuos perfusion through the left femoral vein at a rate of 0.15 ml/min, after a loading dose of 0.8 ml of PAH (12 mg/ml) and 0.8 ml of inulin (2 mg/ml). Blood was withdrawn by intracardiac puncture (2 ml of blood were extracted). Thereafter the animals were euthanized by ether anesthesia.

Representative samples of different kidney portions were taken for histopathological studies and tissue homogenates. Kidney homogenates were obtained using a tissue homogenator Edmund Bulher LBMA at 4 °C. The homogenates were prepared in 50 mM KCl/histidine buffer pH 7.4, 1:10 (w/v) and were spun down with a Sigma Centrifuge 2K15, at 4 °C and 8500 g for 20 min. The supernatants were taken for biochemical determinations.

Biochemical determinations

PAH and inulin were determined in deproteinated plasma and urine samples by cadmium sulfate (15), using for PAH a photocolorimetric technique as modified by Smith and Tinkelstein (16). Inulin was measured by the direct method of resorcinol without alkaline treatment (17).

Kidney homogenates were assayed for total SOD (Cu/Zn and Mn SODs) activity, determining the capacity of the enzyme of inhibiting the autoxidation of pirogallol by 50 % (18). The phospholipase A activity was determined according to a standard procedure (19) and fructosamine by means of a colorimetric procedure (20). The proteins were measured by a standard Coomassie Blue method (21). Ultraspect Plus Spectrophotometer from Pharmacia LKB was used for spectrophotometric methods.

Histological study

Samples of rat kidneys were taken and fixed in 10 % neutral buffered formalin, processed and embedded in paraffin. The histological sections, stained with hematoxylin and eosin, were examined by a pathologist unaware of the treatment schedule.

Statistical analysis

The statistical analysis was started by using the OUTLIERS preliminary tests for detection of error values. Afterward the Anova method (one way analysis of variance) was used followed by homogeneity variance test (Bartlett-Box). In addition, a multiple comparison test was used (Duncan test) and for the comparison of two groups, the Student's t test was done. For the analysis of the biochemical parameters Mann-Whitney test was applied. Results are presented as mean ± standard deviation (SD). Different letters indicate a statistical significance of at least $p < 0.05$.

Results

Table I shows the renal flow measured by means of the clearance of PAH and the GFR measured by means of plasma clearance of inuline. A significant decrease of PAH and Inuline clearances were observed in groups 2 (ischemia) and 3, 4 and 5 (O_2-5, 10 and 15 d + ischemia, respectively) in comparison with either groups 1 (control) or 6, 7 and 8 (O_3-5, 10 and 15 d + ischemia, respectively). Among either groups 2, 3, 4 and 5 or groups 1, 6, 7 and 8 there were no statistically significant differences.

Table I. Plasmatic clearance of p-amino-hippurate (PAH) and Inulin in the different experimental groups.

Experimental Groups (n=10)	PAH ml/min/100g	Inuline ml/min/100g
1-Control	3.08 ± 0.73^a	0.60 ± 0.20^a
2-Ischemia	1.66 ± 0.82^b	0.32 ± 0.26^b
3-O_2(5d)+ischemia	1.58 ± 0.70^b	0.33 ± 0.27^b
4-O_2(10d)+ischemia	1.49 ± 0.67^b	0.35 ± 0.23^b
5-O_2(15d)+ischemia	1.16 ± 0.61^b	0.43 ± 0.20^b
6-O_3(5d)+ischemia	2.45 ± 0.81^a	0.49 ± 0.15^a
7-O_3(10d)+ischemia	2.66 ± 1.06^a	0.53 ± 0.18^a
8-O_3(15d)+ischemia	3.51 ± 0.90^a	0.72 ± 0.35^a

Values represent mean ± SD. Statistical significance between a and b of at least $p<0.05$

Table II shows the biochemical parameters measured in kidney homogenates. The phospholipase A activity (PLA2) in the groups 6, 7 and 8 (O_3-5, 10 and 15 d + ischemia, respectively) did not differ from the control group and values were significantly lower in comparison with the group 2 (ischemia) and groups 3, 4 and 5 (O_2-5, 10 and 15 d + ischemia, respectively). No difference was observed among the groups 2, 3, 4 and 5. A similar trend was observed in regard to the fructosamine concentration, that yielded significantly lower values in control and O_3-5, 10 and 15 d + ischemia groups in comparison with the ischemia and O_2-5, 10 and 15 d + ischemia groups. No statistical differences among groups 1, 6, 7 and 8 and among groups 2, 3, 4 and 5 were observed. As was expected, the renal SOD activity showed a significant increase in the O_3-5, 10 and 15 d + ischemia groups in comparison with control, ischemia and O_2-5, 10 and 15 d + ischemia groups, No statistical differences among groups 6, 7 and 8 and among groups 1, 2, 3, 4 and 5 were observed.

Table II. Results of phospholipase A2 (PLA2), fructosamine and superoxide dismutase (SOD) in the different experimental groups.

Experimental Groups (n=10)	**PLA_2 U/L/min**	**Fructosamine Δ D.O**	**SOD U/mg proteins**
1-Control	22.43 ± 12.15^a	0.013 ± 0.010^a	742 ± 182^a
2-Ischemia	104.74 ± 33.77^b	0.020 ± 0.005^b	964.48 ± 184.00^a
3-O_2(5d)+ischemia	109.40 ± 24.60^b	0.016 ± 0.001^b	856.99 ± 103.80^a
4-O_2(10d)+ischemia	100.91 ± 23.12^b	0.017 ± 0.001^b	877.73 ± 103.21^a
5-O_2(15d)+ischemia	92.54 ± 22.61^b	0.019 ± 0.001^b	996.12 ± 128.67^a
6-O_3(5d)+ischemia	47.71 ± 5.70^a	0.014 ± 0.002^a	1032.0 ± 99.6^b
7-O_3(10d)+ischemia	44.57 ± 6.40^a	0.014 ± 0.001^a	1251.61 ± 132.72^b
8-O_3(15d)+ischemia	33.66 ± 9.20^a	0.015 ± 0.002^a	1407.67 ± 142.12^b

Values represent mean ± SD. Statistical significance between a and b of at least $p<0.05$

The histhological study of the kidney was in accordance with the biochemical findings. Table III shows the percent of kidney without lesions in the different experimental groups. The groups O_3-5, 10 and 15 d + ischemia presented higher percent of kidneys without lesions in comparison with the ischemia and O_2-5, 10 and 15 d + ischemia groups. Best results were achieved with 10 and 15 days of ozone treatment before the ischemia.

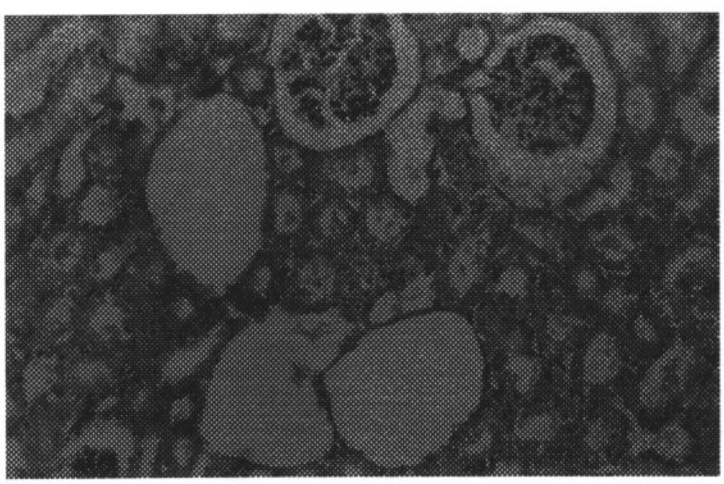
Table III. Results of the histological study showing the percent of kidneys without lesions.

Experimental Groups (n=10)	**Kidneys without lesions (%)**
1-Control	100
2-Ischemia	10
3-O_2(5d)+ischemia	37
4-O_2(10d)+ischemia	20
5-O_2(15d)+ischemia	14
6-O_3(5d)+ischemia	64
7-O_3(10d)+ischemia	80
8-O_3(15d)+ischemia	77

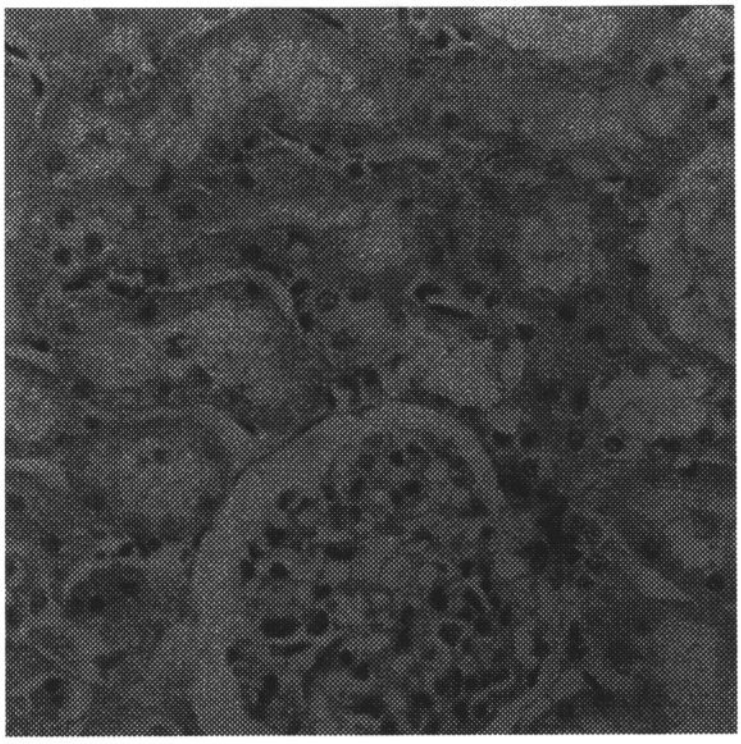
No lesions were present in the control group (Figure 1A). Kidney samples of the animals subjected to ischemia (Figure 1B) showed subcapsular hemorrhage, dilatation of convoluted tubules and of the Bowman's capsule and glomerular compression. The same was achieved in the kidney samples of animals treated with oxygen, during 10 days, before the ischemia (Figure 1C), but in addition, capsules without glomeruli were found. In contrast, kidney samples of the animals subjected to ischemia with previous ozone treatment (10 sessions) showed minimal lesions which consisted of dilatation of convoluted tubules, preserving the glomeruli and capsule in good shape (figure 1D).

1A

1B



1C



1D

Figure 1. Histological study. (A): Control group, no apparent kidney lesions. (B): Ischemia, subcapsular hemorrhage, dilatation of convoluted tubules and of the Bowman's capsule and glomerular compression. (C): O2(10d) + ischemia, the same as ischemia group, but in addition, capsules without glomeruli. (D) O3(10d) + ischemia, minimal lesions which consisted of dilatation of convoluted tubules, preserving the glomeruli and capsule in good shape. A, B and C (100x); D (250x).

Discussion

It has been demonstrated that a repeated and non-lethal stress is able to confer protection against a prolonged and severe stress (8,10-13,22). In these conditions, oxidative preconditioning is analogous to other phenomena such as ischemic preconditioning (23), thermal preconditioning (24) and chemical preconditioning (25). Ozone oxidative preconditioning is a somewhat paradoxical cellular mechanism where an induction of tolerance to O_3 and ROS generated by toxic agents is achieved. ROS production, occurring during the ischaemia-reperfusion phenomenon, seems to be a major mechanism of tissue injury (3-7, 26,27). These results, as previous reports (2,8,12,13,28,29), support the idea that ozone, being an oxidant, could promote organ stress inducing an enhancement of the endogenous antioxidant defense system, in order to preserve the organ undergoing ischemia. Therefore, the development of tissue injury depends upon the balance between generation of ROS and tissue antioxidant defense mechanism. ROS have been recognize increasingly as potential mediators of inflammatory cells injury during glomerulonephritis (30). Our study have demonstrated that repeated daily administrations of the gas mixture (O_2 + O_3), by rectal insufflation in rats, generated a sort of tolerance to free radicals released after the induced ischemia reperfusion phenomenon. Nevertheless, taking into account the histological study, best results were achieved started from 10 d of O_3 treatment. On the contrary, in groups 2

(ischemia), 3, 4 and 5 (O_2-5,10 and 15 d + ischemia, respectively) a significant cellular damage was documented by functional, biochemical and morphological criteria.

The decrease in SOD activity levels, could be associated with their inhibition or inactivation related to ischemia-reperfusion process or free radical attack itself. The significant stimulation of SOD in the O_3 (5,10 and 15 d) + ischemia groups suggests that cellular protection is most likely achieved through the reduction in the availability of superoxide anion. The interaction between hypoxanthine and xanthine oxidase results in generation of superoxide. Once formed, superoxide is rapidly converted to hydrogen peroxide (H_2O_2), by SOD and then H_2O_2 is converted to water (by glutathione peroxidase) or to water and oxygen (by catalase). However, in the presence of various transition metals, H_2O_2 is rapidly converted to hydroxyl free radical. A delicate balance must, therefore, be maintained among the availability of superoxide, hydrogen peroxide and reduced iron to minimize the highly toxic hydroxyl radical formation. Superoxide can also react with nitric oxide producing peroxynitrite, a highly toxic product. Nitric oxide acts as a potent vasodilator readily degraded by superoxide anion, therefore, antioxidants, particularly SOD (through their ability to scavenge superoxide anion) can maximize the renal protective action of nitric oxide. It have been demonstrated (31,32) the increase activities of SOD, catalase and glutathione peroxidase after chronic O_3 exposure. Moreover, in patients treated with ozone by autohemotherapy, an overexpression of glucose-6-phosphate dehydrogenase, glutathione peroxidase and a reduction of the lipid peroxidation in plasma were obtained (10). In vitro observations (33) provide evidence that elevated SOD protein level confer resistance against ROS damage in mammalian cells and in vivo experiments with transgenic mice, expressing enhanced MnSOD protein levels, demonstrated protection against otherwise lethal hyperoxia (34). Administration of SOD or catalase was highly effective in attenuating the functional and structural abnormality (proteinuria and glomerular lesions) in rats submitted to a damage induced by puromycin aminonucleoside (35,36). Thus, an increase in the antioxidant enzyme activities produced by the ozone treatment, protect kidney cells against the toxic effect of ROS.

Phospholipase A activation generates lysophospholipids and other metabolites responsible of cellular lysis. The increased phopholipase A activity in groups 2, 3, 4 and 5 (ischemia and oxygen groups) suggests that the enzyme may be partly responsible for the kidney damage noted in the morphological study. On the contrary, the figures in the ozone groups and control (groups 1, 6, 7 and 8) remained without significance differences, indicating that O3 exerted indirectly a protection against the cellular disruption. The same was obtained in the evaluation of fructosamine, as an indirect measure of the oxidative stress. Groups 2, 3, 4 and 5 with figures significantly higher in comparison with the control and ozone groups, suggesting an accelerate protein glycosylation (37).

Conclusions

Enhanced antioxidant enzymes activities induced by ozone treatments provided kidneys with an effective defense system against the toxic effect to ROS. Histological findings demonstrated that the best results were obtained from 10 days of ozone treatment before the ischemia-reperfusion damage. Therefore, ozone therapy may contribute to minimize the oxidative injuries produced by the oxygen radicals released during an organ transplantation.

Keywords

Ozone; kidney ischemia/reperfusion; oxidative stress; preconditioning; antioxidant defense system.

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