EFFECTS OF OZONE OXIDATIVE POSTCONDITIONING ON RENAL TUBULAR EPITHELIAL CELLS AGAINST ISCHEMIA/REPERFUSION INJURY IN RATS.

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ABSTRACT
Ischemia/reperfusion injury is seen in renal surgery or transplantation. It is a major cause of acute renal failure. Since ozone oxidative preconditioning attenuates renal ischemia/reperfusion injury, morphology of tubular epithelial cells was investigated in order to prove that ozone oxidative postconditioning reduces renal ischemia/reperfusion injury. Twenty adult Wistar rats were divided into four groups: control, ischemia/reperfusion damaged (60 min of ischemia, with 10 days of reperfusion), and two groups of rats submitted to ischemia and postconditioned during reperfusion: one group with ozone (0.5 mg/kg body weight) and another one with oxygen (13 mg/kg body weight). Kidneys were fixed in buffered formalin, paraffin-embedded and stained with HE and PAS. Five variables were analyzed in renal proximal tubules in 10 fields per section, by means of a mathematical model designed for this study. Groups were compared taking into account the percentage of damaged proximal tubules per field for each variable by applying the statistical program Graph Pad Prism version 5.00 for Windows. Normal structure was observed in control group. Loss of brush border, discontinuity and denudation of tubular basement membrane, presence of peritubular inflammatory cells and cell necrosis was noticed in ischemia/reperfusion damaged group. Remarkable conservation of proximal tubules was observed in ozone-postconditioned. By contrast, in oxygen-postconditioned group, tubular morphology was similar to ischemia/reperfusion damaged group.

Keywords: ozone oxidative postconditioning, ozone therapy, renal proximal tubules, ischemia reperfusion, computerized morphometry.

EFECTO DEL POSTCONDICIONAMIENTO OXIDATIVO CON OZONO SOBRE LAS CÉLULAS EPITELIALES TUBULARES RENALES CONTRA EL DAÑO POR ISQUEMIA/ REPERFUSIÓN EN RATAS.

RESUMEN
El daño por isquemia/reperfusión ocurre durante la cirugía o el trasplante renal. Es una causa importante de insuficiencia renal aguda. El precondicionamiento oxidativo con ozono disminuye este daño. En este trabajo se estudió la morfología de las células epiteliales de los túbulos proximales renales para probar que el postcondicionamiento oxidativo con ozono protege del daño por isquemia/reperfusión. Veinte ratas Wistar adultas se dividieron en cuatro grupos: control, sometidas a isquemia/reperfusión (60 minutos de isquemia, con 10 días de reperfusión) y dos grupos de ratas sometidas a isquemia y postcondicionadas durante la reperfusión: un grupo con ozono (0,5 mg / kg de peso) y otro con oxígeno (13 mg / kg de peso). Los riñones se fijaron con formol tamponado, se incluyeron en parafina y se colorearon con HE y PAS. Se estudiaron cinco variables en los túbulos proximales renales en diez campos por sección, por medio de un programa matemático diseñado para este estudio. Los grupos se compararon teniendo en cuenta el porciento de túbulos proximales dañados por campo para cada variable mediante el programa Graph Pad Prism versión 5.00 para Windows. El grupo control mostró estructura normal. El grupo dañado por isquemia/reperfusión presentó pérdida del borde en cepillo, discontinuidad y denudación de la membrana basal tubular, presencia de células inflamatorias peritubulares y necrosis celular. Las ratas postcondicionadas con ozono mostraron conservación notable de los túbulos proximales renales. Por el contrario, en el grupo postcondicionado con oxígeno, la morfología celular tubular fue similar al grupo dañado por isquemia/reperfusión.

Palabras claves: poscondicionamiento oxidativo con ozono, terapia de ozono, túbulos proximales renales, isquemia reperfusión, morfometría computarizada**.**

INTRODUCTION

Ischemia-reperfusion (I/R) damage occurs under several clinical conditions [1] and it is one of the most frequent causes of acute renal failure (ARF). ARF is due to toxic or ischemic injury to the kidney and clinically it is called acute tubular necrosis (ATN) [2].

ARF occurs in around 5% of the hospitalized patients, and around 10% of them need hemodialytic therapy [3]. Two to ten percentages of patients that survive to ARF develop terminal renal failure and need to be dialyzed for long periods of time [4,5]. The occurrence of ARF is associated with an increment of the mortality and with high costs of health care [5,6].

Kidneys are especially sensible to ischemic damage in situations such as: renal transplant, repair of suprarenal aneurysm, reconstruction of renal artery, contrast-induced nephropathy, heart failure and shock. [7,8,9].

The events conducting to I/R damage are complex: ischemia begins a sequence of interrelated events that produce lesion and cell death due to the suppression of the blood flow, deoxygenation and further reperfusion. Although reperfusion is the most effective strategy to limit the damage of the organ, it could cause cell death due to the production of reactive oxygen species (ROS) [10]. Renal proximal tubules epithelial cells are especially susceptible to I/R, producing an ATN [11].

Experimentally, the production of ROS in renal tissue submitted to I/R has been reduced by means of previous exposures to ischemia (ischemic preconditioning) [12] or using drugs (pharmacological preconditioning) [13,14].

As recently demonstrated, short repetitive cycles of ischemia during reperfusion significantly reduce the extension of heart attacks in dogs [15]. Other authors have reported that ischemic postconditioning limits mitochondrial failure and prevents ROS production in isolated hearts, concluding that this methodology reduces the general functional damage and protects cell structure [16].

Nowadays these new protective strategies (ischemic postconditioning [17,18] and pharmacological postconditioning) [19] are being applied during reperfusion of ischemic kidney.

Ozone exerts a protective effect against renal I/R damage in rats when it is applied as preconditioning [20,21]. Recently the beneficial effect of ozone postconditioning was demonstrated by means of biochemical and metabolical markers of renal function and by morphological qualitative studies of renal tissue [22].

The main histopathological characteristic of renal I/R is the lesion of proximal tubules, due to epithelial cell death by necrosis or apoptosis [23]. The main cause of the development and progression of acute renal injury after I/R is the dysfunction of the epithelial cells of proximal tubules [24].

The aim of this study was to investigate the effect of ozone oxidative postconditioning on lesions indicating acute tubular necrosis in epithelial cells of proximal tubules of kidneys from rats submitted to I/R, using a mathematical program designed for this study.

MATERIALS AND METHODS

Animals

Twenty healthy female Wistar rats from the National Center for the Production of Laboratory Animals (CENPALAB), weighting 220-250 g were used in this study. Rats were fed with standard commercial diet and water ad libitum. Animals were randomly divided into four groups of 5 rats each one: negative control group (non-treated), I/R: positive control group (submitted to I/R), P-Oz: ozone-treated group (0,5 mg/kg body weight) and P-Ox:oxygen-treated group (13 mg/kg body weight).

Surgical procedure for renal I/R and treatments

Rats were anesthetized with sodium pentobarbital (30 mg/kg body weight, i.p) and were given heparine (50 UI, i.p). Laparotomy was them 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 ischemia, the vascular renal packages were exposed, and both renal arteries were isolated and cross-clamping, provoking an ischemia during 60 min. The animals were placed under an overhead lamp in order to avoid anesthetic hypothermia. The temperature was monitored continually by using a clinical thermometer in the rectal area. Then the clamps were removed, allowing the reperfusion of the kidneys and the treatments were administered during 10 days. The P-Oz group was treated with ozone/oxygen gas mixture, administered by rectal insufflations performed with a plyethylene 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). The ozone was generated using OZOMED equipment (Ozone Research Center, Havana, Cuba), 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 [22,25]. The P-Ox group was treated in the same way as the P-Oz group, but insufflating with oxygen alone (13/kg, bw). This group was included to determinate the effect of the oxygen in the oxygen/ozone gas mixture. I/R group received no further treatment. In the eleventh day, the left kidney of each animal was removed for the histological study. Finally, the animals were sacrificed by ethyl ether inhalation.

Histological study

Kidney samples from all animals were fixed in 10% buffered (phosphate buffer 0.01 mmol/L, ph 7.4) formalin, dehydrated and paraffin-embedded. Then, 3 µm-thick sections were cut, deparaffinized, hydrated and stained with hematoxylin and eosin and with periodic acid- Schiff (PAS) reagents. The sections were observed by Light microscopy using a Nikon 50i, at 1000 X. The images were obtained by means of a high resolution digital camera DS-5M-U1 connected to the microscope.

Morphological and morphometric analysis

The presence of the following variables was determined in transversely cut tubules:

1. Necrotic cells (cells with cytoplasm vacuolization, nuclear alterations (fragmented, ballooned and pyknotic nuclei) or with loss of the cellular integrity),
2. Loss of the brush border,
3. Discontinuity and denudation of the tubular basement membrane,
4. Detached cells and tubular casts in the lumen,
5. Peritubular inflammatory cells.

One histological section per animal was studied, 10 fields of the deep cortex region from the superior to the inferior kidney pole were evaluated, with an average from 30 to 35 proximal tubules per animal (150 to 165 tubules for study group). The evaluation of the state of each variable was carried out (normal histological condition was considered 0 and pathological condition was considered 1.

For the quantification of these variables a program developed for .Net platform, using the integrated development environment Visual Studio 2008 and c# language, was designed for this study. For each variable the percentage of damaged tubules per field was determined by means of this program.

Statistical analysis

For each variable the percentage of damaged tubules per histological field was compared between groups by applying the statistical program Graph Pad Prism version

5.00 for Windows. The mean values and standard deviations were determined by means of descriptive statistic. For the analysis of the differences among groups, the non parametric t tests Kruskal-Wallis and Test of multiple comparisons of Dunn were carried out.

RESULTS

Morphological results

In negative control group the normal structure of renal cortex proximal tubules was observed (Fig. 1).

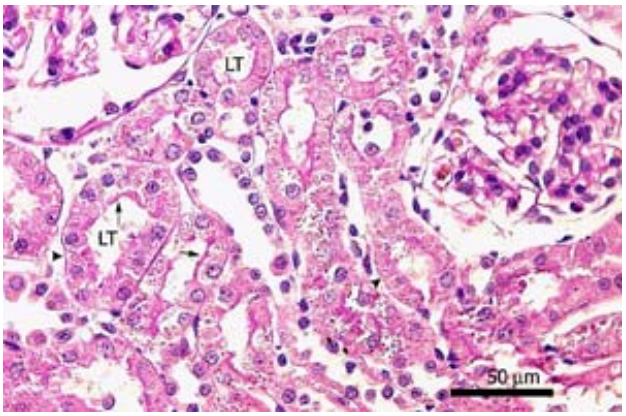

Fig. 1. Control group. Renal tubules, Arrow: brush border, LT: tubular lumen.

In I/R group renal cortex showed patchy damage related to: loss of brush border, discontinuity and denudation of the tubular basement membrane, necrotic cells and presence of peritubular inflammatory cells (Fig. 2).

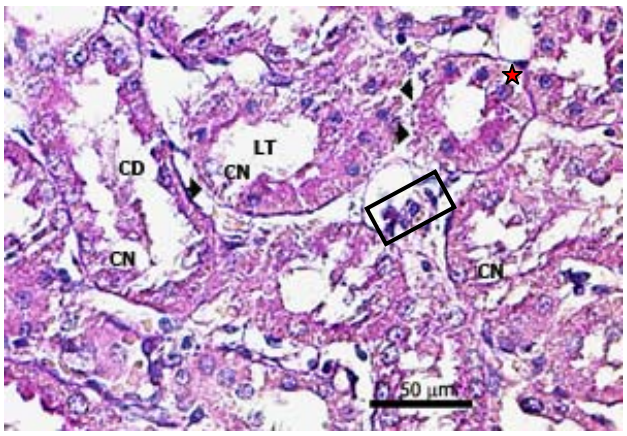

Fig. 2. Ischemia/Reperfusion group. Renal tubules, Arrow tips: discontinuous tubular basement membranes, ★ tubular denudation, CN: residual necrotic cells, LT: tubular lumen, CD: necrotic cells within tubular lumens rectangle: inflammatory cells.

In P-Oz group preservation of normal tubular morphology was seen in most of the tubules, regarding to: presence of brush border, continuity of tubular basement membranes. However slight deterioration of some proximal tubules was observed (Fig. 3).

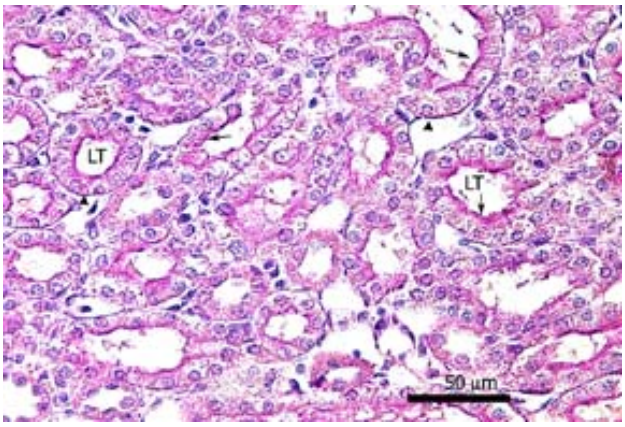

Fig. 3. Ozone postconditioned group. Renal tubules, Arrows: brush borders, arrow tips: continuous tubular basement membranes, LT: tubular lumen.

The kidneys of the group P-Ox exhibit large areas of damaged proximal tubules (Fig. 4).

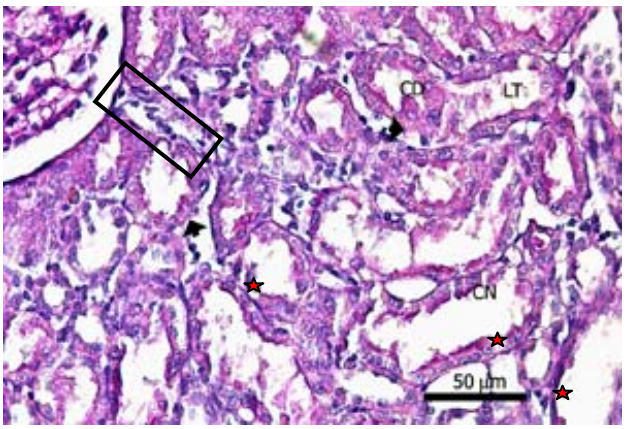

Fig. 4. Oxygen postconditioned group. Renal tubules. Arrows: brush borders, arrow tips: discontinuous tubular basement membranes, ★ tubular denudation, CN: residual necrotic cells, LT: tubular lumen, CD: necrotic cells within tubular lumens, rectangle: inflammatory cells.

Morphometric results

In control animals a low percentage of damaged tubules showing necrotic cells, loss of the brush border, discontinuity and denudation of the tubular basement membrane and presence of peritubular inflammatory cells was obtained. I/R significantly increased the percentage of tubules with: necrotic cells, loss of the brush border, discontinuity and denudation of tubular basement membrane, detached cells and casts in tubular lumen, and presence of peritubular inflammatory cells (Fig. 5).

Ozone postconditioning after I/R damage induced significant reduction in the percentage of tubules showing loss of brush border and presence of peritubular inflammatory cells to values approaching those of control group; whereas necrotic cells and discontinuity and denudation of the tubular basement membrane significantly decreased, but the values were not close to those of the control group. However, this treatment induced only a slight decrease of the percentage of tubules with detached cells and casts with respect to I/R group (Fig. 5).

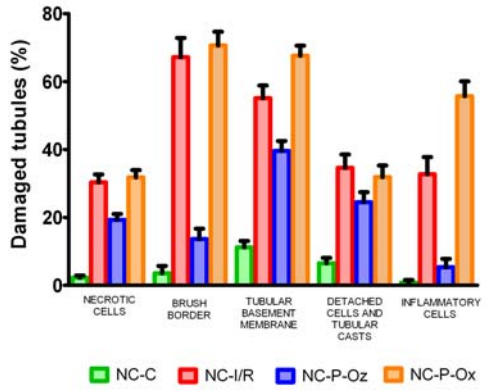

Fig. 5. Morphological variables analysed in renal cortex proximal tubules. Means and standard deviations, $p< 0.05$.

DISCUSSION

ROS play a decisive role during functional and structural deterioration of the kidney submitted to ischemic damage, mainly when this damage exceeds 45 min of abolition of the renal circulation [21]. The increase of ROS has been correlated with a decrease of the renal function in models of renal I/R [20,21].

Ozone has a direct influence on the metabolism of oxygen. This achieves it for the increment of the partial pressure of oxygen in blood and tissues; for their effect of stimulating the metabolism of erythrocytes and to avoid the disposition of the erythrocytes in piles of coins, improving this way the blood flow and therefore, the oxygen transport to the tissues [26]. Ozone-therapy induces the activation of enzymes involved in the degradation of peroxides and free radicals [25].

Ozone oxidative preconditioning has showed a cytoprotective effect on liver and kidney submitted to ischemia/reperfusion, when inducing the activation of the anti-oxidative endogenous defense processes [27,21]. The protective effect of ozone oxidative preconditioning against I/R damage has been observed on renal proximal tubules´ morphology, in experiments of this group [28].

Recently the beneficial effect of ozone oxidative postconditioning was demonstrated by means of biochemical (fructosamine, A2 phospholipase, catalase, peroxide dismutase and thiobarbituric acid reactive substances) markers and morphological qualitative studies of the structure of renal tissue [19].

Previous findings have been confirmed in this work, since the treatment with ozone induced recovery of proximal tubule epithelial cells of renal cortex. It was evidenced in a significant decrease of the percentage of damaged tubules regarding I/R group, in contrast to the oxygen-treated group.

In group P-Ox, the values were similar to those found in the group submitted to I/R, except for the variable peritubular inflammatory cells that appeared higher than in I/R group. In oxygen postconditioned animals, the lesions due to I/R were increased by the liberation of more ROS and products of the peroxidation, with a depletion of the anti-oxidant defense system to counteract the increase of ROS [22].

CONCLUSIONS

Ozone postconditioning protected the morphology of the renal proximal tubules, after 60 min of ischemia and 10 days of reperfusion. Therefore, ozone postconditioning could be considered as an efficient tool against renal ischemia-reperfusion damage in rats, when administered, not only before the injury (ozone preconditioning) but also after the insult (postconditioning). Postconditioning treatments, in contrast with the preconditioning ones, could be clinically used in vascular surgery or renal transplant services.

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