Short Comunications

Ozone Therapy on Rats Submitted to Subtotal Nephrectomy: Role of Interleukin 6 and Antioxidant System

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ABSTRACT

Chronic renal failure (CRF) represents a world health problem. Ozone increases the endogenous antioxidant defense system, preserving the cell redox state. The aim of this study is to evaluate the effect of ozone/oxygen mixture in the renal function, morphology, and biochemical parameters, the rol of cytokine number 6 in an experimental model of CRF (subtotal nephrectomy). Ozone/oxygen mixture was applied daily, by rectal insufflation (0.5 mg/kg) for 15 sessions after the nephrectomy. Renal function was evaluated, as well as different biochemical parameters, at the beginning and at the end of the study(10 weeks). Renal plasmatic flow (RPF), glomerular filtration rate (GFR), the urine excretion index, and the sodium and potassium excretions (as a measurement of tubular function) in the ozone group were similar to those in Sham group. Nevertheless, nephrectomized rats without ozone (positive control group) showed the lowest RPF, GFR, and urine excretion figures, as well as tubular function.. Serum creatinine values and protein excretion in 24 hours in the ozone group were decreased compared with nephrectomized rats, but were still higher than normal values. Histological study demonstrated that animals treated with ozone showed less number of lesions in comparison with nephrectomized rats. Proinflammatory cytokine (IL-6) was decrease in renal tissue after ozone by rectal insunnflation. However, ozone/oxygen mixture induced

a significant stimulation in the enzymatic activity of CAT, SOD, and glutathione peroxidase, as well as reduced glutathione in relation with Sham and positive control groups.

In this animal model of CRF, ozone rectal administrations produced a delay in the advance of the disease, protecting the kidneys against vascular, hemorheological, and oxidative mechanisms. This behavior suggests ozone therapy has a protective effect on renal tissue by down regulation of the oxidative stress and proinflammatory cytokine shown in CRF.

Key words: Chronic renal failure (CRF); cytokine (IL-6); endogenous antioxidant; subtotal nephrectomy.

INTRODUCTION

Chronic Renal Failure (CRF) represents a world health problem; once established, it 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 . 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 increase in glomerular capillary pressures and flows. Eventually these adaptations prove mal adaptive, predisposing to sclerosis of the residual glomerular population.Reactive oxygen species (ROS) play a key intermediary role in the pathophysiologic processes of a wide variety of clinical and experimental renal diseases. It ranges from acute to chronic injuries, making the kidney the site in which several unrelated diseases involve ROS.

Some investigations showed that the patients with nephropathy manifested increased plasma concentrations of pro-inflammatory cytokines such as TNF-α, IL-6 and IL-18, all of them correlating positively with increase urine albumin: serum creatinine ratio, which is a marker of renal damage inflammation. In order hands to eliminate toxic ROS, cells are equipped with various antioxidant defense systems. Among various antioxidant systems equipped within aerobic cells, three antioxidant enzymes, superoxide dismutase (SOD), glutathione peroxidase (GSH-Px), and catalase (CAT), are major mechanisms to reduce local levels of ROS.CRF is associated with depressed SOD and elevated NADPH

oxidase expression, which can contribute to oxidative stress by increasing superoxide anion

Taking into account some of the ozone therapeutic properties, such as antiplatelet activity, rol over immune system, enhancement of cell energy and the increase of the antioxidant defense system the aim of this paper is to evaluate the effect of ozone therapy in the renal function, morphology, level of cytokine 6 and biochemical parameters that measure oxidative stress in an experimental model of CRF.

METHODS

Thirty young female Wistar rats (180-200 g) were fed with standard laboratory chow and water ad libitum and were kept under an artificial light/dark cycle of 12 hours The experiments were performed in accordance with the ethical guidelines for investigations in laboratory animals and were approved by the Ethical Committee for Animal Experimentation of the National Center for Scientific Research, Havana, Cuba. Treatment schedule and surgical procedure Ozone (O3) was generated by OZOMED 01 equipment manufactured by the Ozone Research Center (Cuba). Ozone was obtained from medical-grade oxygen by means of a silent electric discharge, representing about 3% of the gas mixture (ozone/oxygen).

Ventral laparotomy was performed under aseptic conditions after anesthesia (sodium pentobarbital, 30 mg/kg intraperitoneal route). The right kidney was then removed, while two-thirds of the left kidney underwent acute infarction by ligation of two first-order branches of the main renal artery. Recovery from anesthesia and from the surgical procedure was complete within 24 hours**.** Animals were allocated randomly to 3 experimental groups of 10 animals each:(1) Sham group (negative control group), where rats underwent a ventral laparotomy under anesthesia as described above; however, only handling of the renal pedicle without the removal of renal mass was performed;(2) positive control group, where rats were subjected to 5/6 renal ablation as described above; and (3) ozone group, where rats were handled as in group 2 but also received, after the damage, 15 sessions of the gas mixture, composed of oxygen (O2) + O3 (2 .5−2.6 mL at a concentration of 50 μ g/mL, representing a dose of 0.5 mg/kg weight), by rectal insufflation, once per day.In all animals, the weight and the systolic arterial pressure (SAP) in the tail. All these procedures and measurements were repeated, after

the partial nephrectomy, once a week, for 10 weeks, the 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 paminohippurate (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 . Creatinine in serum was determined in the final blood sample obtained by intracardiac puncture (2 mL of blood were extracted)

Biochemical assays

Proteins were calculated by the Biuret photocolorimetric technique using a Shimadzu spectrophotometer.Potassium and sodium urine concentrations were measured for the calculation of the excretions of both substances in a Corning flame photometer using the method described by Oser Creatinine in plasma was measured by Brot method.Kidney homogenates were obtained using a tissue homogenator Ultra-Turrax T25 polytron at 4C for GSH, TBARS, GSH-Px, and SOD determinations. GSH was determined by a slightly modified version of Beutler method, using a spectrophotometer. Enzymatic activity of SOD was determined by a modified version of Minami and Yoshikawa method, CAT was determined according to Evans and Diplock method ,To estimate TBARS levels, a method described by Ohkawa et al was used. Histological study Samples of rat kidneys were taken, fixed in 10% neutral buffered formalin, processed, and embedded in paraffin. A pathologist unaware of the treatment schedule examined the histological sections, stained with hematoxylin and eosin. Statistical analysis: the one-way analysis of variance (ANOVA) then homogeneity variance test (Bartlett-Box) were applied. In addition, Duncan's multiple range test and the Student *t* test, for the comparison of two groups, were done. Interleukin -6 Bioassay: Interleukin (IL) 6 levels in the kidney tissue were determined by immunohistochemistry technique.(Hirano).

RESULTS

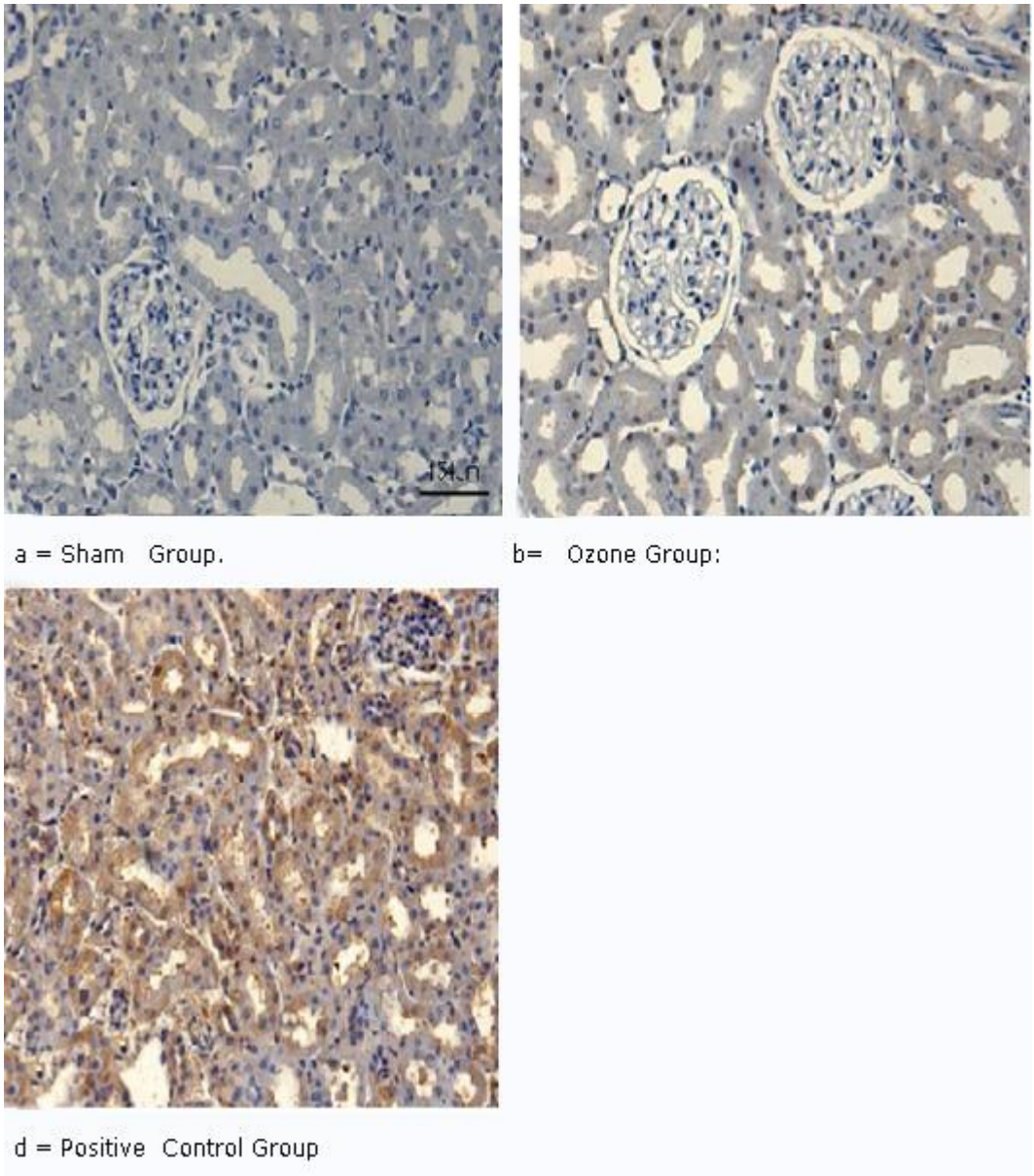
At the end of the study (10 weeks after the partial nephrectomy), animals treated with ozone showed SAP figures lower than those in the positive control group, but higher values compared to those in Sham group (negative control group). Compared to protein excretion and serum creatinine, figures, in the ozone group, were lower than the positive control group Potassium and sodium excretion values in negative control and ozone groups were similar, RPF and GFR in ozone and Sham groups showed similar figures. Histological renal injuries (RI) were 0%, 25 %, and 100% in the Sham, ozone, and positive control groups, respectively. The histological findings for ozone and positive control groups were glomerular collapse (GC),tubule degeneration (TD), and cortical-medullary hemorrhages (CMH). Ozone group showed 20% of GC compared with positive control group with 100%, the TD ozone group showed figures lower than positive control group, CMH was higher in the positive control group compared with ozone group. CMH cortical-medullar hemorrhages; and RI renal injury.

Table 1. Behavior of the systolic arterial pressure (SAP); protein, potassium, and sodium excretions; creatinine figures; renal plasmatic flow (RPF); glomerular filtration rate (GFR) and Histological findings in the residual renal mass, due to the partial nephrectomy, in the different experimental groups, where; GC glomerular collapse; TD tubule degeneration; CMH cortical-medullar hemorrhages; and RI renal injury. Denotes statistical significance of at least P <.05 at the end of the study, in the different groups. Data are mean ± SD. a, b, and c denote statistical significance of at least P <.05

Measurements	Sham Group	Positive Group	Ozone Group
SAP (mm Hg)	110±14 a	175±18 b	128±19 c
Protein Excretion (mg/24h/100gr rat)	1.28±0.22 a	9.70±2.05 b	3.08±0.75 c
Potassium Excretion (mEq/24h/100gr rat)	32.4±11.5 a	23.4±9.3 b	31.6±8.6 a
Sodium Excretion (mEq/24h/100gr rat)	485±57 a	345±60 b	470±41 a
Plasma Creatinine (µmol/l)	94±18 a	170±14 b	108±10 c
RPF (ml/min/100gr rat)	1.81±0.024 a	1.20±0.06 b	1.87±0.023
GC %	0 a	100 b	20 c
TD %	0 a	100 b	10 c
CMH %	0 a	100 b	20 c
RI %	0 a	100 b	25 c

The immunoreactivity of the interleukin number 6 (IL-6) in the renal tissue for the sham group was negative, nevertheless in the positive control group showed a big immunoreactivity for IL-6 at tubules, glomerular, renal interstitium and renal cortex level respectively. The Ozone group at 0,5 mg/kg, showed very scarce and diffuse immunoreactivity for the IL-6 in most tubules.

The immunoreactivity of the interleukin number 6 (IL-6) in the different groups: Sham group (a) not immunoreactivity for IL-6, ozone group (b) scarce and diffuse immunoreactivity for the IL-6, positive control group (d) with strong immunoreactivity.



a = Sham Group.

b= Ozone Group:

d = Positive Control Group

Subtotal nephrectomy induced a significant increase in TBARS (P = .0253) , whereas applications of ozone/oxygen gaseous mixture after subtotal nephrectomy . the levels of positive control group (Table 2). SOD activity was significantly decreased to 55% (P = .0143) in positive control group, but, in ozone group, 39% of the enzymatic activity was recovered (P = .0253). Ozone therapy after subtotal nephrectomy induced a total increase in SOD activity of 94% (P = .0281). A similar behavior was observed for CAT enzymatic activity. In positive control group there was a significant decrease , whereas in ozone group a recovery was observed (P = .0143) in the enzymatic activity of CAT, indicating total increase in CAT activity of 268% (P = .0034). GSH concentration and GSH-Px enzymatic activity were not significantly affected by subtotal nephrectomy in this animal model, but when fifteen intrarectal applications of ozone/oxygen gaseous mixture were applied we observed a significant stimulation of GSH-Px, as well as an increase in GSH concentration.

Table.2. Renal concentration of different biochemical parameters, thiobarbituric acid reactive substances (TBARS), catalase (CAT), superoxide dismutase (SOD), reduced glutathione (GSH), and glutathione peroxidase (GPx), at the end of the study in the experimental groups. One unit of SOD enzymatic activity is equal to the amount of enzyme that diminishes the initial absorbance of nitroblue tetrazolium by 50% and CAT activity is described as the enzymatic activity quantity that transforms 1 mol of H2O2, at room temperature, at 15 min/g of wet tissue. International units are expressed as μmol of transformed hydroperoxides/min/mL of GPx. a, b, and denote statistical significance of at least $P < .05$

Biochemical Parameters	Sham Group	Positive Control Group	Ozone Group
TBARS(nmol/mg/protein)	0.22±0.02 a	0.67±0.025 b	1.18± 0.32 c
CAT (K15/g of wet Tissue)	7.80±0.39 a	2.47±0.35 b	13.12±3.8 c
SOD (units/mg protein)	8.73±1.16 a	3.77±0.23 b	16.65±0.33 c
GSH (nmol/mg protein)	8.85±1.90 a	8.82±1.85 a	15.29±3.20 c
GPx (IU/mg protein)	6.02±0.25 a	7.05±0.94 a	11.47± 2.23 c

DISCUSSION

Chronic Renal Failure (CRF) in the kidney prove maladaptive in renal tissue , predisposing to sclerosis of residual glomerular population where the intrarenal vasculature is the most affected, therefore, improvement of rheological properties of blood could delay the progression of CRF.It has been clearly demonstrated that ozone therapy has a cytoprotective effect in kidneys submitted to CRF, beneficial effects of

using ozone oxidative post-conditioning have also been reported in different animal models such as diabetes or using adriamicine or cisplatin as nephrotoxic drugs.

The mechanism of protection induced by ozone therapy by rectal insufflation in the CRF in rats, has been shown to target the important mediators of lethal renal injury for example: by reducing oxidative stress as demonstrated in this study, such as decreasing calcium overload, improving endothelial function, increasing adenosine levels, and attenuating the apoptosis of renal cells, among others. 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.

Analyzing the results of renal function, significant decreases in RPF and GFR occurred in the positive control group compared with the ozone and

sham control groups. Ischemia damages the endothelial cells and when the blood flow decreases, shearing forces increase, producing more ROS release, damaging even more of the endothelium, ozone therapy increase tissue oxygenation via its haemorheological effects, increasing erythrocyte pliability, possibly diminishing blood viscosity and erythrocyte aggregation, allowing a higher renal blood flow in an organ previously submitted to a lack of blood.

The Protein Excretion was higher in positive control group compared with the ozone group, plasma creatinine concentration was lower in ozone group compared with the positive control group, The SAP behavior in ozone group was similar to sham group. In our opinion the ozone therapy has antiplatelet activity, diminishing blood viscosity, that could produce a decrease in the friction between the blood and the glomerular vascular walls, beside the flow rise contributes to diminishing the endothelial injuries and the glomerular collapse, avoiding the tubular hypoxia, the hemorrhages, and the release of several proinflammatory cytokines such as IL-6,IL-1 and TNFα .The pathological levels of ROS have been demostrated to be capable of degrading glomerular basement membrane and inducing glomerular injury chacterized by impaired glomerular filtration and increase of protein excretion.

With respect to the behavior of antioxidant enzymes in residual kidney mass, ozone therapy after subtotal nephrectomy induced a total increase in SOD,CAT,GPx activity and GSH levels, causes reduced in radical damage mediated by ROS. The ozone postconditioning may be linked to Nrf2/EpRE (nuclear factor erythroid 2/electrophile-responsive element) activation pathway in vivo. Lamberto R demonstrated that levels of Nrf2 in peripheral blood mononuclear cells (PBMC) were found to increase immediately

after ozone/oxygen exposure (35 mg/ ml, prior to reinfusion)These data demonstrate for the first time in vivo the activation of the Nrf2 pathway by a low dose of ozone and the promotion of the feedback mechanism that induces the synthesis of proteins which collectively favors cell survival. Ozone can increase the level of nuclear translocated Nrf2, which is associated with an increase in Nrf2 protein translocation from the cytoplasm to the nucleus. As a consequence Nrf2 increased the activity of the antioxidant enzymes.Hui Chen showed some of mechanisms associate to the effects of ozone therapy in the prevention of renal injury during the ischaemia reperfusion phenomenon. In these work ozone therapy was able inhibit inflammation by reduce of caspase activity and proinflammatory interleukins (TNFα and IL-1).In our study ozone therapy was able to reduce the interleukin number 6 (IL-6) immunoreactivity in renal tissue compared to positive control group. It is very important because of IL-6 is a proinflammatory interleukins associated with damage of glomerular capillaries and kidney tubules in CRF.
Histological studies showed that lesions were most severe in the positive control group (glomerular collapse (GC), tubule degeneration (TD) and cortical-medullary hemorrhages (CMH), correlating well with the groups that did not show a cytoprotective effect, in the other hand lesions were moderate in the ozone group, proving that the renal damage is minor compared with the positive control group. From a structural point of view, ozone treatment favours kidney recovery.

CONCLUSIONS

Ozone Therapy favours the functional and structural recovery of renal tissue submitted to CRF through mechanisms that promote the maintenance of adequate cellular redox balance, improvements in blood circulation and oxygen metabolism and decrease proimflammatory interleukin such as IL-6 in renal tissue after CRF.

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