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Ozone Treatment Reduces Blood Oxidative Stress and Pancreas Damage in a Streptozotocin-Induced Diabetes Model in Rats

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SUMMARY. In spite of the fact that ozone has been used as a therapeutical agent and beneficial effects have been observed, so far only a few biochemical and pharmacodynamic mechanisms have been studied. We have demonstrated that controlled ozone administration may promote an oxidative preconditioning or adaptation to oxidative stress, preventing the damage induced by Reactive Oxygen Species (ROS) through preservation of antioxidant endogenous systems. Taking into account that STZ produces ROS generation, which promotes pancreas damage with loss of its function, we studied ozone effects on blood oxidative stress and its relationship with pancreas injury mediated by STZ. Five groups of rats were classified as follows: (1) Non-diabetic control group treated only with citrate buffer solution; (2) positive control group using as a diabetes inductor; (3) Ozone group, receiving 10 treatments (1.1 mg/kg) one per day after STZ-induced diabetes; (4) Oxygen (26 mg/kg) one per day, as in group 3 but using oxygen only; (5) control ozone, as group 3, but without STZ. Ozone + STZ treatment improved glycemic control with regard to STZ group (16.1 ± 1.45 vs 27.12 ± 2.12 mmol/L). Blood oxidative stress was controlled by ozone as it was showed in the reduction of malondialdehyde, total hydroperoxides and peroxidation potential. In addition, antioxidant endogenous systems were increased (superoxide dismutase, catalase, glutathione peroxidase and reduced glutathione). In line with these results, there was a decrease in the percentage of damaged pancreatic islets by ozone treatment. Ozone antioxidant properties preserved β-cells functions and reduced hyperglycemia. Taken together, these results suggest that this complementary medical approach may represent a potential alternative in the treatment of diabetes and its complications.

RESUMEN. "La Ozonoterapia reduce el Estrés Oxidativo en Sangre y los Daños en Páncreas in la Diabetes inducida por Estreptozotocina". A pesar de que la ozonoterapia se ha utilizado con éxito en la terapia con efectos beneficiosos, se han realizado pocos estudios bioquímicos o farmacodinámicos dirigidos al estudio de su mecanismo de acción. Hemos demostrado previamente que la administración controlada del ozono promueve el precondicionamiento oxidativo o adaptación al estrés, fenómeno que previene del daño causado por las Especies Reactivas del Oxígeno (ERO) mediante la preservación de los sistemas antioxidantes endógenos. Teniendo en consideración que la estreptozotocina (STZ) es un ageneradosr de ERO que promueve el daño pancreático, estudiamos el efecto del ozono sobre marcadores sanguíneos del estés oxidativo y su relación con el daño tisular originado por STZ. Se trabajó con 5 grupos experimentales: (1) control no diabético, tratado solo con solución amortiguadora citrato, (2) Control positivo, al que se le inoculó el agente inductor de daño STZ, (3) grupo tratado con ozono, que recibió 10 tratamientos (1,1 mg/kg) antes de inducir el daño con STZ, (4) grupo tratado con oxígeno Oxygen (26 mg/kg) con la misma frecuencia de tratamientos que el grupo (3) y (5) control ozono, que recibió un tratamiento similar a grupo 3 pero no se indujo daño con STZ. El grupo tratado con ozono + STZ produjo una mejoría del control glicémico en comparación con el grupo tratado con STZ (16,1 ± 1,45 vs 27,12 ± 2,12 mmol/L). El ozono controló el estrés oxidativo en plasma al reducir las concentraciones de malondialdehido, hidroperóxidos totales y el potencial de peroxidación. Adicionalmente, el sistema de antioxidantes endógenos se incrementó (superóxido dismutasa, catalasa, glutatión peroxidasa y glutatión reducido). En correspondencia con estos resultados se observó una disminución en el porcentaje de daño de los islotes pancreáticos en el grupo tratado con ozono. Las propiedades antioxidantes del ozono preservaron la función de las células β-pancreáticas y redujeron la hiperglicemia. Este conjunto de resultados sugiere que el ozono como terapia complementaria representa una alternativa potencial en el tratamiento de la diabetes y sus complicaciones.

KEY WORDS: Oxidative stress, Ozone, Streptozotocin-induced diabetes.
PALABRAS CLAVE: Diabetes inducida por estreptozotocina, Estrés oxidativo, Ozono.

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INTRODUCTION

There is a clear link between diabetes and oxidative stress. Hyperglycemia leads to Reactive Oxygen Species (ROS) generation and alteration of endogenous antioxidants [1]. These events are associated with diabetes complications such as the development of diabetes-specific microvascular pathology in the retina, renal glomerulus and peripheral nerve. Diabetes is also related with accelerated artherosclerotic macrovascular disease, affecting arteries that supply the heart, brain and lower extremities. As a result, patients with diabetes have a much higher risk of myocardial infarction, stroke and limb amputation [2,3].

Four main hypotheses about how hyperglycemia causes diabetes complications have generated a large amount of data as well as several clinical trials based on specific inhibitors of these mechanisms. The four hypotheses are: increased polyol pathway flux, increased advanced glycation end-product (AGE) formation; activation of protein kinase C (PKC) isoforms and increased hexosamine pathway flux [4].

It has been proposed that an important role for radical anion superoxide ($O_2^{\cdot -}$), generated during the mitochondrial electron-transport chain, is as the unifying factor that links the four pathological events [4]. There seems to be a threshold above which $O_2^{\cdot -}$ production is markedly increased. It has been found that hyperglycemia increases the proton gradient above this threshold value as a result of overproduction on electron donors by the tricarboxylic acid (TCA) cycle [5]. Superoxide anions can be dismutated to hydrogen peroxide which generates very reactive hydroxyl radicals by Fenton chemistry. This ROS is able to oxidize lipids, proteins and DNA, with concomitant changes in their structures and function.

Ozone employed for therapeutic purposes is a gas (O_2 + O_3) mixture in which ozone represents only about 3%. Its beneficial actions have been demonstrated at experimental and clinical levels [6-11]. Ozone administered by rectal insufflation in dose and number of controlled treatments has shown protective effects against the damage induced by carbon tetrachloride and hepatic and renal ischemia-reperfusion. The postulated protective mechanism was “Oxidative Preconditioning” which confers protection by stimulation of antioxidant endogenous systems, decreasing glycogen depletion and lactate production. Ozone protected Ca^{2+}-ATPase from inactivation by oxidative stress, regulated accumulation of adenosine and blocked the xanthine/xanthine oxidase pathway for ROS generation [6-10]. In addition, it has been demonstrated that there is a decrease of blood cholesterol and a stimulation of antioxidative response in cardiopathy patients with intravenous ozone therapy [11].

Recently, we have shown in pancreas homogenates the effects of ozone on the streptozotocin-induced diabetes model. Ozone treatment improved glycemic control, regulated the redox balance, reduced markers of polyol pathway and the non-enzymatic glycosylation of proteins, and increased the levels of nitrates and nitrites as a measure of nitric oxide (NO) production [12].

Diabetes and its complications are associated with oxidative stress, ozone treatment regulates the redox balance and markers of oxidative and endothelial damage in pancreas homogenates are present in a model of experimental diabetes. Because of this, we investigated the actions of ozone on systemic mediators of oxidative injury in plasma of streptozotocin-induced diabetes rats and their correspondence with the pancreatic damage since streptozotocin (STZ) has been implicated in ROS generation associated to experimental diabetes [13].

MATERIALS AND METHODS

Animals

Male Sprague-Dawley rats with an initial body weight of 250-278 g were obtained from CENPALAB (Bejucal, Havana, Cuba). Animals were housed in temperature and light-controlled rooms and allowed free access to normal diet pellet and tap water. All procedures were performed as approved by the International Animal Care Committees (ARCA N° 012) and in accordance with the European Union Guidelines for animal experimentation.

Induction of experimental hyperglycemia

Experimental diabetes was induced by a single intraperitoneal (i.p.) injection of 45 mg/kg streptozotocin (Sigma, St. Louis, MO, USA) to overnight food-deprived rats [14]. STZ was dissolved in citrate buffer solution (0.1 M, pH 4.5) and freshly prepared immediately before each injection. Animals were considered hyperglycemic when non-fasting serum glucose levels were higher than 20 mM after 48 h of STZ injection [15]. Afterward, the different treatments were started as mentioned below. Blood glucose was measured using a diagnostic kit obtained from Sigma 315-100 (Sigma, St. Louis, MO, USA) based on a colorimetric reaction.

Treatment schedule

The protocol consisted of five experimental groups (n =10, each): (1) Non-diabeatic control group treated only with citrate buffer solution; (2) positive control group using STZ as a diabetes inductor; (3) Ozone group, receiving 10 treatments (1.1) mg/kg, dose of ozone in which the phenomenon of oxidative preconditioning is achieved without appreciable toxicity [6-10] one per day after STZ-induced diabetes; (4) Oxygen, vehicle of O_3 (26 mg/kg), dose equivalent to the O_2 concentration present in one O_3 dose) one per day, as in group 3 but using oxygen only; (5) Control ozone, as group 3, but without STZ. The ozone concentration in the O_3/O_2 mixture was 50 µg/mL.

Ozone was generated by OZOMED equipment, manufactured by the Ozone Research Center (Cuba), and was administered by rectal insufflation. Ozone was obtained from medical grade oxygen, was used immediately and it represented only about 3% of the gas (O2 + O3) mixture. The ozone concentration is measured by using a built-in UV spectrophotometer at 254 nm (accuracy, 0.002 A at 1A, repeatability 0.001 A and calibrated with internal standard). The ozone dose is the product of the ozone concentration (expressed as mg/L) by the gas (O2 + O3) volume (L). By knowing the body weight of the rat the ozone dose is calculated as mg/kg as in our previous papers [6-10].

After 11 days of diabetic induction, blood glucose was measured, body weight of the animals was monitored and then they were euthanized by diethyl ether anesthesia. Immediately after, blood samples were obtained from the abdominal aorta and mixed with 3.8% sodium citrate, used as an anticoagulant, for biochemical determinations. The samples were maintained at -70 °C.

Biochemical determinations

The biochemical parameters were evaluated in plasma 11 days after STZ-induced diabetes and 24 hafter the last treatment with ozone or oxygen, as it corresponded.

The different parameters were determined by spectrophotometric methods using an Ultrospect Plus Spectrophotometer from Pharmacia LKB. Catalase activity was measured by following the decomposition of hydrogen peroxide at 240 nm at 10 sec intervals for one minute 16. Superoxide dismutase (SOD) and glutathione peroxidase (GSH-Px) were measured using kits supplied by Randox Laboratories Ltd., Ireland (Cat. N° SD125 and N° RS505). Concentrations of malondialdehyde (MDA) were analyzed using the LPO-586 kit obtained from Calbiochem (La Jolla, CA). In the assay, the production of a stable chromophore after 40 min of incubation at 45 °C was measured at a wavelength of 586 nm. For standards, freshly prepared solutions of malondialdehyde bis [dimethyl acetal] (Sigma St. Louis, MO, USA) were employed and assayed under identical conditions [17]. Quantification of total hydroperoxides was measured by Bioxytech H2O2-560 kit (Oxis International Inc., Portland, OR, USA) using xylenol orange to form a stable colored complex, which can be measured at 560 nm. Total protein concentration was determined by the method of Bradford with bovine serum albumin as standard [18]. After precipitation of thiol proteins using TCA 10%, the reduced glutathione (GSH) were measured according to the method of Sedlak and Lindsay [19] with the Ellman's reagent (5,5' dithiobis (2-nitrobenzoic acid) 10-2M (Sigma St. Louis, MO, USA), the absorbance was measured at 412 nm . Peroxidation Potential was determined by incubating the serum with copper sulphate at 37 °C for 24 hours. The final concentration of copper sulphate in the medium was 2 mM. Serum Peroxidation Potential (PP) was estimated by taking the difference between lipid peroxide values (MDA) at 24 hand 0 h [20].

Histopathology

Since the anatomy of the pancreas of the rats is diffuse, the organ was removed and fixed in 10% buffered formaline and the samples were taken at random. The paraffin sections were stained with hematoxilin-eosin and Gomori's aldehide fuschin for the histopatholicical study identification of pancreatic β-cells [21].

A total count of pancreatic islets was made in the sections of animals corresponding to STZ-induced diabetes and ozone preconditioned groups (n = 10) to determine the percentage of damaged islets.

The qualitative morphological indicators taken in consideration for the damage were apparent absence of β-cells and vacuolar degeneration or necrosis of such cells [22].

Statistical analysis

The OUTLIERS preliminary test for detection of error values was initially applied for statistical analysis. Afterward, the homogeneity variance test (Bartlett-Box) followed by ANOVA method (single way) was used. In addition, a multiple comparison test was employed (Duncan test). The Mann-Whitney U test was used to compare

the percentages of damage pancreatic islets. Data were expressed as the mean ± standard deviation of 10 animals. The level of statistical significance employed was at least $p < 0.05$ for all experiments.

RESULTS

Body weights and blood analysis

Rats treated with streptozotocin (STZ) and STZ + O2 were hyperglycemic (27.12±2.12 26.19±1.34 mM of plasma glucose concentration 11 days after STZ-induced diabetes, respectively) and lost weight over the experimental period (- 30.26±14.59 and - 16.27±14.40 g, changes between End and the Start of the experiment, respectively). Ozone treatment reduced hyperglycemia by 40% in comparison with STZ-treated rats (16.1±1.45 mM, 11 days after STZ-induced diabetes). Body weight of these rats increased in similar way as non-diabetic control (Ozone +38.20±16.15, Non-diabetic control + 41.52 ±18.16 g).

Blood Redox Balance

Concentrations of SOD, CAT, GSH-Px, and GSH were measured (Fig. 1A, B, C, D).

In ozone-treated rats SOD and CAT activities showed a similar trend (Fig. 1A, B). Nevertheless SOD activity increased and didn't show differences with non-diabetic control, STZ + O_2 or STZ groups while CAT raised with regard to control but it decreased in comparison with STZ + O_2 and STZ groups.

Ozone treatment increased GSH-Px activity with regard to the remaining groups (Fig. 1C). Similar patterns in GSH concentrations was observed (Fig. 1D).

High levels of MDA and total hydroperoxides are indicators of oxidative stress and they may be considered as indicators of injury (Table 1).

Ozone treatment ameliorated the increase in lipid peroxidation products induced by STZ. There were increases of total hydroperoxides and PP in STZ + O_2 and STZ groups. In contrast, these oxidative mediators were maintained at control level in STZ-induced diabetic rats treated with ozone (Table 1).

Histopathology

Percentages of damaged pancreatic islets in STZ and STZ + O_3 groups are shown in Table 2.

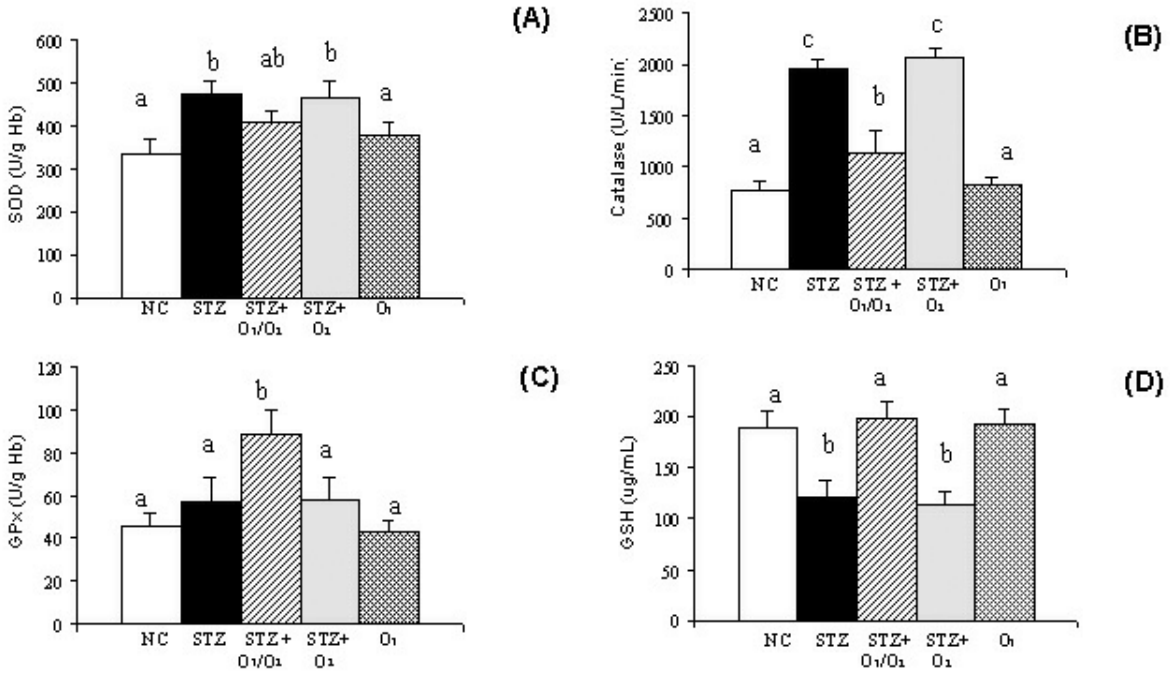

Figure 1. Antioxidant system markers in non-diabetic and diabetic rats: NC, non-diabetic controls; STZ, diabetic group induced by streptozotocin 45 mg/kg i.p.; STZ + O3/O2, diabetic group treated with ozone (1.1 mg/kg) 10 treatments by rectal insufflation; STZ + O2, diabetic group treated with oxygen, vehicle of ozone (26 mg/ kg) 10 treatments by rectal insufflation. Data are means ± SEM. Means having different superscript letters indicate significant difference ($p < 0.05$) between groups. (A) Superoxide Dismutase (SOD); (B) Catalase (CAT); (C) Glutathione Peroxidase (GSH-Px); (D) Glutathione (GSH).

Experimental Groups	MDA	TH	PP
Non-Diabetic Control	4.69 ± 0.39 ^{a}	9.99 ± 0.59 ^{a}	24.33 ± 3.49 ^{a}
Diabetic (STZ)	10.74 ± 0.38 ^{b}	15.14 ± 0.58 ^{b}	54.67 ± 5.47 ^{b}
STZ + Ozone	7.30 ± 0.34 ^{c}	9.40 ± 0.43 ^{a}	29.66 ± 4.22 ^{a}
STZ + Oxygen	12.41 ± 0.46 ^{b}	14.67 ± 0.66 b	52.66 ± 6.80 ^{b}
Ozone	5.03 ± 0.30 ^{a}	9.95 ± 0.43 ^{a}	22.93 ± 4.05 ^{a}

Table 1. Levels of Lipid Peroxidation Products (MDA), Total Hydroperoxides (TH) concentrations and Peroxidation Potential (PP) in non-diabetic and diabetic rats (μM). Data are means ± SEM. Means having different superscript letters indicate significant difference ($p < 0.05$) between groups.

It was observed a significant decrease ($p < 0.05$) in the percentage of damaged islets for STZ-induced diabetes rats treated with ozone with regard to STZ group.

Light microscopy micrographs showing Gomori's aldehyde fuschin staining of pancreas sections from non-diabetic and diabetic rats are shown in Figure 2 (A,B,C,D). It was clear β cells loss and the cellular vacuolization in STZ-diabetic rats (Fig. 2B). Similar morphological appearance was found in STZ + O_2 group with atrophic of pancreatic islets and severe injury (Fig. 2C). Ozone-treated rats were protected against pancreas damage induced by STZ (Fig. 2B). It was observed a normal islet's appearance with α and β cells preserved in a similar way to non-diabetic control group (Fig. 2A).

DISCUSSION

There is emerging evidence that diabetes leads to the depletion of the cellular antioxidant defense system and increased levels of ROS. The concept of oxidative stress, being an important trigger in the onset and progression of diabetes and its complications, may offer a unique therapeutic option for the treatment of diabetes and its complications using antioxidants or nutrients with high antioxidant capacity. Antioxi-

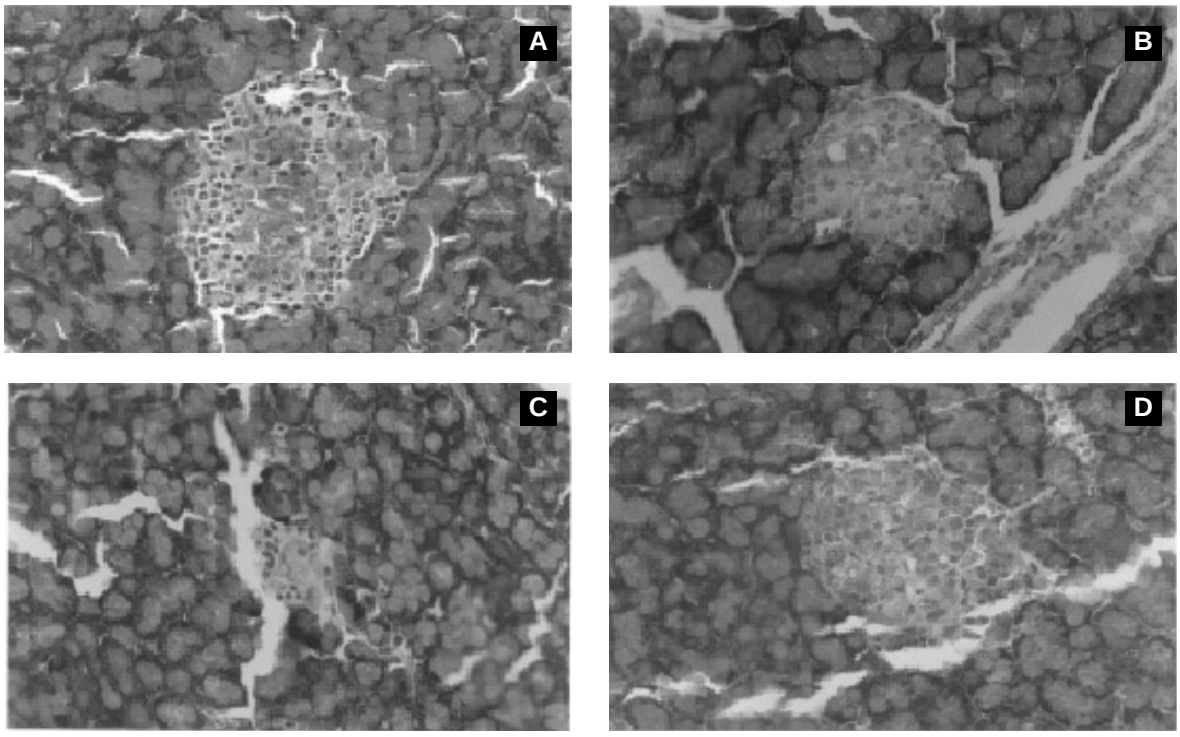

Figure 2. Histological results in non-diabetic and diabetic rats: (**A**) Non-diabetic control group. Normal appereance of β-cells (dark blue) and (-cells (red); (**B**) STZ-induced diabetes. Loss of β-cells and cellular vacuolization; (**C**) STZ-induced diabetes + Oxygen. Severe damage with atrophic of pancreatic islets; (**D**) STZ-induced diabetes + Ozone. Normal aspect of the islets (β-cells, blue; α-cells, red). Gomori, 400 x.

dants have been shown to reduce indices of oxidative stress measures in experimental disease model and -most importantly- also in humans [23].

There is much evidence from experimental studies that the formation of ROS is a direct consequence of hyperglycemia [23]. The oxidative stress due to ROS generation may play an important role in the initiation of the pathophysiological cascade of events leading to vascular and other diabetic complications [24]. In this way, loss of antioxidant-pro-oxidant balance represents a linking between diabetes and its complications.

The group treated with oxygen (vehicle of the ozone) didn't differ from the STZ-induced diabetic rats and the group treated only with ozone didn't show differences with regard to non-diabetic control.

Blood redox balance was favored by ozone treatment. SOD and Catalase enzymes showed a light increase while GSH-Px activity raised by two-fold in STZ + O_3 in comparison with non-diabetic control group (Fig. 1 A, B, C). There seems to be a correspondence between these results and GSH concentrations (Fig. 1 D). It has been found that the regeneration of GSH is delayed in the presence of high glucose causing an impairment of the antioxidant defense [25,26].

In this model, the decrease of hyperglycemia by ozone may be explained, in part, through the integrity preservation of pancreatic β-cells (Fig. 1D). In addition, the maintenance of GSH levels may be the result of ozone regulation on aldose reductase (AR) activity [12] since it uses NADPH as a co-factor. NADPH is also a co-factor for the Glutathione Reductase enzyme which is responsible of GSH regeneration by reduction of oxidized glutathione (GSSG).

On the other hand, GSH-Px catalyzes the elimination of hydrogen peroxide formed when $O_2^{\cdot -}$ are scavenged by SOD. In line with the above results, total hydroperoxides were maintained at non-diabetic control levels by ozone treatment (Table 1). H_2O_2 is produced during glucose autoxidation and it is not only toxic to cells but is also able to permeate cell membranes. In the extracellular environment H_2O_2 reacts with transition metals like iron and copper to generate highly reactive hydroxyl radicals ($^{\cdot}OH$) which can react with macromolecules in the vicinity and can cause damage [27]. Lipids are targets of $^{\cdot}OH$ radicals, thus generating the process of lipid peroxidation. In correspondence, ozone treatment reducing oxidative stress in STZ-induced diabetic rats ameliorated MDA production (Table 1).

In line with the above results, PP (Table 1) was not significantly different with regard to non-diabetic control group in STZ-induced diabetic rats treated with ozone. It is important to note that PP represents a balance between antioxidant and pro-oxidant influences on serum lipids 20 indicating that ozone treatment promotes a prevalence of antioxidant effects over those pro-oxidants improving the redox cellular status.

There was a correspondence between the decrease of hyperglycemia, the reduction of oxidative stress and the histopathological results. It is known that STZ penetrating into the organism generates an oxidative stress, which is associated with the destruction of β-cells [28]. Ozone treatment was able to protect β-cells against STZ damage (Table 2, Fig. 2D) demonstrating how its antioxidant properties [6-12] preserved β-cells function and the reduced hyperglycemia induced by STZ.

Experimental Groups	n	Damaged Pancreatic Islets (%)
DIABETES (STZ)	10	36.54 ± 18.08 [a]
STZ + Ozone	10	16.98 ± 15.14 [b]
STZ + Oxygen	10	38.47 ± 16.50 [a]

Table 2. Percentages of damaged pancreatic islets in STZ-induced diabetes and STZ-induced diabetes treated with ozone in rats. Data are means ± SEM. Means having different superscript letters indicate significant difference ($p < 0.05$) between groups.

CONCLUSIONS

In summary, it has been demonstrated that hyperglycemia and oxidative stress in blood by STZ-induced diabetes is controlled by ozone administration. Moreover, pancreas damage was avoided with this treatment. Taken together these results suggest that this complementary medical approach may represent an alternative in the treatment of diabetes and its complications. Other works studying the effects of ozone in diabetic patients are in progress.

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