

# Ozone: A Possible Agent that Delays the Chronic Renal Failure

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## Abstract

Chronic renal failure (CRF) represents a world health problem. The aim of this paper is to evaluate the effect of ozone therapy, in the renal function and morphology, in an experimental model of CRF. Rats were divided into 4 groups: 1-negative control, rats without any treatment; 2-positive control, rats submitted to 5/6 reduction of the total renal mass (right kidney nephrectomy, with 2 branches of the renal arteria, of the left kidney, clamped); 3-ozone, as group 2, but receiving, rectally, 7 sessions of ozone (0.5 mg/kg), previously to the surgery procedure and twice per week (during 10 weeks) after the partial nephrectomy; 4-oxygen, as group 3, but receiving oxygen instead of ozone. Ozone oxidative preconditioning effect protected the tissues against the oxidative stress present in the CRF, being in correspondence with the positive histological results obtained.

## Introduction

Chronic renal failure (CRF) represents a world health problem. CRF, once established, goes irreversibly to a final stage, provoking the patient death. In contrast with the capacity of the kidneys to regain function following acute renal injury, renal injury of a more prolonged nature often leads to progressive and irreversible destruction of nephron mass (1). Such reduction of renal mass, in turn, causes structural and functional hypertrophy of surviving nephrons. This compensatory hypertrophy is due to an adaptive hyperfiltration mediated by increases in glomerular capillary pressures and flows. Eventually, these adaptations prove maladaptive, in that they predispose to sclerosis of the residual glomerular population (1-4). The intrarenal vasculature is the most affected structure, preventing an appropriate blood flow, favoring the glomerular sclerosis (1-5). For that reason, the improvement of the rheological properties of the blood could delay the progression of the CRF.

Glomerulonephritis was the most common initiating cause of CRF in the past. Possibly because of more aggressive treatment of glomerulonephritis, diabetes mellitus and hypertensive renal diseases are now leading causes of CRF. The inexorable course to renal failure often is accompanied by anemia, malnutrition, impaired metabolism of carbohydrates, fats and proteins, impaired platelet function and defective utilization of energy (1).

Reactive oxygen species (ROS) play a key intermediary role in the pathophysiologic processes of a wide variety of clinical and experimental renal diseases. These range from acute to chronic injuries, making the kidney as the site in which several unrelated diseases involves ROS (6). ROS have been demonstrated to be capable of degrading glomerular basement membrane and inducing glomerular injury characterized by impaired glomerular filtration and sieving function (7,8). In order to degrade toxic ROS, cells are equipped with various antioxidant systems. Therefore, the development of tissue injury depends upon the balance between ROS generation and tissue antioxidant defense mechanism (9).

Taking into account some of the ozone therapeutic properties, such as, antiplatelet activity (10), enhancement of the cell energy (11) and the increase of the antioxidant defense system (12-16), the aim of this paper is to evaluate the effect of ozone therapy in the renal function and morphology in an experimental model of CRF.

## **Materials and Methods**

### **Animals**

Forty young male Sprague Dawley rats (180-200 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. The studies were performed in concordance with the European Union regulations for animal experiments.

### **Treatment Schedule and Surgical Procedure**

Ozone (O<sub>3</sub>) 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<sub>3</sub>+O<sub>2</sub>). 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<sub>2</sub> + O<sub>3</sub>) volume (L). By knowing the body weight of the rat, the ozone dose is calculated as 0.5 mg/kg.

Animals were allocated randomly to 4 experimental groups, of 10 animals each: 1-negative control group, normal rats without any procedure or treatment; 2-positive control group, animals were anesthetized using sodium pentobarbital, at doses of 30 mg/Kg of weight and then submitted to 5/6 reduction of the total renal mass (right kidney nephrectomy, with 2 branches of the renal arteria, of the left kidney, clamped); 3-ozone group, as the positive control group, but receiving 7 sessions of the gas composed of O<sub>2</sub> + O<sub>3</sub> (2.5-2.6 ml with O<sub>3</sub> concentration of 50 µg/ml, representing a dose of 0.5 mg/kg weight), by rectal insufflation, once per day, previously to the surgery procedure and twice per week (during 10 weeks) after the partial nephrectomy; 4-oxygen group, as the ozone group, but using rectal oxygen (13 mg/kg weight) instead of the gas mixture composed of O<sub>2</sub> + O<sub>3</sub>.

## Sample Preparation

A day before the partial nephrectomy, all animals were housed in metabolic cages during 24 h, without food and water *ad libitum*. In all animals, the weight and the systolic arterial pressure (SAP) in the tail of the animals (17) were measured. Water volume ingested and the volume of urine eliminated were determined. Also, the urine excretion index (urine volume/water volume ingested) and the osmotic, protein, sodium and potassium excretions were measured. All this procedure and measurements were repeated, after the partial nephrectomy, once a week, during 10 weeks, time during which, the CRF continued its evolution. The time of the study was not prolonged for more than 10 weeks, avoiding the unpredictable death due to the final stage of the CRF. In the last day of evolution, plasmatic clearance of p-amino-hippurate (PAH) and inulin, in order to know the renal plasma flow (RPF) and the glomerular filtration rate (GFR), respectively, were determined using the method of unique injection (no urine) and the multicompartimental analysis of the plasmatic concentration curves in 9 blood samples (18). Creatinine and potassium in serum were determined in the final blood sample obtained by intracardiac puncture (2 ml of blood were extracted). Thereafter the animals were euthanized by ether anesthesia.

## Biochemical determinations

PAH and inulin were determined in deproteinated plasma samples by cadmium sulfate (19), using for PAH a photolorimetric technique as modified by Smith and Tinkelstein (20). Inulin was measured by the direct method of resorcinol without alkaline treatment (21). Urine osmolality was determined using a digital automatic microosmometer (22). Sodium and potassium urine concentrations, for the calculation of the excretions of both substances, as well as plasma potassium were measured in a Carning flame photometer (model 400), using the corresponding calibration curves (23). Proteins were calculated by the Biuret photolorimetric technique using a Spectrophotometer (Shimadzu) (24). Creatinine in plasma was measured in deproteinated filtrates by the method of sodium tungstate, using for its valoration the method of picric acid modified by Brot (26).

## 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. Results are

presented as mean  $\pm$  standard deviation (SD). Different letters indicate a statistical significance of at least  $p < 0.05$ .

## Results

At the end of the study (10 weeks after the partial nephrectomy), animals treated with ozone presented: SAP figures lower than the positive control and oxygen groups, but higher values with respect to negative control group. Urine excretion index, in the ozone group, was similar to the negative control group. However, positive control and oxygen groups presented higher values of the urine excretion index in regards to negative control and ozone groups (Table I).

Table I. Behavior of the systolic arterial pressure (SAP) and urine excretion index, at the end of the study, in the different experimental groups.

Groups	SAP	Urine excretion index	Osmotic excretion mOsm/24 h/100g-rat
Negative Control	111 $\pm$ 13 <sup>a</sup>	1.26 $\pm$ 0.20 <sup>a</sup>	4.83 $\pm$ 0.56 <sup>a</sup>
Positive Control	165 $\pm$ 13 <sup>b</sup>	0.89 $\pm$ 0.31 <sup>b</sup>	4.08 $\pm$ 0.91 <sup>a</sup>
Oxygen	167 $\pm$ 15 <sup>b</sup>	0.89 $\pm$ 0.14 <sup>b</sup>	3.90 $\pm$ 0.65 <sup>a</sup>
Ozone	157 $\pm$ 9 <sup>c</sup>	1.20 $\pm$ 0.25 <sup>a</sup>	4.06 $\pm$ 0.92 <sup>a</sup>

Data are mean  $\pm$  SD. Statistical significance among different letters of at least  $p < 0.05$

Protein excretion figures, in the ozone group, were higher than the negative control group, but lower than the positive control and oxygen groups, at the end of the study. Potassium and sodium excretion values, in the negative control and ozone groups were similar, but lower in the positive and oxygen groups with respect to negative and ozone groups (Table II).

Table II. Protein, potassium and sodium excretions, at the end of the study, in the different experimental groups.

Groups	Protein excretion mg/24h/100g-rat	Potassium excretion mEq/24h/ 100g-rat	Sodium excretion mEq/24h/ 100g-rat
Negative Control	1.31 $\pm$ 1.71 <sup>a</sup>	34.26 $\pm$ 12.50 <sup>a</sup>	575.26 $\pm$ 54.86 <sup>a</sup>
Positive Control	8.78 $\pm$ 2.06 <sup>b</sup>	26.07 $\pm$ 8.30 <sup>b</sup>	449.30 $\pm$ 63.29 <sup>b</sup>
Oxygen	7.93 $\pm$ 1.97 <sup>b</sup>	23.40 $\pm$ 4.27 <sup>b</sup>	451.75 $\pm$ 165.40 <sup>b</sup>
Ozone	4.08 $\pm$ 0.85 <sup>c</sup>	30.55 $\pm$ 9.25 <sup>a</sup>	507.48 $\pm$ 40.3 <sup>a</sup>

Data are mean  $\pm$  SD. Statistical significance among different letters of at least  $p < 0.05$

At the end of the study, RPF and GFR presented higher figures in the ozone group respect to the others. The lowest values of RPF and GFR were achieved in positive control and oxygen groups. Potassium and creatinine figures in plasma, in the negative control and ozone groups were similar, but with decrease values in the positive control and oxygen groups (Table III).

Table III. Creatinine and potassium figures in plasma, as well as renal plasmatic flow (RPF) and glomerular filtration rate (GFR), at the end of the study, in the different experimental groups.

Groups	Plasma potassium mEq/L	Plasma creatinine mg/100ml	RPF mL/min/100g- rat	GFR ML/min/100g- rat
Negative Control	5.23 ± 1.66 <sup>a</sup>	1.29 ± 0.30 <sup>a</sup>	1.71 ± 0.25 <sup>a</sup>	0.34 ± 0.01 <sup>a</sup>
Positive Control	4.99 ± 1.30 <sup>b</sup>	0.99 ± 0.18 <sup>b</sup>	1.22 ± 0.67 <sup>b</sup>	0.26 ± 0.06 <sup>b</sup>
Oxygen	4.68 ± 1.21 <sup>b</sup>	0.93 ± 0.36 <sup>b</sup>	1.37 ± 0.20 <sup>b</sup>	0.24 ± 0.09 <sup>b</sup>
Ozone	5.43 ± 1.83 <sup>a</sup>	1.08 ± 0.27 <sup>a</sup>	1.95 ± 0.81 <sup>c</sup>	0.39 ± 0.22 <sup>c</sup>

Data are mean ± SD. Statistical significance among different letters of at least p<0.05

Histological injuries were 13, 94 and 100 % in the ozone, oxygen and positive control groups, respectively (Table IV). The histological findings were glomerular collapse, tubule degeneration, cortical-medullar hemorrhages, dilatation of convoluted tubules, glomerular capsule dilatation and renal injury among others. Positive control and oxygen groups presented those manifestations in 80 - 100 % of animals, however in the ozone groups were presented in 10 -20 %.

Table IV. Histological findings in the residual renal mass (1/6), due to the partial nephrectomy, in the different experimental groups.

Groups	GCD %	GC %	DCT %	TD %	CMH %	IR %
Negative Control	0	0	0	0	0	0
Positive Control	100	100	100	100	100	100
Oxygen	100	80	100	100	90	94
Ozone	100	20*	100	10*	10*	13*

GCD- glomerular capsule dilatation; GC- glomerular collapse; DCT- dilatation of convoluted tubules; TD- tubule degeneration; CMH- cortical-medullar hemorrhages; IR- renal injury

\* Statistical significance of at least p<0.05.

## Discussion

Animals submitted to the subtraction of 5/6 of the total renal mass moved forward the installation of the CRF, demonstrated by the increase of SAP, the proteinuria and the presence of renal damage in the histological study. This behavior is still more pronounced in the positive and oxygen control groups, where the renal damage achieved 100 and 94 %, respectively.

The results have shown, at the end of the study, that the animals treated with ozone had the highest figures of RPF and GFR, as well as lower figures of proteinuria, higher urine excretion and lower SAP in comparison with the positive and oxygen control groups. These

results can be linked to the ozone antiplatelet activity (10), diminishing blood viscosity, that could produced a decrease in the friction between the blood and the glomerular vascular walls, decreasing the flow resistance, increasing the RPF and GFR The flow rise contributes to diminish the endothelial injuries and the glomerular collapse, avoiding the tubular hypoxia, the hemorrhages and the release of several proinflammatory cytokines (26-29).

The vasoactive properties of the arachidonic acid metabolites are one of its important actions (30). Thromboxane A<sub>2</sub> (TxA<sub>2</sub>), a platelet product of arachidonic acid, stimulates platelet activation and secretion, being a potent vasoconstrictor. In contrast, prostacyclin (PGI<sub>2</sub>), an endothelial cell product of arachidonic acid metabolism, inhibits platelet activation by raising intraplatelet levels of cyclic adenosine monophosphate, being a vasodilator. It is known (30), that thromboxanes, adenosin diphosphate (ADP), trombine and collagen stimulate platelet activation. It had been demonstrated that ozone treatment diminished Tx<sub>B2</sub> and increased 6 Keto PGF<sub>1</sub> in patients with different pathologies (31). It had also been proved, in *in vitro* studies, in platelet-rich plasma (PRP) and in washed platelet suspension (WPS) of healthy persons, that ozone therapy improved the antiplatelet activity induced by ADP and collagen in (PRP) and the induced by collagen and thrombine in WPS in a dose dependent way (10). The ozone antiplatelet activity could be in relation with the inhibitory effect that ozone therapy has in thromboxane production. Several papers (32-34) referred the ozone antiplatelet activity in patients with different diseases (diabetes, atherosclerosis, subacute ischemia).

In the other hand, it had been demonstrated that ozone is able to regulate the calcium levels, maintaining its homeostasis, avoiding any damage to the cell structure (35). Also, it is possible that the ozone oxidative preconditioning effect, with the stimulation of the antioxidant defense system (12-16), protected the tissues against the oxidative stress present in the CRF (6), being in correspondence with the positive histological results obtained.

## Conclusions

In this animal model of CRF, rectal administrations of ozone produced a delay in the advance of the disease, protecting the kidneys against the deleterious effects present in the CRF. Consequently, whenever possible, ozone therapy may become an important therapy to improve the quality of life of patients suffering of CRF.

## Key Words

Ozone; chronic renal failure; antiplatelet activity; reactive oxygen species.

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