

## ***N*-acetylcysteine supplementation remodels thiol-related biochemical pathways towards decreased oxidation in diabetic submandibular glands**

A suplementação de *N*-acetilcisteína remodela as vias bioquímicas relacionadas ao tiol em direção à redução da oxidação nas glândulas submandibulares diabéticas

La suplementación con *N*-acetilcisteína remodela las vías bioquímicas relacionadas con el tiol hacia una oxidación disminuida en las glândulas submandibulares diabéticas

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### **Abstract**

Secondary disorders in consequences to diabetes involves the development of several diseases in the oral cavity, as periodontitis, xerostomy, infection by diverse pathogens and dysfunctions on the salivary secretion. These alterations occur partially, in consequence of the oxidative stress occasioned by hyperglycemia, and are important in patients undiagnosed or that have flaws in their therapeutic process. The aim of this work was to evaluate biochemical alterations of submandibular glands in response to oxidative stress during diabetes mellitus, and verify the effects of *N*-acetylcystein supplementation to diabetic rats, specially on the regulation of modifications related to glutathione and thiol proteins. For this purpose, the levels of some oxidative stress markers and the occurrence of the post-translational event of S-glutathionylation were evaluated. The  $\alpha$ -amilase degranulation by isolated acinar cells and glandular relative weight was also measured for each experimental group. The compound was able to decrease the lipoperoxidation and proteic oxidation observed in the submandibular gland of diabetic rats, preventing the decrease of the tecidual reducing power and increasing the occurrence of the post-translational process of S-glutathionylation. The diabetic condition increases the degranulation of  $\alpha$ -amilase and the glandular weight, but the supplementation with *N*-acetylcystein did not affect these events. Together these findings may help to elucidate the status of oxidative

stress on salivary glands and suggest new therapeutic strategies employing antioxidants of low molecular weight to prevent oral and systemic dysfunctions related to diabetes.

**Keywords:** Diabetes Mellitus; Submandibular gland; Oxidative stress; N-acetylcystein.

### Resumo

Desordens secundárias em consequências da diabetes envolvem o desenvolvimento de diversas doenças na cavidade oral, como periodontite, xerostomia, infecção por diversos patógenos e disfunções na secreção salivar. Essas alterações ocorrem parcialmente, em decorrência do estresse oxidativo ocasionado pela hiperglicemia, e são importantes em pacientes não diagnosticados ou que apresentam falhas no processo terapêutico. O objetivo deste trabalho foi avaliar as alterações bioquímicas das glândulas submandibulares em resposta ao estresse oxidativo durante o diabetes mellitus, e verificar o conjunto farmacológico da suplementação de N-acetilcisteína em ratos diabéticos, especialmente na regulação das modificações relacionadas às proteínas glutatona e tiol. Para tanto, foram avaliados os níveis de alguns marcadores de estresse oxidativo e a ocorrência do evento pós-translacional de S-glutationilação. A degranulação da  $\alpha$ -amilase por células acinares isoladas e o peso relativo glandular também foram medidos para cada grupo experimental. O composto foi capaz de diminuir a lipoperoxidação e oxidação protéica observadas na glândula submandibular de ratos diabéticos, evitando a diminuição do poder redutor tecidual e aumentando a ocorrência do processo pós-translacional de S-glutationilação. A condição diabética aumenta a degranulação da  $\alpha$ -amilase e o peso glandular, mas a suplementação com N-acetilcisteína não afetou esses eventos. Juntos, esses achados podem ajudar a elucidar o estado do estresse oxidativo nas glândulas salivares e sugerir novas estratégias terapêuticas empregando antioxidantes de baixo peso molecular para prevenir disfunções orais e sistêmicas relacionadas ao diabetes.

**Palavras-chave:** Diabetes Mellitus; Glândula submandibular; Estresse oxidativo; N-acetilcisteína.

### Resumen

Los trastornos secundarios que resultan de la diabetes implican el desarrollo de diversas enfermedades en la cavidad bucal, como periodontitis, xerostomía, infección por diversos patógenos y disfunciones en la secreción salival. Estos cambios ocurren parcialmente, debido al estrés oxidativo causado por la hiperglucemia, y son importantes en pacientes que no están diagnosticados o tienen fallas en el proceso terapéutico. El objetivo de este trabajo fue evaluar las alteraciones bioquímicas de las glándulas submandibulares en respuesta al estrés oxidativo durante la diabetes mellitus, y verificar la combinación farmacológica de la suplementación con N-acetilcisteína en ratas diabéticas, especialmente en la regulación de cambios relacionados con el glutatión. y proteínas de tiol. Para ello, se evaluaron los niveles de algunos marcadores de estrés oxidativo y la ocurrencia del evento de S-glutatilación postraducciona. También se midieron la desgranulación de la  $\alpha$ -amilasa por las células acinares aisladas y el peso relativo de la glándula para cada grupo experimental. El compuesto fue capaz de disminuir la lipoperoxidación y oxidación proteica observada en la glándula submandibular de ratas diabéticas, evitando la reducción del poder reductor tisular y aumentando la ocurrencia del proceso postraducciona de S-glutatilación. La condición diabética aumenta la desgranulación de  $\alpha$ -amilasa y el peso glandular, pero la suplementación con N-acetilcisteína no afectó estos eventos. Juntos, estos hallazgos pueden ayudar a dilucidar el estado de estrés oxidativo en las glándulas salivales y sugerir nuevas estrategias terapéuticas que utilizan antioxidantes de bajo peso molecular para prevenir los trastornos orales y sistémicos relacionados con la diabetes.

**Palabras clave:** Diabetes Mellitus; Glándula submandibular; Estrés oxidativo; N-acetilcisteína.

## 1. Introduction

There are several secondary disorders classically described as a diabetes mellitus consequence (Rahman et al., 2007; Heydary et al., 2010). Although cardiovascular diseases, nephropathies, neuropathies and retinopathies are the principal focus of the technical literature and academic studies, the diseases in the oral cavity have to be seen as an important aspect related to the needs in health care of a patient with diabetes mellitus. In spite of the multifactorial mechanisms involved in the pathogenesis of oral diseases during diabetes, it has been described that the increased oxidative stress on salivary glands is, in part, responsible by the deficient salivary secretion and is related to the development of xerostomy, also establishing conditions that may increase the susceptibility to other oral pathologies, mainly periodontitis and infections (Anderson et al., 1994; Sandberg et al., 2000; Reznick et al., 2006; Soell et al., 2007; Yuen et al., 2009). The comprehension of the biochemical alterations in response to oxidative stress occurred on salivary glands during diabetes mellitus and the search for substances

that are able to control these modifications are crucial for planning new palliative and preventive therapeutic strategies that may promote an increase in the quality of life for patients with this metabolic disorder.

The high production of reactive oxygen and nitrogen species and the alteration of some redox-dependent signaling pathways, is a common event observed on many tissues during hyperglycemia (Atli et al., 2004; Choi et al., 2008; Pasupathi et al., 2009). In consequence, thiol compounds related-pathways, mainly those containing glutathione and thiol proteins, may be strongly affected during diabetes mellitus oxidative stress, showing a depletion of the glutathione reduced/oxidized (GSH/GSSG) redox-buffer, an increase on S-glutathionylation post-translational event levels. These alterations were not previously described on salivary glands and are an important data for the development of the strategies previously discussed.

The low molecular weight, *N*-acetylcystein is an antioxidant substance that is able to act as a source of cysteine for glutathione synthesis that was able to protect experimental models of oxidative damage (Pocernich et al., 2000; Liu et al., 2007; Caylak et al., 2008). This molecule also is able to react directly with some oxidants like hypochlorous acid and hydroxyl radical and hydrogen peroxide (Aruoma et al., 1989).

However, despite the fact that the NAC molecule has powerful antioxidant properties, according to recent studies this mechanism is still uncertain, as they shown by the study by Ezerina et al., 2018 that the treatment of cells with NAC leads to the production of mitochondrial sulfur, and this production may explain its antioxidant properties.

These properties sustained the hypothesis that this compound is able to minimize or reverse the biochemical and functional alterations that commonly are present in result of diabetes mellitus-induced oxidative stress.

## 2. Methodology

This study is of an experimental laboratory quantitative nature (Pereira A.S. et al., 2018).

### 2.1 Treatment of animals

Adult male Wistar rats were used in the experiments (*Rattus norvegicus*), with body mass between 290 g and 390 g. The animals were divided into groups: control (C, n = 4), diabetic (D, n = 4), control supplemented (CS, n = 4) and diabetic supplemented (DS, n = 4). Only for the experiments for the separation of proteins by Western blotting and determination of the activity of alpha-amylase, was used n = 8.

The animals were kept in individual cages for 28 days of experiment, with free access to water and standard ration. After the experimental period were killed by exsanguination and cardiac perfusion with saline (NaCl 0.9% w / v) under general anesthesia with diethyl ether. All experiments were performed in accord to Helsinki Declaration and with approval of Alfenas Federal University Ethics Committee (protocol number 64/2005).

### 2.2 Induction of diabetes mellitus

After fasting for 12 hours, the animals received intraperitoneal injection of alloxan (120mg/kg body weight) dissolved in sterile saline (NaCl 0.9% w / v). The control group received only the vehicle. The glycemia of the mice were given one week after induction by Accu-Chek Blond Glucose Meter system. Animals were considered diabetic when they had blood glucose level above 250 mg glucose / dL of blood. Blood glucose was measured again on the day of sacrifice by the same system and confirmed by enzymatic colorimetric method of glucose oxidase (500 nm, kit BT 10008, Biotécnica<sup>®</sup>) from blood samples taken by cardiac perfusion of the animals.

### 2.3 Supplementation of animals

From the first day of the experiment until 28<sup>th</sup> day, when the animals were sacrificed, the control group and diabetic supplemented received N-acetylcysteine (100mg/kg body weight) by gavage.

### 2.4 Collection of sample glandular

After general anesthesia with ethyl ether, the rats underwent exsanguination and cardiac perfusion with isotonic saline. Then, the submandibular glands were removed and stored at -18 °C in Tris-HCl (150 mM, pH 7.2) until its use. On the day of use, the glands were weighed after thawing and removal of adjacent tissue. The relative glandular mass (RGM) was determined by the formula: gland mass X 100 / body mass (Nogueira, 2004).

### 2.5 Determination of lipoperoxidation index

To determine the oxidative damage to lipid, we performed the analysis of the index of lipoperoxidation. During the process of lipid peroxidation are produced different aldehydes, mainly malondialdehyde which represents about 80% of the oxidized products produced by the ROS attack cellular lipids. These aldehydes, that react with thiobarbituric acid, were determined following the method of Esterbaue & Cheeseman, 1990. Gland samples were homogenized in Tris-HCl (150 mM, pH 7.2) and then centrifuged at 500xg for 10 minutes. Trichloroacetic acid (TCA, 20% w / v in 0.25 N HCl) was added in the supernatant to precipitate the proteins. Butylated hydroxytoluene (BHT, 4% w / v) was also added to prevent lipid peroxidation *ex vivo*. The mixture was kept on ice for 30 minutes and then centrifuged at 2400xg for 2 minutes, followed by addition thiobarbituric acid (TBA, 0.7% w / v) in the supernatant.

This solution obtained was heated to 100 ° C for 30 minutes, for the development and the stabilization of the color resulting from the reaction of TBA with reactive substances. After cooling, the optical density was measured at 535 nm in spectrophotometer, the values were expressed relative to total protein concentration using  $\epsilon_{535 \text{ nm}} = 153 \text{ mM}^{-1} \cdot \text{cm}^{-1}$ .

### 2.6 Determination of oxidative damage to protein structures

The damage caused by protein oxidation were estimated by protein carbonyl content in the samples (RICE-EVANS et al., 1991). For this purpose, gland tissue was homogenized in Tris-HCl buffer (150 mM, pH 7.2). The mixture was centrifuged at 500xg for 10 minutes and was added 2.4-dinitro-phenyl hydrazine (DNP, 10 mM) in the supernatant, incubating for 1 hour at room temperature. Then, to complete the reaction, proteins were precipitated with TCA (20% w / v). The precipitate was washed 3 times with ethanol / ethyl acetate (1:1 v / v) and dissolved in a solution of guanidine (6 M). The carbonyl content was assessed by measurement of optical density at 370 nm on a spectrophotometer and expressed in relation to total protein concentration ( $\epsilon_{370 \text{ nm}} = 22.000 \text{ M}^{-1} \cdot \text{cm}^{-1}$ ).

### 2.7 Determination of GSH and GSSG

The cellular redox state was determined through the quantification of GSH in relation to its oxidized form GSSG (SIES, 1999). The glands were homogenized in Tris-HCl (150 mM, pH 7.2) and centrifuged at 500xg for 10 minutes. To the fractions of supernatant was added perchloric acid (HClO<sub>4</sub>, 0.5 M) containing EDTA (6.3 mM) and bipyridine (0.06% w / v). After centrifugation at 10000xg for 10 minutes, aliquots of supernatant were mixed with 5-5 dithiobis-2-nitrobenzoic acid (DTNB, 2 mM) in NaH<sub>2</sub>PO<sub>4</sub> (80 mM) / EDTA (6.3 mM) at pH 7.5. After 40 minutes incubation at room temperature, was determined by absorbance ( $\lambda = 412 \text{ nm}$ ) due to formation of acid, 2-nitro-5 thiobenzoic (TNB). The result was compared to the respective standard curve obtained with GSH (Griffith, 1980). For determination of GSSG, the samples were incubated with

glutathione reductase with its cofactor NADPH (30 minutes at room temperature). With this, all GSSG was reduced. DTNB (2 mM) was added in solution and after 40 minutes was read to 412 nm on a spectrophotometer, determining the total concentration of glutathione by interpolation on its respective standard curve. The GSSG was calculated subtracting the value of GSH from the total concentration of glutathione and dividing the result by 2. The reducing power was calculated through the ratio  $GSH / GSH + 2GSSG$ .

## 2.8 Determination of protein s-thiolation

The post-translational protein modifications were verified by determining the occurrence of protein S-thiolation. Binding of thiols with cysteine residues in the structure of a protein, causes a post translational modification due to the action of ROS / RNS. To verify this modification, the samples were homogenized in Tris-HCl (150 mM, pH 7.2) and centrifuged at 500xg for 10 minutes. Then, the supernatant was recovered and added HClO<sub>4</sub> (0.5 M) containing EDTA (6.3 mM) and bipyridine (0.06% m / v), centrifuging again at 1000xg for 10 minutes. The supernatant was removed and the protein precipitate was resuspended in SDS (1% w / v). Thereafter, was added dithiothreitol (DTT, 50 mM) and waited 30 minutes to reduce the thiol. Then added HClO<sub>4</sub> (0.5 mM) containing EDTA (6.3 mM) and bipyridine (0.06% m / v) and the mixture was centrifuged at 500xg for 10 minutes. The supernatant was incubated for 40 minutes with DTNB (2 mM) in NaH<sub>2</sub>PO<sub>4</sub> (80 mM) / EDTA (6.3 mM) at pH 7.5. The absorbance was determined at 412 nm and the results interpolated from the standard curve of glutathione (Mills & Lang, 1996).

## 2.9 Determination of total protein concentration

The protein concentrations were determined in all samples by the method of Bradford, 1976 using bovine serum albumin (BSA) as standard for the calibration curve.

## 2.10 Separation of acinar cells

For the separation of acinar cells was added to the solution with amino acids and proteolytic enzymes (trypsin, collagenase, hyaluronidase) in homogenized glandular. This mixture was incubated for 30 minutes at 37°C. The solution with the dispersion cellular and fragments of connective tissue not hydrolyzed, were filtered using nylon net washed with distilled water and EDTA. The filtrate containing only dispersed cells, was centrifuged at 1000xg for 5 minutes. The supernatant was removed and the precipitate was washed with a solution of bovine serum albumin (BSA, 2% w / v), to resuspend cells. This step was performed two times and after the third centrifugation, the cells were dispersed in phosphate buffer (0.05 M pH 6.9) at 37°C.

## 2.11 Determination of the activity of alpha-amylase

The solution with acinar cells dispersed was incubated at 37°C for 10 minutes and homogenized. The sample was divided into five portions: T1 = baseline flow, without adrenergic stimulation; T2 = flow stimulated with 0.1 μM of beta-adrenergic agonist isoproterenol (ISO); T3 = flow stimulated with 0.05 μM of ISO; T4 = flow stimulated with 0.02 μM of ISO and T5 = flow stimulated with 0.01 μM of ISO. After incubation at 37 ° C for 15 minutes and centrifuged at 1000xg for 10 minutes, the supernatant was recovered, to determine total protein concentration by Bradford method, 1976. Alpha-amylase's substrate solution (1% w/v) were added to the assay. After 5 minutes, was added 3,5-dinitrosalicylic acid, reagent that stains the disaccharide maltose (result from the hydrolysis of starch by alpha-amylase). The temperature was raised to 100 ° C and maintained for 15 minutes. After cooling, we performed a reading of absorbance spectrophotometry at 540 nm and the

concentration of maltose was calculated by interpolation of the calibration straight line previously built. The activity of alpha-amylase was defined as the amount of maltose released / minute / mg protein at 37 °C and pH 6.9 (Specific activity, U).

## 2.12 Statistical analysis

All results were expressed as mean  $\pm$  SD of at least three experiments performed in triplicate. The results were statistically analyzed for variance tests using the ANOVA (ZARR, 1996). Values of p equal to or less than 0.05 were considered statistically significant.

## 3. Results

### 3.1 Induction of diabetes mellitus and measurement glandular mass

The induction of diabetes mellitus in response to the intraperitoneal alloxan injection was effective, since the glucose detected on the seventh day after the application of this compound was, on average,  $311 \pm 8.8$  mg glucose / dl blood, and control animals showed an average glucose value at  $84 \pm 21$  mg glucose / dl blood. Supplementation with NAC for 28 days did not cause significant decrease in control groups blood glucose ( $110 \pm 12.5$  mg glucose / dl blood), but also did not significantly alter this parameter in diabetic animals supplemented ( $279 \pm 13$  mg glucose / dl blood). Table 1 shows the values of body mass, mass of the gland (GW) and relative gland mass (RGM) of the submandibular.

**Table 1** – Body weight, glandular mass (GM) and relative glandular weight (RGW) of submandibulars of diabetics rats (D), control (C), supplemented control (SC) e supplemented diabetics (SD). The values expressed the mean  $\pm$ sd.

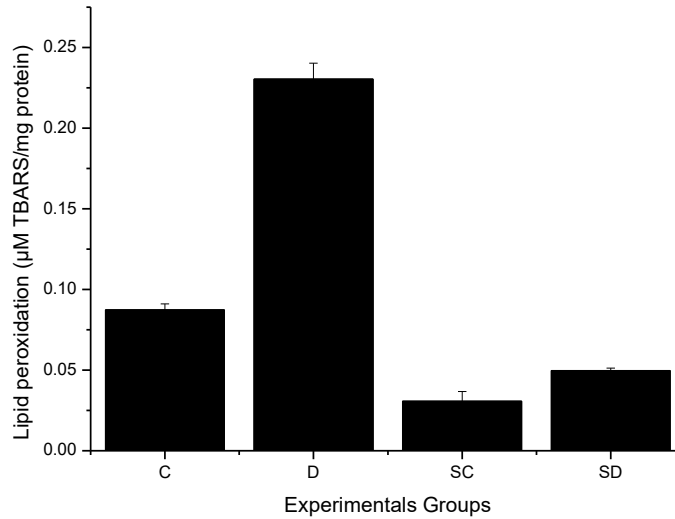
Groups	n	Body weight(g)	GW(g)	RGW
C	4	296 $\pm$ 5,8	0,324 $\pm$ 0,021**	0,115 $\pm$ 0,010
D	4	297 $\pm$ 5,0	0,447 $\pm$ 0,030	0,154 $\pm$ 0,006*
SC	4	369 $\pm$ 20,0	0,398 $\pm$ 0,026	0,105 $\pm$ 0,010
SD	4	388 $\pm$ 28,0	0,455 $\pm$ 0,051	0,116 $\pm$ 0,012

\*  $p < 0,001$  vs C vs SD; \*\* $p < 0,001$  vs D e  $p < 0,01$  vs SC. Source: Authors.

### 3.2 Index of lipid peroxidation in submandibular glands

Figure 1 shows the concentration of TBARS from lipid peroxidation, expressed per mg of protein. This index increased 300% ( $p < 0.001$ ) in diabetic compared to control and has performed 50% higher than the supplemented diabetic. The control supplementation presented a reduced basal level of lipid peroxidation of 87% ( $p < 0.001$ ).

**Figure 1.** The index of lipid peroxidation on each experimental group was determined by spectrophotometric quantification of TBARS.  $p < 0.01$  vs C vs D,  $p < 0.01$  vs. SD vs. C,  $p < 0.01$  vs SC vs C,  $p < 0.05$  vs SC vs SD.

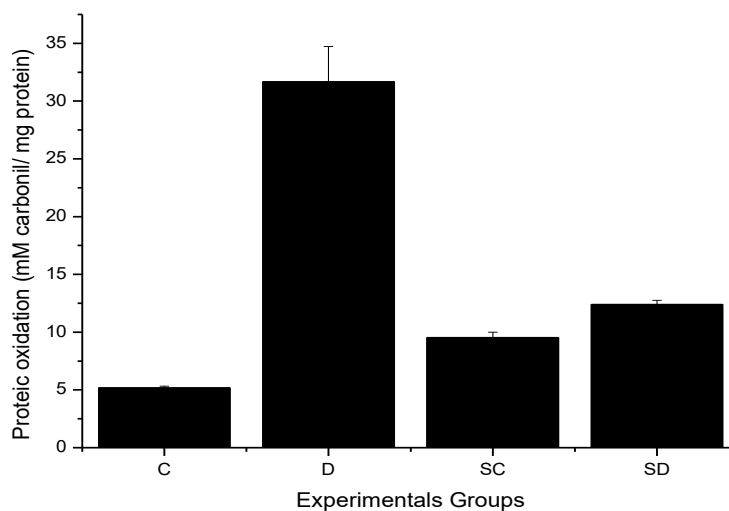


Source: Authors.

### 3.3 Protein oxidation in submandibular glands

Figure 2 shows the rate of protein oxidation in submandibular glands determined by reaction of carbonyl groups, formed by the action of reactive species to protein structure, with 2,4 DNP and expressed in mmol/mg protein. In this figure we can see that the diabetic group showed an increase of 500% ( $p < 0.0001$ ) at this level compared to the control and 150% ( $p < 0.01$ ) compared to diabetic supplemented. Moreover, comparing control and control supplemented the latter showed an increase of 76% ( $p < 0.05$ ).

**Figure 2.** The occurrence of protein carbonyls was detected by reaction with 2,4 dinitrophenylhydrazine and quantified in relation to total protein content. Results expressed as mean  $\pm$  SD.  $p < 0.01$  vs C vs D,  $p < 0.05$  vs. C vs. SC,  $P < 0.01$  vs. C vs. SD,  $P < 0.01$  vs SC vs D,  $p < 0.01$  vs D vs SD.



Source: Authors.

### 3.4 Determination of GSH and GSSG in submandibular glands:

To evaluate the reducing power of submandibular glands under the different experimental conditions we determined the concentration of the main redox couple ages cellular GSH and GSSG. Table 2 shows a decrease of three times in the concentration of GSH in the diabetic state ( $p < 0.001$ ), as well as evidence that supplementation of the diabetic rats with N-acetylcysteine increased, on average, 118% concentration of this tripeptide ( $p < 0.01$ ). We can also see a greater concentration of oxidized glutathione (GSSG) in diabetic, in the order of 185% ( $p < 0.05$ ) higher than controls, suggesting a heightened production of ROS / NRS in this condition.

**Table 2.** Glandular homogenates were subjected to quantitative spectrophotometric analysis of GSH and GSSG to 412 nm. The concentrations of GSH and GSSG were calculated as mean  $\pm$  SD of the differences detected between trials in the absence or presence of glutathione reductase from three independent experiments performed in triplicate.

Experimentals groups	Oxidad or reduced form ( $\mu\text{g}/\text{mg}$ protein)	
	GSH	GSSG
C	9,6 $\pm$ 3,0	0,14 $\pm$ 0,04
SC	10,1 $\pm$ 3,7	0,12 $\pm$ 0,03
D	3,2 $\pm$ 0,7*	0,4 $\pm$ 0,17**
SD	7,0 $\pm$ 1,7	0,3 $\pm$ 0,08

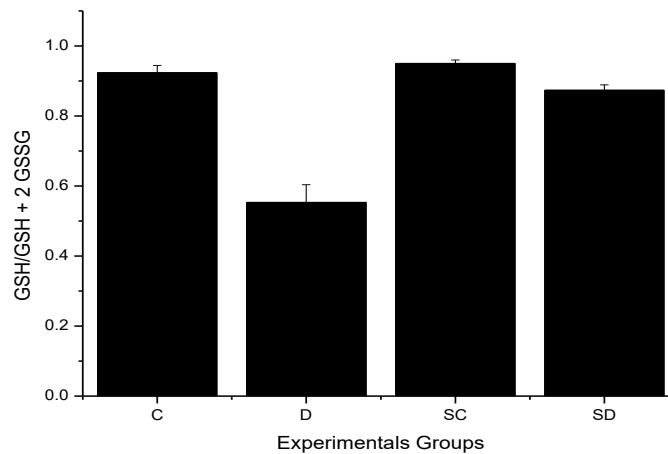
\*  $p < 0,001$  vs C e  $p < 0,01$  vs SD; \*\* $p < 0,05$  vs C. Source: Authors.



### 3.5 Reducing power in submandibular glands

Figure 3 shows a decrease of 45% ( $p < 0.01$ ) in the ability of submandibular glands tissues to reduce oxidant molecules produced in response to diabetes. Supplementation with NAC was effective, occasioning a recovery of 43% ( $p < 0.05$ ) in the reducing power of the DS group as compared with group D.

**Figure 3.** Ratio GSH / GSH + 2 GSSG was determined and compared among groups.  $p < 0.01$  vs C vs D,  $p < 0.01$  vs SC vs D,  $p < 0.01$  vs. D vs. SD,  $P < 0.05$  vs SC vs SD.

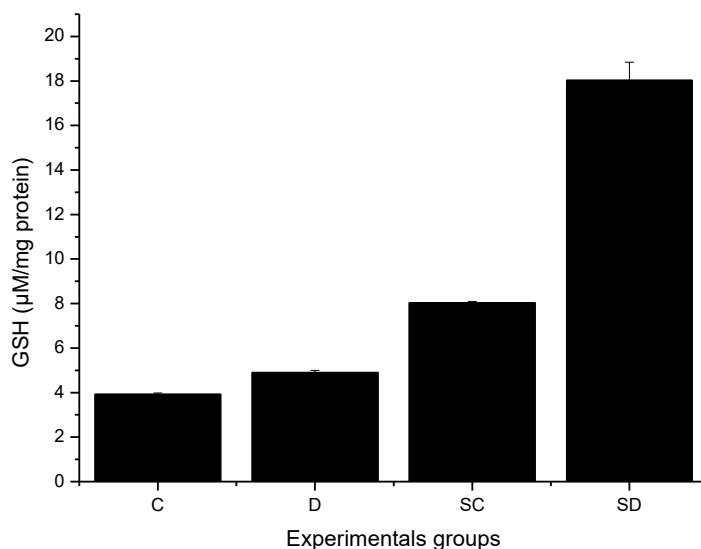


Source: Authors.

### 3.6 Protein S-Thiolation

The results presented on Figure 4 shows that the supplemented groups had higher binding of thiols to protein structures when compared to their respective controls. Comparing C and CS, it can be detected that the levels of S-tiolation in the second group increased 2.16 times ( $p < 0.01$ ) when compared with the first one. The DS had a rate 3.8 times greater than the D ( $p < 0.001$ ). Furthermore, the D group showed an increase of 21.09% ( $p < 0.01$ ) compared to control. Together this results can, at least in partial way, explain the depletion of GSH without proportional increase in the oxidized form, as previous described.

**Figure 4.** Concentrations of thiols bound to protein structure determined in tissue homogenates were determined by reacting with DTT and subsequently with DTNB. Results were calculated as the concentration of GSH bound to protein structure per protein mass, and were expressed as the Mean  $\pm$  SD.  $p < 0.01$  vs. C vs. SC,  $p < 0.01$  vs. C vs. SD,  $p < 0.01$  vs. SC vs. D,  $p < 0.01$  vs. D vs. SD,  $p < 0.01$  vs. SC vs. SD.



Source: Authors.

### 3.7 Activity of $\alpha$ -amylase by degranulating salivary gland acinar cells

The results showed that the acinar cells of submandibular glands respond differently, compared to the induction of diabetes and in front of NAC supplementation (Table 3).

**Table 3.** The enzyme activity (U) was determined in basal flow conditions or under  $\beta$ -adrenergic stimulation. Results expressed as mean  $\pm$  S.D. of three experiments,  $n = 4$ .  $p < 0.01$ ,  $p < 0.05$  vs. respective control groups.

Submandibular Gland		
Experimentals groups	Basal	IPR
C	14,0 $\pm$ 06	68,0 $\pm$ 10
D	19,0 $\pm$ 07	76,0 $\pm$ 09
SC	38,0 $\pm$ 09 <sup>b</sup>	112 $\pm$ 18 <sup>a</sup>
SD	37,0 $\pm$ 17 <sup>b</sup>	123 $\pm$ 12 <sup>a</sup>

Source: Authors.

## 4. Discussion

The recognition of changes in the biochemical pathways that occur during diabetes provides support for the development of new therapeutic strategies that aim to promote the improvement of patients' quality of life through the implementation of new preventive and palliative activities. Although the consequences of oxidative stress during diabetes have

already been widely discussed in the technical literature, the etiology of the damage caused by this pathology in the oral cavity has not yet been fully elucidated. Once the etiology and the mechanisms that cause changes are established, it is necessary to look for molecules capable of attenuating these manifestations.

The thiols groups present in cells and tissues have been used for a long time as indicators of their redox profile, but current studies demonstrate that thiols chemistry has a regulatory effect on the physiological and pathophysiological activity of cells, being profoundly altered in situations in which organic homeostasis is disrupted. The results obtained during this work offered new information about the alterations of the metabolic pathways regulated by thiols that occur in the submandibular glands in response to hyperglycemia. The increase in GSSG to the detriment of GSH, observed in parallel with the increase in protein s-thiolation and expression of calreticulin represents important changes in this glandular tissue in response to diabetes. The thiol compound n-acetylcysteine was efficiently used as a source of available thiol groups to prevent depletion of the redox buffer, which culminates in the reduction of tissue reducing power. It should be noted that treatment with NAC was not able to prevent increases in blood glucose, but it was able to prevent important metabolic changes.

The group of diabetic animals had a glandular mass 38% greater than the control, this value differs from that reported by a Nogueira, 2004 who in his study found a 20% reduction in the submandibular glandular mass in diabetic rats. When comparing the control and supplemented control groups, the latter showed an increase of 23%. Despite these findings, these results have little clinical significance since the glandular mass does not take into account that the greater the animal's body mass, the greater its corresponding gland. As can be seen in Table 1, the Supplemented Control group has an average body mass 24% greater than the Control. The results of Relative Glandular Mass (MGR) confirm those already observed by Anderson et al., 1994 who find an increase in MGR in diabetic rats, suggesting a glandular increase caused by hyperplasia or hypertrophy of their cells. On the other hand, the decrease in MGR with N-acetylcysteine supplementation in diabetic rats exerts protection in the submandibular glands from possible biochemical changes that produced hypertrophy and/or hyperplasia. The increase in MGR in submandibular cells suggests that diabetes determines oxidative lesions in glandular cells and leads to a physiological increase due to hyperplasia and / or hypertrophy, with alteration in their function, in an attempt to compensate for the loss determined by oxidation of biomolecules and / or death cell.

The TBARS results indicate that an oxidative attack occurred in the lipid molecules in this gland, showing that in diabetes, the increase in the production of ROS / ERN is not balanced by a proportional increase in endogenous antioxidant defenses. It is clear that NAC supplementation was effective in reducing lipid damage, both in diabetic conditions (pathological conditions) and in the control group (physiological conditions). Some studies have found an increase in lipoperoxidation due to the higher concentration of species reactive to thiobarbituric acid (TBARS), in the diabetic state. Miranda-Filho et al. 2020 they evaluated the efficacy of the hydroethanolic extract of *Raphanus Sativus* leaves in reducing the damage of oxidative stress under the submandibular glands of diabetic rats, for this measured the oxidative damage in the gland through the concentration of malondialdehyde and carbonylated protein levels, and concluded study that the extract reduced the glycemia of the animals and also the effects of diabetes mellitus that involve the oral cavity. Kakkar et al. (1995) found an increase in this index in the heart of diabetic rats induced by streptozotocin. Limaye, Raghuram and Silvakami, 2003 they observed a 50% increase in the concentration of malonaldehyde in the kidneys of diabetic rats. In other organs of diabetic rats it was also possible to find an increase in lipoperoxidation as in the liver (Feillet-Coudray et al., 1999) and blood (Dominguez et al., 1998; Qujeq et al., 2004).

In analyzes of salivary glands, Nogueira, 2004 found an increase in submandibular lipid peroxidation, confirming the results found. The increase in damage to lipid molecules is associated with greater production of ROS, especially of the radicals  $O_2^{\cdot-}$  and  $\cdot OH$  in the diabetic state. On the other hand, the results point to a high oxidative stress in the submandibular, a

fact that can be explained by the fact that this gland is not so prepared to defend itself against the exacerbated production of ROS, since its metabolism is predominantly anaerobic, and, consequently, the activities of antioxidant enzymes are not as expressive (Pedraza-Chaverrí et al., 2005). Accordingly, the submandibular individuals responded well to supplementation with N-acetylcysteine, which, serving as a substrate for the synthesis of GSH, favors the reduction of oxidative stress.

The result of protein oxidation reinforces the statement that there is an increase in the attack of ROS to the biomolecules of the submandibular gland in diabetics, and that supplementation with NAC is effective only in the case of oxidative stress, since when administered chronically to the control there is an increase in the formation of protein carbonyls.

The concentrations of GSH and GSSG in the submandibular gland are shown in Tables 2 and Figure 3. From their analysis, it is noted that the induction of diabetes causes a significant drop in the concentration of GSH, without a corresponding proportional increase in the concentration of GSSG. Similar results to that study were reported by Chai et al. 1994 in human neutrophils and by Brigagão et al. 2004 in neutrophils of stimulated mice, which presented strong evidence that this process is due to the exacerbated production of ROS / ERN by cells. The results of determination of GSH and GSSG in submandibular individuals confirm the previous ones shown in table 2 and figure 3, where the data indicate a higher occurrence of lipoperoxidation and protein oxidation in submandibular individuals, in addition to showing that the supplementation partially recovered these levels of oxidative lesions. The  $\gamma$ -glutamyl-cysteinyl-glycine (GSH) tripeptide due to its free thiol group in the cysteine residue, has the ability to react with ROS / ERN and, thus, prevent injuries to biomolecules. Several studies confirm the reduction of the GSH content in the diabetic state: in the liver (Saxena et al., 1993; Wohaiab & Godin, 1987) and in the blood of humans with both type I and type II diabetes (Godin et al., 1988; Seghrouchni et al., 2002). On the other hand, Nogueira, 2004 found an increase in the concentration of this tripeptide in submandibular. The decrease in GSH found in the submandibular in our study does not necessarily mean a reduction in the speed of its synthesis, but probably its use at high speed by the analyzed tissues. The accumulation of the oxidized form of glutathione in the diabetic submandibular can be explained by a possible increase in the activity of the enzyme glutathione peroxidase, since according to Kakkar et al. 1995 there is an increase in the activity of this enzyme in the presence of oxidative stress, which uses GSH to detoxify organic peroxides. Some studies in other tissues confirm the increased activity of this enzyme in kidney diabetes (Dohi et al., 1988; Limaye; Raghuram; Silvakami, 2003) and in the blood of diabetic rats (Godin et al., 1988; Qujeq et al., 2004).

The results of reducing power are consistent with those of the lipoperoxidation and protein oxidation analyzes, which showed a higher level of oxidative damage to biomolecules in the submandibular gland.

Based on these results, which point to a depleting action of the reduced form GSH during diabetes mellitus, without stoichiometric accumulation of the oxidized form GSSG, we postulate the occurrence of protein S-thiolation, mainly the protein S-glutathation process in the salivary glands. The mobilization of soluble thiols to the protein fraction during oxidative stress, as a way of protecting susceptible cysteine residues in protein structures, as well as enzymatic regulation for adaptive response to stress and other metabolic changes, has already been reported in different tissues (Klantt & Lamas, 2000).

The binding of thioic compounds to cysteine residues occurs mainly due to the action of ERO / ERN on cysteines present in the protein structure or on thiols, oxidizing them and making them more reactive, thus favoring the formation of mixed protein disulfides. This modification may be indicative of the occurrence of oxidative stress, as the increase in S-thiolated hemoglobin (specifically S-glutathione, HbSSG) has been recently reported, with a clear correlation between the glycemic rate and the installation of microangiopathies and the accumulation of TBARS in blood samples of diabetic individuals (Sampathkumar et al., 2005).

As observed in the studied salivary gland, diabetes caused an increase in the occurrence of S-thiolation. These results confirm the previously analyzed parameters, where the submandibular was susceptible to oxidative stress, caused by the diabetic state. On the other hand, it can be seen that the CS and DS groups had a higher occurrence of S-thiolation, a hypothesis for this phenomenon is that N-acetylcysteine (NAC), used in supplementation, being a thiol compound, would be linking to the structure protein. In the DS group, this increase may be due to the sum of the oxidative stress action and greater to the availability of RS• radical compounds.

These results, analyzed together, suggest that NAC is an efficient supplement to contain oxidative stress, but, similarly to other thiolic compounds, it can act as a pro-oxidant in conditions where there is no exacerbation of the production of ROS / ERN in fabrics. The pro-oxidant potential of this modified amino acid has been previously reported in the literature, where the supplementation of healthy individuals, without a significant oxidative stress, led to a decrease in the speed of GSH synthesis and the accumulation of GSSG. In addition, DNA damage in the presence of copper II, detected both in vitro and in isolated cells, was clearly associated with a high concentration of NAC (Ercal, Gurer-Orhan, 2002).

The results of this study make clear the need for a detailed study of the installation of oxidative stress and the effect of antioxidants on it, since indiscriminate supplementation with exogenous compounds can be harmful to the body. It is worth mentioning that the submandibular gland and the pancreas have the same embryological origin and it can be speculated that the effects demonstrated in this work may be valid for both glands, suggesting a focus for further studies.

## 5. Conclusion

The results allow us to conclude that diabetes determines an increase in the relative glandular mass in the submandibular and the administration of N-acetylcysteine (NAC) prevents this process (i), the installation of diabetes mellitus causes oxidative metabolic changes in lipids and proteins, which were significantly reduced by oral supplementation with NAC (ii), diabetes causes a reduction in the reducing power in the analyzed salivary gland, and supplementation with NAC recovers the same (iii), there is a decrease in the concentration of reduced glutathione in submandibular individuals as a result of the condition diabetic, without concomitant and proportional increase in GSSG, a process partially recovered by N-acetylcysteine (iv), there is an increase in protein S-thiolation associated with the onset of diabetes mellitus (v), oral supplementation of normoglycemic animals with NAC caused an increase in Protein S-thiolation. It is emphasized the need to be studied in more detail in other experimental models, in order to elucidate the additional effects that may come to collaborate with our findings.

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