Superoxide Dismutase Ameliorates the Bowel Alterations Induced by Diabetes Mellitus – An Experimental Study

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ABSTRACT

Introduction: The gastrointestinal system is frequently affected in patients with diabetes mellitus (DM). Some of these alterations are due to oxidative stress and the production of free radicals. The present study was designed to evaluate whether treatment with superoxide dismutase (SOD) exerts protection on established bowel alterations in experimental diabetes mellitus induced by streptozotocin. We measured the lipid peroxidation, the superoxide dismutase activity and the DNA damage. **Materials and Methods:** We used the anorectal pressure to evaluate the nitrosative stress and used an inflammatory score to measure the macroscopic and microscopic bowel alterations. **Results:** The oxidative stress and the DNA damage was elevated in DM group and reduced with the SOD administration. The use of SOD also ameliorates the inflammatory bowel alterations and the anorectal pressure. **Conclusion:** SOD administration showed beneficial effects in all parameters of large bowel alterations in DM rats.

Keywords: oxidative stress; diabetes; superoxide dismutase

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INTRODUCTION

Diabetes mellitus is a metabolic endocrine disorder that affects many systems. The complications may arise in several organs in different moments and situations, the gastrointestinal often being among the affected systems. Diabetes mellitus is one of the most common metabolic diseases, affecting about 2.5-3% of the world's population and 7% or more in some countries.[1] A considerable amount of evidence suggests that oxidative stress may play an important role in the pathogenesis and complications of diabetes. Different mechanisms can contribute to the enhanced oxidative stress in diabetic patients, in particular in subjects with poor glycaemic control and hypertriglyceridaemia.[2] Diabetic patients may have reduced antioxidant defenses, such as diminished activity of glutathione peroxidase, catalase and superoxide dismutase, as well as decreased levels of non-enzymatic antioxidants. In consequence, they may suffer from an increased risk of oxidative stress-related diseases, including atherosclerosis, the most common reason for premature death.[3]

The accumulation of oxidative stress products can cause damage to biological molecules: proteins, lipids and DNA. The production of reactive oxidative species (ROS) is elevated in diabetes, particularly among those who have poor glycaemic control. It has been hypothesized that increased ROS generation in long-standing diabetes may result in oxidative damage to DNA. Studies with comet assay have shown increased levels of DNA breakage in peripheral blood cells of type I diabetic patients with poor glycaemic control, but not in patients with normal glycaemia.[4,5] Dinçer et al. found increased strain breakage and formamidopyrimidine DNA glycosylase (Fpg)-sensitive sites by the comet assay in DNA from leukocytes of patients with type I diabetes.[6]

Lipid peroxidation of cellular structures, a consequence of free radical activity, is thought to play an important role in late complications of diabetes mellitus.[2,7]

The intestinal mucosa exhibits numerous morphological and functional alterations during diabetes, such as hyperplasia and hypertrophy of the epithelial cell, elevated levels of digestive enzymes, increase absorption of sugars, amino acids, enhanced endogenous synthesis of cholesterol and triglycerides and decreased fluidity of the brush border membrane. The intestinal mucosa is also vulnerable to oxidative stress on account of the constant exposure to ROS generated by luminal contents such as oxidized food debris, transition metals like iron and cooper, bacterial metabolites, bile acids and salivary oxidants.[8] There is poor information regarding DNA damage in intestinal mucosa during diabetes or its antioxidant status.[9]

The effects of the oxidative stress in the large bowel affect not only the mucosa but also the intestinal motility. Lower anorectal pressures are well documented in Wistar rats with diabetes and manometry is a good method to evaluate this alterations. Products of the oxidative stress such as the nitric oxide seem to be the responsible for this alterations.[10,11]

To regulate overall ROS levels, the intestinal mucosa possesses a complex of antioxidant systems, of which the superoxide dismutases (SOD) are the initial enzymes, converting superoxide anion to H_2O_2. SOD expression in patients with active intestinal bowel disease seems to be altered. In particular, decreased protein activity and levels of cytoplasmic Cu/Zn-SOD have been reported consistently.[12,13]

While lymphocytes are good indicators of the systemic burden by exposure factors, the results obtained for potential target tissues of induced injuries by diseases as diabetes are considered to be of higher relevancy. Mammalian cells are equipped with both enzymatic and non-enzymatic antioxidant defenses to minimize the cellular damage caused by interaction between cellular constituents and ROS.[14]

The superoxide radicals are formed in inflammatory processes by phagocytic cells and are highly reactive having great capacity to promote tissue damage. The cells contains endogenous enzymes as superoxide dismutase, glutathione peroxidase and catalase to convert toxic forms of oxygen into molecular oxygen and water, but these functions are exceeded when the cells liberate these radicals in to the extracellular space, since the extracellular SOD is usually found in very low concentrations.[15]

Recent studies demonstrate that this cycle of toxicity can be stopped by the administration of exogen superoxide dismutase (Orgoteína/Ontosein) eliminating the extracellular superoxide radicals and allowing to reduce the consequence of inflammatory effects.[15,16] Several experimental strategies have been used to address the importance of the enhanced production of superoxide in the pathogenesis or bowel diseases, but inconsistent findings have left this issue largely unresolved. Therefore, further investigation about the effects of SOD on bowel diseases seems warranted, especially elucidating the value of this therapeutic approach in established bowel alterations in diabetes mellitus.

Based in these observations, the present study was designed to evaluate whether treatment with SOD exerts protection on established bowel alterations in experimental diabetes mellitus induced by streptozotocin and if so, highlight possible mechanisms through which SOD may confer protection, specifically its effects on oxidative stress and DNA damage.

MATERIAL AND METHODS

Induction of Diabetes Mellitus

Male Wistar rats weighting 200-250 g were obtained from the Experimental Animals Facility of the Basic Sciences Institute of the Federal University of Rio Grande do Sul (UFRGS). They received water and food *ad libitum*. Diabetes Mellitus was induced by a single intraperitoneal injection of streptozotocin (STZ) at a dose of 70 mg/Kg of body weight (Sigma Chemical Company, St. Louis, MO, EUA).[17] The control animals received, intraperitoneally, only sodium citrate buffer at the same volume. All procedures related to the rats were carried out according to the guidelines of the Ethical Research Commission in Health of the Research and Graduate Group of the Hospital de Clínicas of Porto Alegre (HCPA).[18]

Treatment groups

Forty male Wistar rats were randomized in four groups: CO = Control Group (n = 10), CO + SOD = Control Group that receives SOD, DM = Diabetes Mellitus Group (n = 10) and DM + SOD = Diabetes Mellitus Group that receives the drug. Glycemia was determined in all animals before the induction of DM and at the day of their death. Groups of animals were treated with daily subcutaneous (s.c.) doses Cu/Zn SOD 13 mg/Kg/day or vehicle (saline). For the current study, a preparation of SOD commercially available (Ontosein®, Tedec-Meiji Farma Laboratories, Alcalá de Henares, Spain) was used. The doses of SOD used are based on previous studies about treatments with SOD.[19] The first injection of

SOD was administered 53 days after induction of DM. SOD was administered once daily up to the end of the study at day 60. Anorectal manometry was performed before the induction of DM and 60 days after. Subsequently the rats were killed and intestinal tissue fragments were collected for histopathological and DNA damage analysis. The remaining material was collected, immediately frozen in liquid nitrogen, and after kept in freezer -80 ºC for subsequent biochemical analysis.

Glycemia Determination

The blood was withdrawn through the retro-orbital plexus[20] and placed in test tubes with sodium heparin to avoid coagulation. Glycemia was measured by a colorimetric assay (ENZI-COLOR Kit, Bio Diagnóstica), with a spectrophotometer. Rats were diagnosed as diabetic when their blood glucose level was above 250 mg/dL.[21]

Measurement of Lipid Peroxidation

Oxidative stress was determined by measuring the concentration of thiobarbituric acid reactive substances (TBARS). The amount of aldehydic products generated by lipid peroxidation was quantified by the thiobarbituric acid reaction using 3 mg of protein per sample. Results were referred as TBARS. Spectrophotometric absorbance was determined in the supernatant a 535 nm.[22]

Quantification of the Antioxidant Activity of superoxide dismutase (SOD).

Cytosolic superoxide dismutase (SOD; EC 1.15.1.1) was assayed according to Mirsa and Fridovich[23] at 30 ºC. The rate of autooxidation of epinephrine, which is progressively inhibited by increasing amounts of SOD in the homogenate, was monitored spectrophotometrically at 560 nm. The amount of enzyme that inhibits epinephrine autooxidation at 50% of the maximum inhibition was defined as 1 U of SOD activity.

Macroscopic and microscopic analysis

Macroscopic and microscopic damage of the colonic mucosa was assessed by two blinded observers to the treatment, according to previously established scores (Morris et al;[24] Sandborn et al[25]). The scale for macroscopic damage ranged from 0 to 4 as follows: 0 = normal appearance; 1 = mucosal erythema only; 2 = mild edema, slight bleeding or small erosions; 3 = moderate edema, bleeding, ulcers or erosions; 4 = severe ulcerations, erosions, edema, and tissue necrosis. The fields were scored according to the following scale: 0 = infiltrated normocellular or normal hypercellular lamina, PMNs absent; 1 = diffuse PMNs in lamina propria, occasional cryptitis but few cryptic abscesses, minimal glandular destruction or ulceration; 2 = moderate number of PMNs in lamina propria, cryptitis and prominent cryptic abscesses, some glandular destruction; 3 = numerous PMNs with abundant cryptitis, cryptic abscesses, extensive cellular destruction, prominent ulceration.

Anorectal Manometry

Sphincter pressures were measured before the DM induction and at the sacrifice, 60 days after. A manometer with closed channel was used (Proctossystem 3000 – Viotti – SP), and the values measured in cm H_2O. The catheter with balloon was inserted into the anal canal of the rats and left to rest for 30 sec for muscular and rectal accommodation. Then, the catheter was pulled back and the pressure was recorded. This procedure was repeated three times, and the mean of the recorded pressures was calculated.[11]

Comet assay

The alkaline comet assay was carried out as described,[26] with minor modifications.[27] Each piece of intestine was placed in 0.5 mL of cold phosphate-buffered saline (PBS) and finely minced in order to obtain a cell suspension. Intestine and blood cell suspensions (5 μL) were embedded in 95 μL of 0.75% low melting point agarose (Gibco BRL) and spread on agarose-precoated microscope slides. After solidification, slides were placed in lysis buffer (2.5 M NaCl, 100 mM EDTA and 10 mM Tris, pH 10.0), with freshly added 1% Triton X-100 (Sigma) and 10% DMSO for 48 h at 4 °C. The slides were subsequently incubated in freshly prepared alkaline buffer (300 mM NaOH and 1 mM EDTA, pH > 13) for 20 min, at 4 °C. An electric current of 300 mA and 25 V (0.90 V/cm) was applied for 15 min to perform DNA electrophoresis. The slides were then neutralized (0.4 M Tris, pH 7.5), stained with silver and analyzed using a microscope. Images of 100 randomly selected cells (50 cells from each of two replicate slides) were analyzed from each animal. Cells were also visually scored according to tail size into five classes ranging from undamaged (0) to maximally damaged (4), resulting in a single DNA damage score to each animal, and consequently to each studied group. Therefore, the damage index (DI) can range from 0 (completely undamaged, 100 cells × 0) to 400 (with maximum damage, 100 cells × 4). Damage frequency (%) was calculated based on the number of tailed versus tailless cells.[27]

Micronucleus assay

The micronucleus assay was performed according to the US Environmental Protection Agency Gene-Tox Program.[28,29] Bone marrow from both femurs was suspended in foetal calf serum and smears on clean glass slides were made according to MacGregor et al..[30] Slides were air-dried, fixed in methanol, stained in 10% Giemsa and coded for a "blind" analysis. To avoid false negative results and as a measure of toxicity on bone marrow, the polychromatic erythrocytes (PCE): normochromatic erythrocytes (NCE) ratio was scored in 1000 cells. The incidence of micronuclei (MN) was observed in 2000 PCE for each animal.[27]

Statistical Methods

Data were analyzed using ANOVA. The statistical evaluation of data from Comet assay and micronucleus assay were carried out using the Tukey test. For the others analyses the data were analyzed using analysis of variance with Newman-Keul´s test. Values were expressed as mean ± SE. In all comparisons, statistical significance was set at $P < 0,05$.

RESULTS

Blood glucose

The glycemic levels were significantly increased in the diabetic group with no reduction after treatment with SOD.

Effects of treatment with SOD in lipid peroxidation and antioxidant enzyme activity

The cytosolic concentration of TBARS, marker of lipid peroxidation, increased significantly in the colon of DM group as compared to saline controls. This effect was prevented in DM + SOD animals (Table 1). Superoxide dismutase activity (SOD) was measured as an indicator of the antioxidant status. The increased of intestinal SOD activity in the DM + SOD group was higher than in the group of diabetic animals without SOD treatment (Table 1).

Histology and macroscopy

Treatment with SOD, 13 mg/Kg, resulted in a significant reduction of the macroscopic damage score compared with the diabetic animals untreated with SOD and with those receiving vehicle. Histological damage score was also significantly reduced by the treatment with 13 mg/Kg/day of SOD. We found an important reduction of the inflammatory infiltrate and had no areas of ulcerations in the SOD-treated group when compared with the DM treated animals.

Effect of treatment with SOD on Anorectal function

Demonstration of anorectal manometry has wide acceptance as a helpful method to objectively asses the anorectal sphincter muscles[31]. Anorectal manometry showed a significant decrease in sphincter anal pressure in the DM group when compared with control animals. The decrease of anal pressure observed in the animals of DM groups was partially prevented by SOD administration (Figure 1)

Effect of treatment with SOD on DNA damage

The comet assay was used to measure DNA strand break in peripheral blood and intestine tissues. In the intestine, the damage index significantly decreased in the group CO + SOD in comparison with group CO ($p \leq 0.05$) and in group DM + SOD compared with group DM ($p \leq 0.01$) (Table 2). No significant difference was found

Table 1. Effects of SOD Treatment on Body weight, Glucose level, Lipid Peroxidation (TBARS) and SOD activity in the intestine tissue of DM rats.

	CO	CO + SOD	DM	DM + SOD
Glycemia	198.04 + 28.6	223.9 + 2.11	407.1 + 73.76[a]	461.9 + 99.27[a]
TBARS(nmoles/mg protein)	0.32 + 0.05	0.31 + 0.03	0.66 + 0.20[a]	0.36 + 0.01[b]
SOD (U/mg protein)	3.4 + 2	5.1 + 1.8	4.2 + 1.1	6.7 + 2b
Macroscopic score	0.0 + 0.0	0.0 + 0.0	2.66 + 0.28[a]	1.50 + 0.21[b]
Histologic score	0.0 + 0.0	0.0 + 0.0	2,33 + 0.30[a]	1.25 + 0.18[b]

[a]$P < 0.05$ versus Control; [b]$P < 0.01$ versus DM.

among the groups in the evaluation of DNA damage in peripheral blood (data not shown).

Micronucleus assay results are presented in Table 3, which shows the ratio PCE/NCE and micronucleated polychromatic erythrocytes (MNPCE) values individually and the mean and standard deviation by group. No toxicity in bone marrow was detected and the frequencies of micronuclei were similar in all groups.

DISCUSSION

STZ is probably the most widely used substance in the study of insulin-dependent diabetes mellitus (IDDM), or DM type I, in animals.[32,33] Its mechanism of action is based on the destruction of pancreatic beta cells, and the great advantage of its use is that it has a high affinity for these cells.[34] STZ-induced DM is obtained by a single intravenous or intraperitoneal injection. When STZ is administered at doses of 50-60 mg/Kg, insulin levels decrease by 30% of the normal, leading to hyperglycemia, polyuria, polydipsia, and weight loss.[35,36,37]

In this study we found an important increase in glicemic levels in DM group compared to the controls. SOD administration had no impact in glicemic levels.

We have already shown in a previous study that DM induced by streptozotocin promotes inflammatory damage in the intestinal mucosa.[10] Ettarth et al. (1997) conducted a histological investigation following intraperitoneal injection of STZ and compare these Wistar rats with a control group, the presence of a significant inflammatory process was observed in the mucosa and submucosa of experimental animals.[35] SOD has also been used in some other studies and produced a significant reduction in macroscopic and microscopic scores of inflammation.[10,38]

The current study demonstrates that treatment with Cu/Zn SOD effectively ameliorates the bowel histologic alterations in diabetes mellitus. The beneficial effects of treatment with SOD, 13 mg/kg/day, are of similar magnitude to those previously reported for dexamethasone in the model of experimental colitis.[39]

Cu/Zn SOD is an enzyme widely distributed in the cytoplasm of all mammalian cells and has been shown to exert antiinflammatory effects in a variety of experimental models.[19,40,41] Measurements of lipid peroxidation confirm previous evidence, indicating that development of diabetes is associated with a significant burst in ROS.[42] Panés et al, (2004) showed that treatment

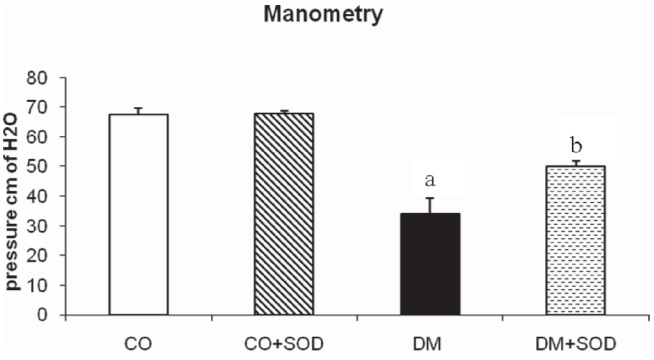

Figure 1. The sphincteric anal pressure was measured through a manometric equipment with a balloon catheter (Proctossystem Viotti, Sao Paulo, Brazil). Values were expressed in cm of H_2O. Group CO: control animals treated with saline (NaCl 0.9%); Group CO + SOD: animals treated with SOD; Group DM: diabetic animals untreated with SOD; Group DM + SOD: diabetic animals treated with SOD.
[a]P < 0.05 versus Control; [b]P < 0.01 versus DM.

Table 2. Comet assay in the intestine from diabetic and non-diabetic rats treated or untreated with superoxide dismutase (SOD).

Tissue	Groups	Damage index	Damage frequency
Intestine	CO	189 ± 64	69 ± 20
	CO + SOD	94 ± 33[A]*	47 ± 21
	DM	248 ± 82	82 ± 14
	DM + SOD	136 ± 69[B]**	59 ± 29

Group CO: control animals treated with saline (NaCl 0.9%); Group CO + SOD: animals treated with SOD; Group DM: diabetic animals untreated with SOD; Group DM + SOD: diabetic animals treated with SOD. Damage index DI: can range from 0 (completely undamaged, 100 cells × 0) to 400 (with maximum damaged 100 × 4); Damage frequency DF(%): was calculated based on number of cells with tail versus those with no tail.
[a]Significant difference from the Group CO: *P < 0.05
[b]Significant difference from the Group DM: **P < 0.01
(ANOVA, Tukey test).

with SOD dose-dependently inhibits peroxidation of lipids in the diabetic intestine.[16] Oxidative stress in diabetics is well-documented as a feature of diabetes, and it has been implicated in the development of complications associated with diabetes.[4,43]

In this study we used the TBARS as a marker of lipiperoxidation. SOD activity was measured as an indicator of the antioxidant status. The cytosolic concentration of TBARS increased in DM group when compared to control group. This effect was prevented by the use of SOD in diabetic animals.

Anorectal pressure measured by manometry is altered in diabetic rats eight weeks after the induction with streptozotocin. The pressure is usually lower than the control groups. These changes are due to oxidative stress, especially by the higher nitric oxide release.[10] The use of antioxidant substances, such as glutamine or SOD, diminishes the oxidative stress and enhances the anorectal pressure.[44]

We found a significant decreased in anorectal pressure in diabetic animals. This results are similar than the others already published.[10,11,31,44] When we used the antioxidant drug the oxidative stress diminished and increased the anorectal pressure to values similar of the control group.

Increased DNA damage in diabetic patients has been shown by various investigators using comet assay.[6,45,46] ROS can attack all types of molecules including DNA. Neither superoxide anion nor hydrogen peroxide reacts directly with DNA. However, transition metal ions, such as Fe^{2+} and Cu^{+}, catalyse their conversion into the highly reactive hydroxyl radical, which in turn provokes a broad spectrum of DNA lesions. These include DNA strand breaks, DNA-protein cross-links and DNA base modifications, which may be measured by the comet assay.[6] At pH of around 13, these lesions are manifested as strand breaks in the form of visible tails in the comet assay.

Streptozotocin (STZ) can induce diabetes mellitus in experimental animals and a possible mechanism underlying its diabetogenic action may involve DNA damage in pancreatic β-cells, activating poly(ADP-ribose) synthetase, leading to depletion of NAD^{+}, and finally to decreased insulin synthesis.[3]

In this study, DNA damages in peripheral blood cells from streptozotocin-induced diabetic rats were not increased in comparison with non-diabetic rats (data not shown), suggesting an adaptive response against systemic oxidative stress protecting DNA. In our study, any nucleated cell in blood was analyzed, not only specific lymphocyte. Furthermore, the frequency of micronucleated PCE in bone marrow was not increased in group DM (Table 2). Interestingly, Anderson et al.[46] demonstrated that the DNA damage measured by the comet assay in lymphocytes was significantly lower in type I diabetic patients by comparison with controls, probably due to adaptation. However in several studies, diabetic patients showed higher levels of oxidative DNA damage with increased ROS generation, when compared with controls.[6,45] Other investigation also concluded that DNA damage observed in the comet assay was higher in type II diabetics than type I diabetics, and overall, higher DNA damage was observed in diabetics in comparison with controls.[47,48]

SOD treatment diminished DNA damage in intestine tissue from diabetic as well as non-diabetic animals (Table 1). SOD decreased baseline DNA damages of intestine, as observed in group CO + SOD and efficiently protected DNA in diabetic animals, which reaching DI values closed to the group CO.

Thus the SOD administration showed beneficial effects probably by decreasing superoxide anion formation and preventing Fenton reaction, inhibiting the generation of hydroxyl radical, which may induce DNA damage.

Impaired antioxidant defense in streptozotocin-induced diabetic rats may be one of the responsible mechanisms for increased DNA damage in intestine tissue.

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