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Effect of streptozotocin on reactive oxygen species and antioxidant enzyme secretion in rat submandibulary glands: A direct and an indirect relationship between enzyme activation and expression

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ABSTRACT

The salivary glands are important exocrine and endocrine organs, whose role in oral health is well recognized. Also these glands contribute to the maintenance of systemic health. During diabetes an impairment of salivary glands is reported. In this work the oxidative stress produced after 10 days of a single dose of streptozotocin administration in rats was observed in submandibulary glands. Under this condition a misbalance of the enzymes with antioxidant activity was observed in glands and in incubation medium, as well as in reactive oxygen species such as hydrogen peroxide (H_2O_2), superoxide anion ($O_2{\bullet}^-$) and nitric oxide (NO). An increase of NO and H_2O_2 and a decrease of $O_2{\bullet}^-$ were found. A direct relationship between peroxidase and nitric oxide synthase (iNOS) activities with enzyme expression was recorded, in contrast an inverse relationship between superoxide dismutase activity and expression was observed. If the high level of H_2O_2 persists in time as well as a low level of peroxidase, oral pathologies are expected to occur. So, under this situation to study the modulation of enzymes involved in reactive oxygen species metabolism during oxidative stress in oral tissues could be very important in the managing of oral pathologies.

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1. Introduction

The salivary glands are important exocrine and endocrine organs, which, through saliva secretion, contribute to the maintenance of oral and systemic health. Peroxidase secreted by salivary glands plays an important role in the protection of oral cavity exerting both antimicrobial activity and protective mechanisms related to hydrogen peroxide (H_2O_2) elimination. The human salivary peroxidase is secreted from the acini of human parotid and submandibular glands (Riva et al., 1978). The antimicrobial activity of peroxidases, is related to the oxidation of thiocyanate (SCN^-) to the antimicrobial hypothiocyanite ($HOSCN/OSCN^-$) in the presence of H_2O_2 (Carlsson, 1987) and allows the conversion of H_2O_2 into water and oxygen (O_2). H_2O_2 is mainly generated by oral microorganism, but it is also produced during normal salivary gland metabolism by the conversion of superoxide anion ($O_2{\bullet}^-$) through the action of superoxide dismutase (Pruitt et al., 1983; Geiszt et al., 2003). As H_2O_2 and $O_2{\bullet}^-$ produce lipid peroxidation, DNA and protein oxidation and also a deregulation of nitric oxide synthesis (Gate et al., 1999) could be highly toxic to many mammalian cells, including fibroblasts (Tenovuo and Larjava, 1984) and epithelial cells isolated from oral mucosal and gingival tissues (Hänström et al., 1983). It is known that, peroxidases are also related to NO (nitric oxide) metabolism (Brennan et al., 2002; Takahama et al., 2003).

Reactive oxygen species not only are produced in salivary glands during microbial infections and physiological situations but also during oxidative stress induced by several pathologies among them diabetes mellitus. Hyperglycemia, a characteristic of diabetes, increases oxidative stress through overproduction of reactive oxygen species (Hunt et al., 1988; Williamson et al., 1993) which are involved in organ injury in heart, liver, and central nervous system (Soko et al., 1996). Oral pathologies such as dental as well as periodontal alterations are frequent clinical manifestations of the diabetic disease (Murrah, 1985). The administration of streptozotocin (STZ), a drug that affects pancreatic cells, is used for induction of diabetes simil type 2 in rats.

But there are few reports about the variation of antioxidant enzymes in submandibulary glands of STZ induced-diabetes animals, Nogueira et al. (2005) and Ibuki et al. (2010) reported an increase of peroxidase, catalase and superoxide dismutase activities depending on the time the animals were euthanized after STZ treatment. Nevertheless there are no studies which relate the activity of the enzymes with the level of reactive oxygen

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species or with antioxidant enzyme expression. Also, the secretion of the antioxidant enzymes during STZ treatment was not studied before.

Taking into account, the purpose of this work was to determine the oxidative status in submandibulary glands of STZ-induced diabetes rats using a model of gland secretion to allow the study of reactive oxygen species (H_2O_2, $O_2^{\bullet-}$ and NO) in gland and in the incubation medium in relation to the enzymes involve in their metabolism such as peroxidase, superoxide dismutase and inducible nitric oxide synthase (iNOS). Moreover, the relation between enzyme activity and expression was studied.

2. Materials and methods

2.1. Animals

Female albino rats of the Wistar strain, weighing between 150 and 200 g were used. They were kept at 22 ± 2 °C in an illumination controlled room (photoperiod 14 h light and 10 h darkness), fed Purina Chow and allowed unrestricted access to water. The day before the experiment, animals were food restricted (24 h fasting). In all cases, vaginal smears (obtained daily between 7.30 and 9.30 a.m.) were used to determine the stage of the estrous cycles for approximately 3 weeks and only rats exhibiting regular 4-day estrous cycles were selected for this study.

Animals were used according to the Guide to the Care and Use of Experimental Animals (DHEW Publication, NIH 80-23). Ten animals were treated with PBS 1% via i.p. and ten animals were injected i.p. with streptozotocin (60 mg/kg) once. Ten days after the animals were euthanized and submandibulary glands dissected for the experiments.

2.2. Biochemical studies

Prior to sacrifice, animals were anesthetized with ether, and blood samples were taken from retro-orbital sinus. Blood was collected into coated EDTA and uncoated containers. Serum was obtained as follows: blood samples were incubated at 37 °C during 10 min, and then centrifuged at 800 × *g* during 10 min. Some of the studies were made in whole blood (gathered in tubes with EDTA) (white blood cells (WBC), RBC (red blood cells), platelets, hematocrit and hemoglobin).

The remaining studies were conducted in serum (obtained from the exudates of the blood gathered in tubes without EDTA): enzymes such as serum glutamate oxaloacetate transaminase (SGOT), serum glutamate pyruvate transaminase (SGPT) to analyze liver functionality, and metabolite markers of the renal functionality: uric acid, blood urea nitrogen (BUN) and serum creatinine. Also cholesterol and triglycerides were measured. The hemoglobin concentration (Hb) was evaluated by cyanmethemoglobin methods using Beckman Model for spectrophotometer. White blood cell counts and red blood cell counts were determined using improved Neubauer hemocytometer. The volume of blood for each count was 20 µl using a dilution factor of 200 for red blood cell counts and 20 for white blood cell counts. The following numbers of fields were used for counting: 5 groups of 16 small squares are counted for red blood cells and 4 large outside squares which contain 16 smaller squares for white blood cells.

2.3. Submandibular gland preparations

All experiments were performed on submandibular gland removed from female control or STZ treated rats. Free connective tissue, fat and lymph nodes were removed under a magnifying glass and the dissected glands were weighed and incubated in Krebs–Henseleit buffer (pH: 7.4) containing (mM): NaCl 125; KCl 4.0; NaH_2PO_4 0.5; $MgCl_2$ 0.1; $CaCl_2$ 1.1 and glucose 5.0; bubbled with 95% O_2 and 5% CO_2 at 37 °C. After an equilibrium period of 10 min, glands from control and STZ treated animals were incubated during 40 min in buffer to allow basal secretion. The enzymes peroxidase and superoxide dismutase were determined in the incubation medium and in glands. For determination of enzyme activity in glands, the glands were homogenized using a Sorvall Omni mixer (DuPont Instruments) in Krebs–Henseleit buffer containing PMSF (10^{-4} M) and EDTA (10^{-3} M), centrifuged at 5000 × *g*, 15 min at 5 °C, the supernatant was used for the enzyme determination (Anesini et al., 2004).

2.4. Peroxidase and superoxide dismutase activity assays

Peroxidase activity was determined by the method described by Herzog and Fahimi (1973). 200 µl of each sample (gland homogenate) was incubated with 775 µl of DAB (5×10^{-4} M) and 25 µl of H_2O_2 (solution of H_2O_2 Parafarm R, 30% v/v diluted 1/86 in distilled water). The reaction was initiated by the addition of H_2O_2; DAB without H_2O_2 was used as blank. The final volume in reaction tube was 1000 µl. The change in absorbance readings was recorded at 30 s intervals for 5 min using a Shimadzu recording spectrophotometer UV-240 (graphic printer PR-1) set at 465 nm, and Δ absorbance/min was calculated. A standard curve with a linear relationship between activity concentration and Δ absorbance/min of horseradish, peroxidase, in the range of 1.95×10^{-3} to 2.5×10^{-5} U/ml was obtained. The activity of samples was calculated by interpolation from the standard curve. Peroxidase activity was assayed spectrophotometrically in duplicate.

Superoxide dismutase was determined by its ability to inhibit the spontaneous oxidation of adrenaline to adrenochrome, which is measured by spectrophotometer at 480 nm. Superoxide dismutase activity was calculated taking into account that, 1 U of superoxide dismutase inhibits the auto-oxidation of adrenaline by 50% (Carrillo et al., 1991).

Results were expressed as total peroxidase or superoxide dismutase activity (U/ml/g gland = activity of homogenized glands + activity found in incubation medium) or as % of secreted enzyme = activity found in incubation medium × 100 / total activity and represent the mean ± SEM of values from ten glands from control and ten glands from STZ treated animals.

2.5. Western blot peroxidase and superoxide dismutase

Gland homogenates (40 µg of protein/lane) were size fractionated in 12% SDS-polyacrylamide gel electrophoresis and transferred to a nitrocellulose membrane. Membranes were incubated for 90 min in Tris buffered saline (TBS, pH 7.5)-3% milk and then overnight with a 1:200 rabbit antibodies against superoxide dismutase and peroxidase (Santa Cruz Biotechnology Inc., Santa Cruz, California, USA). Membranes were washed with TBS-0.05% Tween 20 and incubated with a 1:1000 goat anti-rabbit conjugated horseradish peroxidase (Sigma, CA, USA). Immunodetection was performed using western blot chemiluminescence reagent kit (NEN Life Science, Boston, USA), according to the protocol provided by the manufacturer. The immunoreactive protein bands were analyzed with the Corel photopaint 9.0 program.

2.6. Hydrogen peroxide (H_2O_2) determination

The homogenate of the glands was incubated with 0.56 mM of diaminobenzidine tetrahydrochloride (DAB) in buffer 140 mM NaCl, 10 mM potassium phosphate, 5.5 mM dextrose; and type II horseradish peroxidase 0.01 mg/ml (Sigma, St. Louis, MO, USA). After 1 h incubation the reaction was stopped by the addition of 10 ml of 4 N NaOH and the absorbance was measured at 465 nm in a microplate reader (Microplate Reader Benchmark, Bio-Rad, CA, USA). Results were expressed as H_2O_2 M/g gland. A standard curve of known molar concentrations of H_2O_2 in buffered DAB was run in each test (Davicino et al., 2009).

2.7. Production of superoxide anion

The methods described by Schopf et al. (1984) were employed where the O_2^- anion was evaluated by the reduction of nitroblue tetrazolium (NBT) (Sigma, CA, USA) to formazan. Briefly gland homogenate was incubated with 300 μl of NBT during 30 min. The reaction was stopped with 1 N HCl (Tetrahedron, Buenos Aires, Argentina). Formazan was extracted with dioxane (Dorwill, Buenos Aires, Argentina) and the absorbance was measured in a microplate reader at 525 nm (Microplate Reader Benchmark, Bio-Rad, CA, USA). Results were expressed as mmol NBT reduced/g gland.

2.8. Total nitrite determination and immunoblot analysis of inducible nitric oxide synthase (iNOS)

Total nitrites were determined by Griess reactive (Becherel et al., 1997). The gland homogenate was incubated with Griess reagent for 20 min in the absence of light and measured at 540 nm. Total nitrites were calculated by interpolation in a standard curve made with known concentrations of nitrites.

Salivary gland homogenates extracted from control and STZ-treated animals were dissolved in SDS sample (2% SDS, 10% (vol/vol) glycerol, 62.5 mM Tris–HCl, pH 6.8, 0.2% bromophenol blue, 10 mM 2-mercaptoethanol). Equal amounts of proteins were separated by SDS-PAGE on 10% polyacrylamide gels and transferred PVDF membranes. Nonspecific binding sites in PVDF membranes were blocked with blocking buffer (5% non-fat dry milk containing 0.1% Tween 20 in 100 mM Tris–HCl, pH 7.5, and 0.9% NaCl) for 2 h. The PVDF membrane was subsequently incubated with an antibody specific to iNOS (SIGMA Chemical Co) for 18 h. Anti-actin antibody (Santa Cruz Biotechnology) was used as control for protein loading. After washing with PBS-tween, the membrane was incubated for 1 h with a second antibody anti-rabbit conjugated to HRP (Amersham Biotech) diluted 1:2000 in PBS-tween. The immunoreactive bands were visualized using ECL technology (Amersham Pharmacia). Densitometry analysis was performed by image J (version 5.1 Silk scientific Corporation) software. Densitometry values of iNOS (arbitrary units) in each lane were normalized to corresponding actin densitometry values (Gorelik et al., 2002).

2.9. Determination of lipid peroxidation

Lipid peroxidation was assayed by determining the rate of production of thiobarbituric acid-reactive components that express as malondialdehyde (MDA) by the modified method of Ohkawa (Ohkawa et al., 1979). Briefly, 10 μl of gland homogenate was treated with a mixture of 100 μl of TCA (20%) with thiobarbituric acid (TBA) 0.5% and 100 μl of BHT (4% in ethanol). The reactive mixture was then heated at 90 °C during 30 min. Then it was immediately cooled and centrifuged for 10 min at 800 ×*g*. The content of MDA was assayed by spectroscopy at 532 nm, and 600 nm (unspecific absorbance) and the concentration was calculated by the differences at two λ using the molar extinction coefficient 155 $mM^{-1}\ cm^{-1}$. Finally, the content of MDA was expressed in terms of nmol/g gland.

2.10. Glutathione determination

Reduced glutathione (GSH) level was determined by the method described by Moron et al. (1979). Homogenates were immediately precipitated with 0.1 ml of 25% TCA and the precipitate was removed after centrifugation. Free-SH groups were assayed in a total volume of 200 μl by the addition of 134 μl of 0.6 mM DTNB (5,5′-dithio-bis-(2-nitrobenzoic acid), SIGMA) and 56 μl of 0.2 mM sodium phosphate buffer (pH 8.0) to 10 μl of the supernatant and the absorbance was read at 405 nm using a UV–VIS Systronics spectrophotometer. Glutathione (SIGMA) was used as a standard to calculate μmol GSH/g tissue.

2.11. Histological studies

For these studies, gland samples were fixed in 10% formalin solution at room temperature, they were processed by the standard method. Briefly, tissues were embedded in paraffin, sectioned at 5 μm, and stained with hematoxylin–eosin, and then picked up on glass slides for light microscopy and scanned 40× in an Aperio CS ScanScope.

2.12. Statistical analysis

The Student *t* test for unpaired values was used to determine the levels of significance between values from control and STZ treated animals. Differences between means were considered significant if $P < 0.05$.

3. Results

3.1. Biochemical parameters and general corporal status

First the biochemical parameters of rats submitted to STZ treatment were analyzed. It can be seen in Fig. 1A and B and Table 1 that rats treated with STZ had high level of serum glycemia and low level of protein; no significant differences were observed in red and white blood cells but high significant levels of plasmatic creatinine and urea can be seen (Table 1). High level of triglycerides was also observed. With respect to hepatic enzymes, SGOT was increased in STZ-treated animals. A low significant corporal weight was observed in STZ-treated animals (Fig. 1C).

3.2. Biochemical parameters in submandibulary glands

It can be seen in Fig. 2A, that glands of STZ-treated animals had significant low weight in comparison with control. Moreover, the protein content was lower in STZ-treated glands (Fig. 2B). The histology analysis of the glands revealed that there were no lesions or alterations in gland acini tissues or ducts in comparison with control (Fig. 2C). The level of MDA content was examined in order to estimate the lipid peroxidation production. Fig. 3A shows that, glands from STZ-treated animals presented higher level of MDA when compared to the control group. On the other hand, reduced glutathione was significantly increased in glands from STZ-treated animals (Fig. 3B).

To relate lipid peroxidation with reactive oxygen species level, H_2O_2 and $O_2{\bullet}^-$ were measured in control and STZ treated glands. In Fig. 4 it can be observed that, H_2O_2 was significantly increased in salivary glands of STZ-treated animals in comparison with control, meanwhile $O_2{\bullet}^-$ was lower in the same glands. On the other hand, NO was also significantly increased in salivary glands of STZ treated animals as well as the expression of iNOS (Fig. 5A and B and Table inserted).

To relate the level of reactive oxygen species found with the enzymes involved in their metabolism, peroxidase and superoxide dismutase total activities were assayed. In Fig. 6A, it can be seen that, glands from STZ-treated animals presented low peroxidase activity, meanwhile superoxide dismutase activity was increased in treated animals (Fig. 6B). In order to study the secretion of the enzymes, the enzyme activity in incubation medium was determined and expressed as % secreted. It can be seen in Fig. 6C and D that, in STZ-treated animals the secretion of peroxidase decreased at the same time that the secretion of superoxide dismutase increased. To relate the change in peroxidase and superoxide dismutase activities with a change in enzyme expression, a western blot analysis was done. It can be seen in Fig. 7A and B that STZ-treated animals presented a decrease of peroxidase and superoxide dismutase enzyme synthesis.

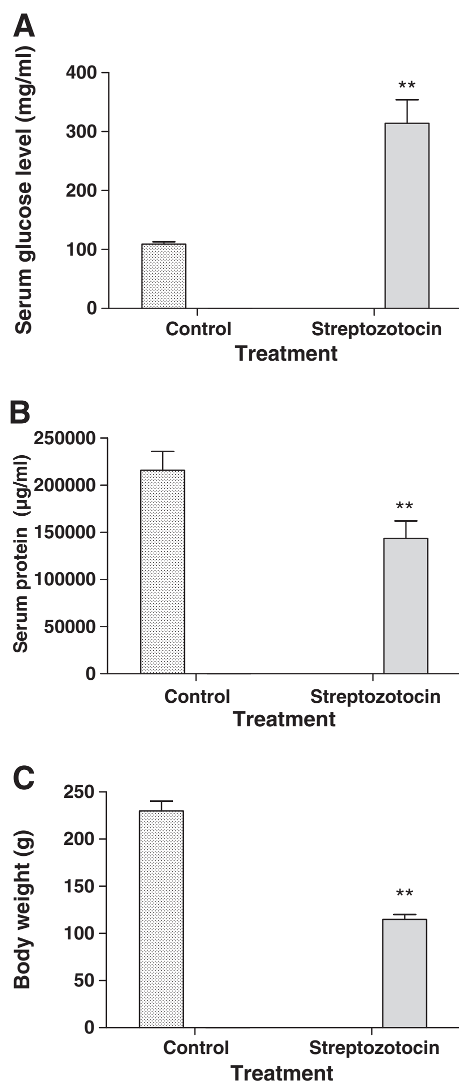


Fig. 1. Effects of streptozotocin on glycemia (A), serum protein (B) and corporal weight (C) of female rats. Rats were treated with streptozotocin and euthanized 10 days after treatment. Glycemia and proteins were determined in serum. Results represent the mean ± S.D. of values from ten control and ten STZ-treated animals. $^{**}P<0.01$ significant differences between control and streptozotocin treated animals in accord with Student *t* test.

4. Discussion

In this work the oxidative stress produced after 10 days of a single dose of streptozotocin in rats was studied in submandibular glands. A misbalance of antioxidant enzyme level as well as in their secretion pattern was observed.

Animals treated with STZ presented high glucose levels as well as low serum proteins; these results are in correlation to the destruction that STZ produces in pancreatic cells creating a diabetes mellitus similar condition. It is known that, the administration of a dose of 60 mg/kg of streptozotocin begins an autoimmune process that results in the destruction of the Langerhans islet beta cells resulting in clinical diabetes within 2–4 days (Weiss, 1982).

The low corporal weight, observed in STZ-treated animals, was related to the decrease in protein content, indicating their catabolism and consequently a reduction of animal corporal mass (in order of 45%) (Fig. 1 and Table 1). STZ-treated animals also presented renal and hepatic failure as it can be seen by the high level of creatinine and urea observed and by the increase level of SGOT activity (Table 1). In addition, STZ-treated rats presented high level of triglycerides, which is common in diabetic status in relation to lipid mobilization. Several authors have reported increases in SGOT and SGPT activities as well as changes in lipid concentrations in the serum of diabetic patients (Singh et al., 2005; Ruzaidi et al., 2005).

The glandular weight of STZ-treated animals was significantly lower than control glands in relation to the low level of protein found in these glands, the decrease observed in salivary gland weight was about 25%. It is known that, STZ-induced diabetes is characterized by severe loss in body weight (Singh et al., 2005); a decrease in body weight was also described by Akbarzadeh et al. (2007) who observed that short time STZ administration (60 mg/kg) can induce a decrease in body weight in adult rats.

The fact that, no histological differences, independent of weight decrease, were found in acini or in ducts of glands from STZ treated animals can be related to the processing time of the salivary glands after STZ treatment; Anderson et al. (1994) observed a reduction in submandibulary acinar cell size after 4–6 month STZ treatment and Cutler et al. (1979) observed an accumulation of secretory material within the cytoplasm and degenerative changes. Meanwhile short term 3–12 week duration does not produce any change in acinar histology.

Submandibulary glands of STZ-treated animals presented high level of malondialdehyde (MDA), an increase of 75% was observed in correlation to high level of reduced glutathione. It is known that, persistent hyperglycemia in a diabetic state may cause high production of free radicals and lipid peroxidation (Wolff and Dean, 1987), so MDA reflects the effects of hydroxyl radical damage of lipid from cells (West, 2000; Babu et al., 2006) and under this situation an increase of a complex reactive oxygen species regulating network composed of antioxidant enzymes and low molecular mass antioxidants such as glutathione is observed (Matés, 2001).

H_2O_2 and NO were increased during STZ treatment. The increase of NO was confirmed by western blot as consequent of an upregulation

Table 1
Biochemical parameters in streptozotocin induced-diabetes rats.

A

Parameters	RBC	WBC	Hb	Ht	Seg N	Bas	Eos	Lym	Mon
Controls	7691428 ± 370649	9616.6 ± 1632	14.75 ± 0.89	43.83 ± 4.16	16.2 ± 6.55	0	1.71 ± 1.38	75.5 ± 6.13	2.71 ± 2
Treated with STZ	7836666 ± 43628	10200 ± 1082	15.3 ± 0.75	47 ± 2.08	22.6 ± 7.68	0	2.3 ± 0.33	69 ± 8.5	6 ± 1

B

Parameters	Urea	Uric acid	Creatinine	Cholesterol	Triglycerides	SGOT	SGPT
Controls	49 ± 5	0.918 ± 0.58	0.34 ± 0.03	72 ± 8	30 ± 3	213 ± 69.22	51.8 ± 1.9
Treated with STZ	78^b ± 6.9	1.05 ± 0.38	0.55^a ± 0065	72.25 ± 4.49	78.5^b ± 16.2	193.2 ± 54.7	78.5^b ± 15.9

A. RBC: red blood cells (mm^3); Hb: hemoglobin (g/dl); Ht: hematocrit (%); WBC (mm^3): white blood cells; Bas: basophils (%); Eos: eosinophils (%); bN: band neutrophils (%); Seg N: segmented neutrophils (%); Lym: lymphocytes (%); Mon: monocytes (%).
B. SGOT: serum glutamate oxaloacetate transaminase (U/l), SGPT: serum glutamate pyruvate transaminase (U/l), urea (mg/dl), uric acid (mg/dl), creatinine (mg/dl), cholesterol (mg/dl) and triglycerides (mg/dl). Values were expressed as a mean ± S.D. of twenty determinations (ten control and ten STZ-treated animals). $^aP<0.05$, $^bP<0.01$ significant differences between control and STZ-treated animals in accordance Student's *t* test.

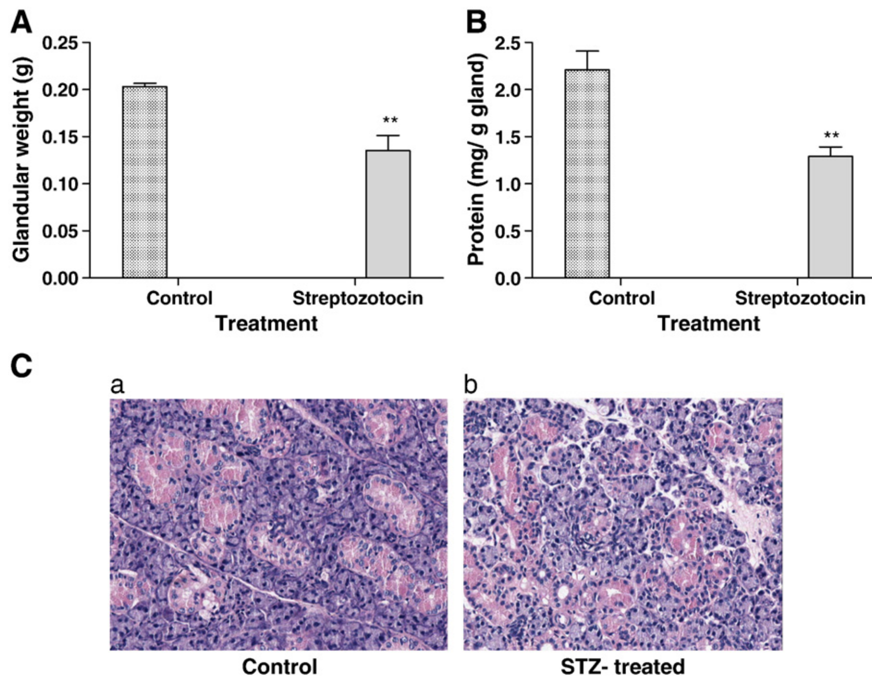


Fig. 2. Effect of streptozotocin on glandular weight (A), protein content (B) and histology (C) of submandibulary glands of female rats. Rats were treated with streptozotocin and euthanized 10 days after treatment. B. Proteins were determined on gland homogenate. C. Representative light micrographs (20×) of rat submandibulary gland lobules, a) gland from control animal, b) gland from STZ-treated animal. Results represent the mean ± S.D. of values from fourteen glands. $^{**}P<0.01$ significant differences between submandibulary glands from control and submandibulary glands from streptozotocin treated animals in accord with Student *t* test.

of iNOS, the enzyme involves in its synthesis (Figs. 4A and B and 5). The increase of iNOS expression could be related to the increase of H_2O_2 as it was observed during inflammatory bowel disease in Caco-cells (Bannan et al., 2001). Also Davicino et al. (2009) determined that an increase of H_2O_2, in a lymphoma cell line (BW5147), was related to iNOS upregulation.

The observed increase in H_2O_2 level was related to a decrease in peroxidase activity and with an increase of superoxide dismutase

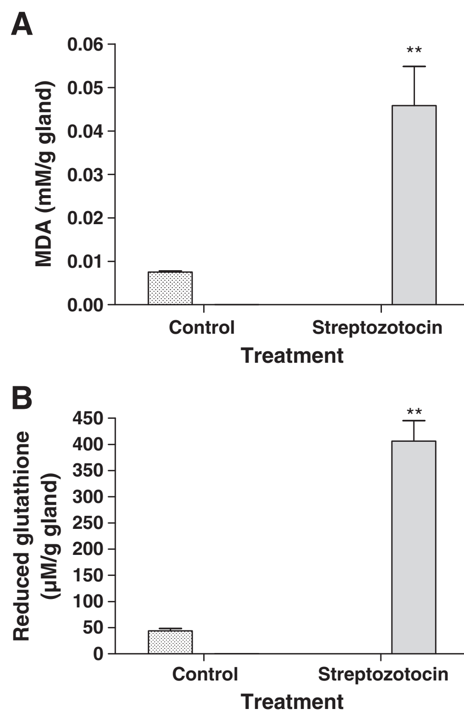


Fig. 3. Effect of streptozotocin on lipid peroxidation (A) and on reduced glutathione (B) in submandibulary glands. Results represent the mean ± S.D. of values from twenty glands. $^{**}P<0.01$ significant differences between submandibulary glands from control and submandibulary glands from streptozotocin treated animals in accord with Student *t* test.

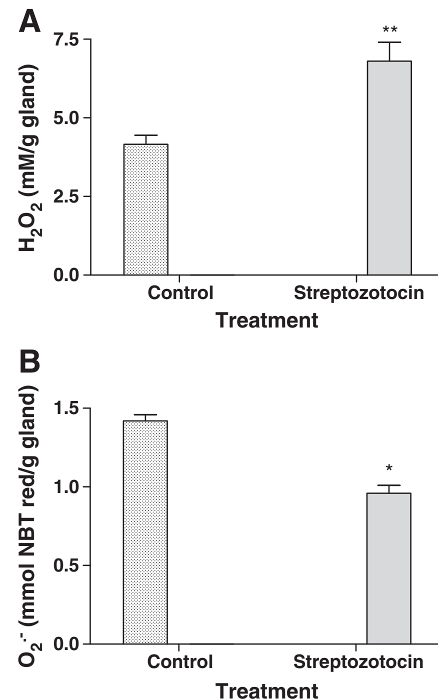
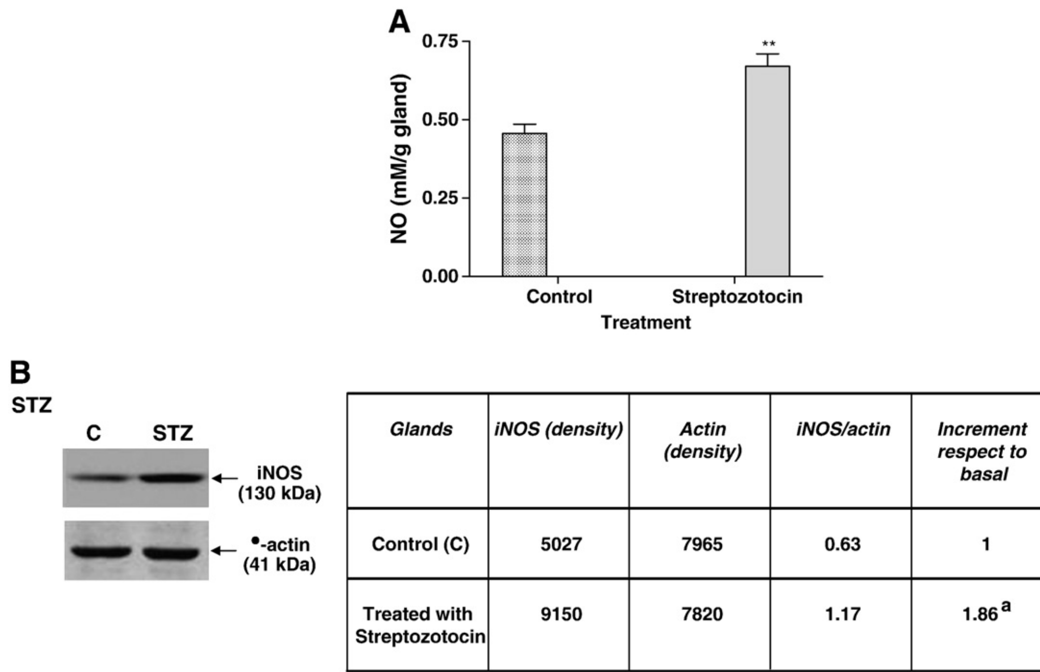


Fig. 4. Level of oxygen species, hydrogen peroxide (A) and superoxide anion (B) in submandibulary glands of streptozotocin treated female rats. Results represent the mean ± S.D. of values from twenty glands. $^{*}P<0.05$, $^{**}P<0.01$ significant differences between submandibulary glands from control and submandibulary glands from streptozotocin treated animals in accord with Student *t* test.



Glands	iNOS (density)	Actin (density)	iNOS/actin	Increment respect to basal
Control (C)	5027	7965	0.63	1
Treated with Streptozotocin	9150	7820	1.17	1.86 [a]

Fig. 5. Effect of streptozotocin on nitric oxide (NO). (A) NO level in glands from control and treated animals; (B) western blot of iNOS. The table inserted shows the density of bands in comparison with actin. Results represent the mean ± S.D. of values from twenty glands. $^{**}P<0.01$, in table inserted $^{a}P<0.01$ significant differences between submandibular glands from control and submandibular glands from streptozotocin treated animals in accord with Student *t* test. The western blot is a representative figure from three determinations.

activity, as peroxidase is an enzyme that participates in the metabolism of H_2O_2, reducing H_2O_2 in water and O_2 and superoxide dismutase catalyzes the decomposition of superoxide generating hydrogen peroxide. So, the decrease of $O_2^{\bullet -}$ level observed could be related to the synthesis of H_2O_2 (Halliwell and Gutteridge, 1990).

On the other hand, the decrease of peroxidase activity was correlated to the downregulation of peroxidase in glands from STZ-treated animals in comparison with control. But the increase of superoxide dismutase activity was not related to an upregulation. In contrast, to what was expected a downregulation of superoxide dismutase was observed by western blot. It is well known that the increase of reactive oxygen species generation can modulate superoxide dismutase activity by both pre- and post translational mechanisms, so it could be possible that the increase of H_2O_2 level produced a repression of superoxide dismutase synthesis. The activation of superoxide dismutase could be related to the presence of catecholamines or to the high level of $O_2^{\bullet -}$ which could be generated early, both are increased in STZ induced diabetes (Adeghate et al., 2001); it was reported that, an agonist of ß-adrenoceptor increases

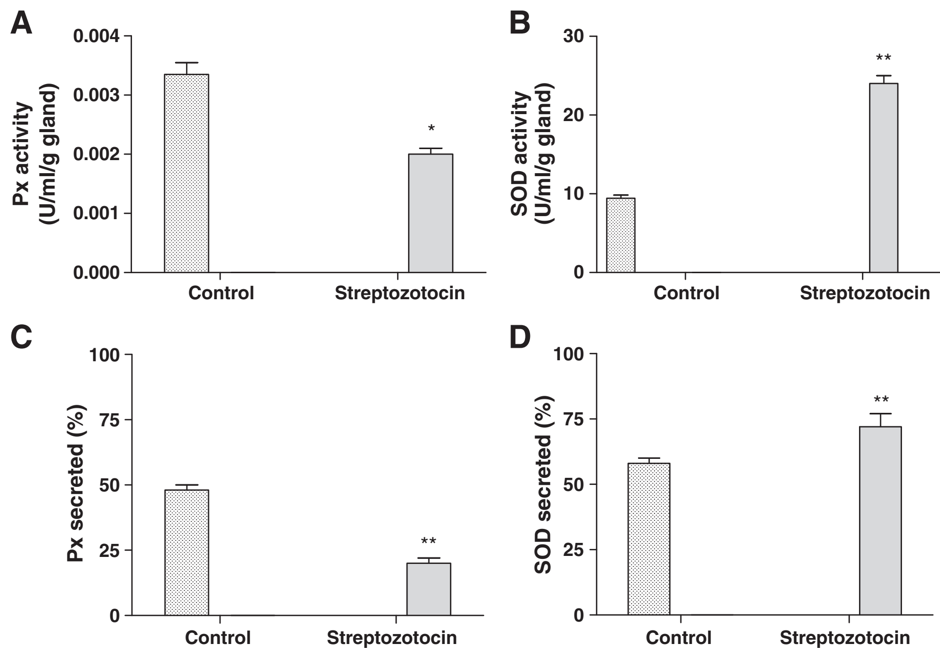
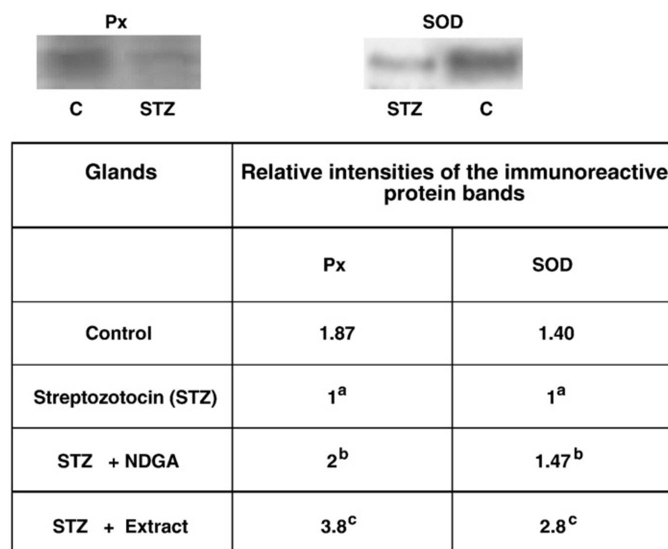


Fig. 6. Effect of streptozotocin on peroxidase and superoxide dismutase total activities of submandibulary glands and on enzyme secretion. A—Effect of streptozotocin on total peroxidase activity and C—on Px secretion. B—Effect of streptozotocin on total superoxide dismutase activity and D—on superoxide dismutase secretion. Black column: control, gray column: STZ-treated glands. Results represent the mean ± S.D. of values from twenty glands. $^{*}P<0.05$, $^{**}P<0.01$ significant differences between submandibulary glands from control and submandibulary glands from streptozotocin treated animals in accord with Student *t* test.



Glands	Relative intensities of the immunoreactive protein bands	
	Px	SOD
Control	1.87	1.40
Streptozotocin (STZ)	1[a]	1[a]
STZ + NDGA	2[b]	1.47[b]
STZ + Extract	3.8[c]	2.8[c]

Fig. 7. Western blot analysis of peroxidase and superoxide dismutase in submandibulary glands of control and STZ-treated animals. The table inserted shows the density of bands. Results represent the mean ± S.D. of values from twenty glands. [a]$P<0.01$ significant differences between submandibulary glands from control and submandibulary glands from streptozotocin treated, [b]$P<0.01$ significant differences between glands from streptozotocin and streptozotocin + NDGA treated animals and [c]$P<0.01$ significant differences between glands from streptozotocin and streptozotocin + extract treated animals in accord with Student *t* test. The western blots are representative figures from three determinations.

superoxide dismutase activity by induction and possible by direct activation (Barroso et al., 2003). Also, it is reported that catecholamines acting on β-adrenoceptor can produce a downregulation of CuZn superoxide dismutase (cupper zinc superoxide dismutase) in myocytes (Srivastava et al., 2007). Furthermore, superoxide dismutase can be downregulated during the increase of H_2O_2 by the induction of P53 which regulates the transcription of proteins involved in redox metabolism (Polyak et al., 1997).

The increase of superoxide dismutase activity found by us was also observed by Ibuki et al. (2010) in a temporal study of antioxidant enzymes in submandibulary and parotid glands from STZ-treated animals. These authors observed that the activity of superoxide dismutase increases in submandibulary glands, in animals which were euthanized 21 and 45 days after STZ-treatment. The same authors revealed that glutathione peroxidase activity was also increased under the same treatment but after 7 and 21 days of treatment. This last result differs from the results obtained by us. This could be possible by the fact that they measured glutathione peroxidase activity meanwhile we measured total peroxidase activity. In accordance with our results, it was shown that, peroxidase activity decreases in submandibulary glands of diabetic rats induced by alloxan (Anderson and Shapiro, 1979).

On the other hand, peroxidase secretion was decreased in STZ-treated animals meanwhile superoxide dismutase secretion was increased. Normal salivary gland function is under the dual control of the parasympathetic and sympathetic nervous systems and norepinephrine and acetylcholine are the principal neurotransmitters that regulate salivary secretion (Garrett and Anderson, 1991). In short, submandibulary glands respond to isoproterenol (adrenaline analog that stimulates ß-adrenoceptor) increasing intracellular level of cAMP followed by protein secretion with oxygen and glucose consumption. The fact that diabetes affects protein expression and exocytosis is well known.

The secretion of peroxidase is mediated by alpha and beta adrenoceptors and is modulated by estrogen level, through α adrenergic control (Anesini and Borda, 2003). In addition, it was shown in alloxan-induced diabetes rats, a positive correlation between estrogen production and peroxidase secretion in uterus, during this state a decrease of estrogen was observed in relation to a decrease of peroxidase secretion (Piyachaturawat et al., 1984). Vatta et al. (2002) demonstrated that submandibulary and parotid glands from STZ treated rats, exhibited diminished neuronal uptake, release and endogenous content of norepinephrine.

In contrast, superoxide dismutase secretion appeared to be related to ß-adrenoceptor stimulation (Barroso et al., 2003). The mechanism by which superoxide dismutase secretion is increased in diabetic animals will be studied later.

5. Conclusions

These results could indicate a deficient action of peroxidase under diabetic condition to counteract the degradation of H_2O_2, not only in surrounding medium but also in submandibulary gland, consequently if the high level of H_2O_2 persists in time as well as the low level of peroxidase activity, oral pathologies are expected to occur. So, under this situation, to understand the mechanisms involved in the modulation of enzymes related to reactive oxygen species metabolism during oxidative stress in oral tissues could be very important in the management of oral pathologies.

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