Alterations of antioxidant biomarkers and type I collagen deposition in the parotid gland of streptozotocin-induced diabetic rats

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1. Introduction

Diabetes mellitus (DM) is a widespread disease that is associated with high morbidity and health care costs. DM is a generalized common chronic metabolic disorder characterized by hyperglycemia resulting from defects in insulin secretion and/or action and certain abnormalities in carbohydrate, fat, electrolyte, and protein metabolism. Because of the high prevalence of DM in humans, a number of...
of diabetic animal models have been developed to study this disease. These models have improved over the years, and rodent models are the most thoroughly described. In these models, injections of alloxan and streptozotocin (STZ) induce high glucose and low insulin plasma levels. In addition, alloxan and STZ cause an insulin-dependent diabetes syndrome, which leads to beta cell destruction with morphological features characteristic of necrotic cell death. Extensive research is still being performed to develop new antidiabetic agents and determine their mechanisms of action. Oral antihyperglycemic drugs have been used to facilitate the study of diabetes, particularly drugs belonging to a class of alpha-glucosidase inhibitors. For example, acarbose has been used in the treatment of patients with diabetes.

Persistent hyperglycemia in the diabetic state may cause considerable production of free radicals in many tissues. Free radicals are generated from the direct auto-oxidation of glucose and during protein glycosylation, and studies have shown a correlation between the status of metabolism control, the duration of DM, and the severity of induced oxidative stress. Interestingly, glucose plays an important role in the accumulation of collagen in tissues. Indeed, the hyperglycemic state leads to advanced glycation end product formation in the components of the matrix and accelerates the crosslinking between collagen fibres. In addition, increased collagen deposition contributes to an increased stiffness of tissues in diabetic subjects because of changes in the structure and organization of the extracellular matrix. Although clinical features of the parotid glands in individuals with diabetes have been identified, the mechanisms underlying the collagen abnormalities in diabetic parotid glands are poorly understood.

Thus, the purpose of the present study was to evaluate the antihyperglycemic effects of acarbose treatment on parotid glands and examine the correlation between antioxidant status and type I collagen deposition in diabetic rats with or without acarbose treatment.

2. Materials and methods

2.1. Animals

Male Wistar rats weighing approximately 160–210 g were acclimated to laboratory conditions for a period of two weeks, which was followed by maintenance under controlled temperature (25 ± 2°C) and light (12/12-h light/dark cycle) conditions. Animals received standard extruded chow and water ad libitum until treatment or sacrifice. All animal procedures followed the guidelines outlined by the Brazilian Society of Laboratory Animal Science (SBCAL 2009) and were approved by the Ethics Committee in Animal Research of the Federal University of Uberlandia, Brazil (CEUA/UFU 051/08).

2.2. Diabetes induction

Diabetes was induced by intravenous injection of STZ (single dose, 40 mg/kg in 0.1 M sodium citrate, pH 4.5) (Sigma–Aldrich, MO, USA). Ten days after diabetes induction, the fasting blood glucose level was monitored, and the blood glucose concentration was measured using reactive strips (Biocheck Glucose Test Strip) (Bioeasy, Minas Gerais, Brazil). Rats having a blood glucose level ≥ 200 mg/dL were considered to be diabetic. The rats were randomly divided into four groups (n = 10 in each group): non-diabetic (NDM); diabetic (DM); diabetic treated with 25 mg/kg acarbose (DMA) and non-diabetic treated with acarbose (NDMA). Treatments were administered once a day by gavage for 20 days. After twenty days, all rats were anesthetized by an intraperitoneal injection of ketamine and xylazine (1:1, v/v), sacrificed by euthanasia, and tissues were harvested and dissected.

2.3. Sample collection

The parotid glands were quickly removed from all rats, washed in chilled 0.9% NaCl, weighed, and immersed in liquid nitrogen or fixed in formaldehyde (see Section 2.6). We calculated the ratio of parotid gland weight (in mg) to body weight (in g). We also collected blood from the portal vein to measure blood glucose and serum biochemical parameters, such as total cholesterol (enzymatic Trinder method), plasma triglycerides (enzymatic Trinder method), total protein (Buret method), creatinine (modified Heinegard and Tiderstram’s method), urea (urease UV kinetic method), aspartate aminotransferase (AST) (IFCC UV kinetic method), alanine aminotransferase (ALT) (IFCC UV kinetic method), γ-glutamyltransferase (γ-GT) (modified Szasz method) and alkaline phosphatase (modified Bowers and McComb method), in a Cobas Mira automatic analyzer (Roche Diagnostic Systems, Basel, Switzerland) at 37°C using commercial kits (Labtest Diagnostica, Minas Gerais, Brazil).

2.4. Homogenate preparation

The parotid glands were homogenized in HEPES buffer (40 mM) containing 100 mM EDTA, 2 mM EGTA, 2 mM DTT, 1 mM benzamidine and 0.5 mM PMSF. Tissue homogenates were centrifuged, and the supernatant was assayed for the activity of oxidative stress markers 10,000 × g for 10 min at 4°C. The total protein concentration was determined using the Bradford method.

2.5. Analysis of oxidative stress markers

Lipid peroxidation products were determined by measuring the content of malondialdehyde (MDA) with the thiobarbituric acid test (TBARS) using a commercial kit (Cayman Chemical Inc., MI, USA). The total antioxidant status (TAS) and the total activities of superoxide dismutase (SOD) and glutathione peroxidase (GPX) were measured using commercial kits (Randox Laboratories Ltd., Crumlin, UK). Catalase (CAT) activity was assayed spectrophotometrically by monitoring hydrogen peroxide decomposition at 240 nm, and the substrate concentration was 20 mM for parotid gland measurements.

2.6. Histology and measurement of collagen

Parotid glands were fixed with 10% formaldehyde in 0.1 M phosphate-buffered saline (pH 7.4) for 24 h, dehydrated in
percentage of type I collagen pixels per field area was measured. Areas with a higher proportion of staining were selected, and the parotid gland weight the relative glandular weight tended to be higher in the DM group compared with the NDM, NDMA and DMA groups. Interestingly, STZ-induced diabetes had no significant effect (p > 0.05) on glandular total protein content; however, total protein was decreased in the DM and DMA rats compared with the NDM and NDMA rats (Table 1).

### 3.2. Biochemical parameters

Alkaline phosphatase values were 359% higher (p < 0.001) in the DM group compared with the NDM and NDMA groups (Table 1). A significant increase was found in DMA rats compared with NDM and NDMA rats (p < 0.001). The DMA rats exhibited values that were reduced by 11% compared with the DM group. We also observed a 61% increase in the levels of γ-GT in the DM rats compared with the NDM and NDMA groups. Treatment with acarbose decreased γ-GT levels by 36% compared with the DM group.

Alanine aminotransferase levels were increased by 94% (p < 0.001) in the DM rats compared with the NDM and NDMA rats. Interestingly, treatment with acarbose was able to decrease ALT levels by 12% (p < 0.001) in the NDM group (Table 1). Likewise, urea values were higher (p < 0.001) in the DM and DMA groups compared with the NDM and NDMA groups. Treatment of the DMA rats with acarbose decreased urea values by 9% compared with the DM group.

The values of creatinine and triglycerides were maintained in all groups. In addition, cholesterol and AST did not show statistical differences between groups (p > 0.05), although a subtle increase in the DM group was observed for both parameters.

### 3.3. Oxidative stress biomarkers

Comparison of oxidative stress biomarkers within groups (Fig. 1) revealed that MDA levels were significantly higher in ethanol, cleared in xylene and embedded in paraffin. To evaluate the morphology of the parotid glands, five-micrometre sections were stained with haematoxylin and eosin (H&E). Sections were also stained with picrosirius red to determine the percentage of connective tissue. Morphometric analysis was carried out using a light microscope (Olympus Ltd., Hertfordshire, UK) with a 40× objective, and the microscope was equipped with an Oly-200 CCD camera linked to a PC and a capture and image analysis system (HL-Image 97, Western Vision Software, UT, USA). Thirty random fields within the areas with a higher proportion of staining were selected, and the percentage of type I collagen pixels per field area was measured.

### 2.7. Statistical analysis

Data were analysed using SigmaStat 3.5 software (Systat Software Inc., IL, USA), and means and standard deviations were calculated. A one-way analysis of variance (ANOVA) was used to compare the values amongst groups, and a p value of < 0.05 was considered significant.

### 3. Results

#### 3.1. Body weight and blood glucose levels

The mean blood glucose concentration of the DM rats was 19% higher than that of the DMA rats (Table 1). Indeed, the average blood glucose level of the DM group was 315% higher (p < 0.001) than the NDM and NDMA rats, whereas the DMA group exhibited a glucose level by 71% lower (p < 0.001) than the DM rats.

Body and parotid gland weights are shown in Table 1. After 20 days of treatment, the mean value of the final body weight of the rats in the NDM group increased, and the mean final body weight of the DM rats decreased by 12% (p < 0.05). The DMA rats exhibited a 6% decrease (p < 0.05) in body weight compared with the NDM group; however, the NDM and NDMA groups maintained their average body weight. Although twenty days of treatment did not result in changes in parotid gland weight the relative glandular weight tended to be higher in the DM group compared with the NDM, NDMA and DMA groups. Interestingly, STZ-induced diabetes had no significant effect (p > 0.05) on glandular total protein content; however, total protein was decreased in the DM and DMA rats compared with the NDM and NDMA rats (Table 1).

#### 2. Anthropometric and biochemical parameters of NDM, DM, DMA and NDMA.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>NDM</th>
<th>DM</th>
<th>DMA</th>
<th>NDMA</th>
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<tbody>
<tr>
<td>Glycemia (mg/dL)</td>
<td>115.60 ± 6.88</td>
<td>479.60 ± 42.07&lt;sup&gt;b&lt;/sup&gt;</td>
<td>401.70 ± 47.63&lt;sup&gt;b&lt;/sup&gt;</td>
<td>118.40 ± 7.92</td>
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<td>Body weight (g)</td>
<td>305.60 ± 6.86</td>
<td>271.60 ± 5.46&lt;sup&gt;c&lt;/sup&gt;</td>
<td>287.60 ± 9.10</td>
<td>305.00 ± 8.09</td>
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<tr>
<td>Gland weight (mg)</td>
<td>92.80 ± 4.07</td>
<td>83.30 ± 4.33</td>
<td>78.20 ± 11.41</td>
<td>85.33 ± 7.41</td>
</tr>
<tr>
<td>Gland/Body weight (mg/g)</td>
<td>0.30 ± 0.01</td>
<td>0.31 ± 0.02</td>
<td>0.27 ± 0.04</td>
<td>0.28 ± 0.03</td>
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<tr>
<td>Protein (µg/ml)</td>
<td>2.23 ± 0.09</td>
<td>2.15 ± 0.12</td>
<td>2.16 ± 0.14</td>
<td>2.29 ± 0.10</td>
</tr>
<tr>
<td>Alkaline phosphatase (U/L)</td>
<td>133.21 ± 14.23</td>
<td>610.92 ± 43.77&lt;sup&gt;b&lt;/sup&gt;</td>
<td>548.98 ± 142.39&lt;sup&gt;b&lt;/sup&gt;</td>
<td>583.47 ± 16.94</td>
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<tr>
<td>Cholesterol (mg/dL)</td>
<td>62.32 ± 4.48</td>
<td>69.46 ± 3.93</td>
<td>69.99 ± 6.03</td>
<td>64.66 ± 3.41</td>
</tr>
<tr>
<td>Creatinine (mg/dL)</td>
<td>0.68 ± 0.04</td>
<td>0.74 ± 0.03</td>
<td>0.63 ± 0.04</td>
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<tr>
<td>γ-GT (U/L)</td>
<td>5.78 ± 1.01</td>
<td>9.28 ± 0.63&lt;sup&gt;b&lt;/sup&gt;</td>
<td>5.96 ± 0.67</td>
<td>4.93 ± 0.41</td>
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<tr>
<td>AST (U/L)</td>
<td>117.90 ± 15.86</td>
<td>130.50 ± 13.60</td>
<td>143.30 ± 22.13</td>
<td>116.40 ± 9.00</td>
</tr>
<tr>
<td>ALT (U/L)</td>
<td>50.30 ± 4.28</td>
<td>97.90 ± 6.28&lt;sup&gt;b&lt;/sup&gt;</td>
<td>86.90 ± 11.44&lt;sup&gt;a&lt;/sup&gt;</td>
<td>59.60 ± 5.34</td>
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<tr>
<td>Triglycerides (mg/dL)</td>
<td>74.05 ± 13.77</td>
<td>157.36 ± 36.11</td>
<td>133.10 ± 32.03</td>
<td>81.16 ± 23.38</td>
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<tr>
<td>Urea (mg/dL)</td>
<td>37.98 ± 2.36</td>
<td>77.31 ± 4.64&lt;sup&gt;b&lt;/sup&gt;</td>
<td>71.11 ± 6.63&lt;sup&gt;b&lt;/sup&gt;</td>
<td>44.88 ± 2.88</td>
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</table>

Data are expressed as means ± S.E.M. n = 10 rats for each group.

* <sup>p</sup> < 0.001 vs. NDM.
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* <sup>p</sup> < 0.001 vs. NDM.
around ducts, acini, nerves, and in the walls of arteries and between the DM and NDM groups. Type I collagen was found stained in the parotid tissue when comparisons were made as the NDM group. The collagen fibers were differentially deposited. The NDMA group showed the same morphology widely spaced and exhibited a higher level of collagen the acini in the gland tissues of the DM group were more between the acini. Moreover, compared with the NDM group, DMA rats showed an intermediate pattern of demarcation

4. Discussion

Diabetes mellitus is a metabolic disease that affects many organs and systems. In the salivary glands, alterations in glycolytic enzyme activity and antioxidant parameters have been reported in association with diabetes. These changes have also been related to increased collagen deposition, which has been described in the heart of diabetic rats.

Biochemical and morphological features of the parotid glands of acarbose-treated and non-treated diabetic rats have been shown to differ from healthy animals. In the present study, we found alterations in the enzymatic antioxidant system (e.g., SOD and GPx enzymes) of the parotid glands after the STZ induction of diabetes. In addition, blood glucose levels in the DM rats remained high, even after acarbose treatment. Non-diabetic rats were significantly heavier than the DM and DMA rats. These observations may be due to a lack of insulin. Indeed, studies have shown that STZ-induced diabetic animals cannot use glucose as an energy source during fasting periods; thus, fat catabolism is activated as an alternative energy source. Consequentially, diabetic animals lose weight.

Based on previous studies, the most marked feature of diabetes mellitus is hyperglycemia. In the present study, there was a greater amount of variation in serum glucose levels in the diabetic group compared with the non-diabetic rats. In addition, the results that we observed for the DMA rats were in agreement with those of Van de Laar et al. who described a clear effect of acarbose treatment on glycemic control. Furthermore, a higher rate of gluconeogenesis was shown to be facilitated by unrestricted proteolysis, which contributes to weight loss. Another striking observation between the NDM and DMA rats was the finding that the diabetic rats lost weight in the same period of time in which the healthy rats gained weight. This may be due to increased proteolysis and a reduction of adipose tissue in the diabetic rats, which was confirmed by the observed loss of fat during dissection. Although parotid gland weight was decreased in the NDMA and DMA groups, there were no significant differences between groups. A similar pattern was observed for body weight, which was probably because there was a positive correlation between gland weight and body weight (r = 0.95) (Fig. 3). Our results were in accordance with those of Nicolau et al. who calculated relative glandular weight and showed a higher value for the parotid gland of diabetic rats compared with non-diabetic rats. Acarbose treatment appeared to decrease parotid gland size and was able to decrease parotid gland weight regardless of STZ treatment. To our knowledge, such findings have not previously been reported and suggest any

![Graph showing oxidative parameters](image)

**Fig. 1** – Effects of STZ-induced diabetes and acarbose treatment on oxidative stress biomarker levels in the rat parotid glands. Bar charts showing the relative percentage values of the oxidative analysis results in the parotid glands of rats in the NDM, DM, DMA and NDMA groups. Data are presented as mean ± S.E.M. Malondialdehyde concentration (MDA), total antioxidant status (TAS), total activity of superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx). Glutathione peroxidase values are ×10^3. (a) p < 0.001 vs. NDM, (b) p < 0.001 vs. NDMA, (c) p < 0.001 vs. DMA and (d) p < 0.05 vs. NDM. n = 10 rats for each group.

the DM group (134%) compared with all other groups (p < 0.001). Acarbose treatment decreased MDA to the levels of the NDM group. The mean value of SOD activity in the DM group was significantly higher (46%) than NDM group (p < 0.05). Furthermore, the DMA group exhibited SOD activity values that were 15% lower than those of the DM group. Similarly, GPx activity was increased in the DM group compared with the NDM by 11% (p < 0.001). Acarbose treatment was also able to reduce by 8% GPx in the DMA group compared with the NDM group (p > 0.001). We did not find any differences between groups for TAS or CAT activity (p > 0.05).

3.4. Type I collagen

Parotid gland tissue stained with H&E was analysed by light microscopy to define the limiting membrane of the acini in more detail (Fig. 2). Compared with the NDM and DM rats, the DMA rats showed an intermediate pattern of demarcation between the acini. Moreover, compared with the NDM group, the acini in the gland tissues of the DM group were more widely spaced and exhibited a higher level of collagen deposition. The NDMA group showed the same morphology as the NDM group. The collagen fibers were differentially stained in the parotid tissue when comparisons were made between the DM and NDMA groups. Type I collagen was found around ducts, acini, nerves, and in the walls of arteries and veins. The staining pattern in the DM group revealed thick and densely stained collagen bundles around ducts, whereas thin and delicate fibres were observed around acini and nerves. Increased collagen staining was more evident in the DMA group compared with the NDM group, and the staining showed intermediate levels of deposition compared with the DM group.

Fig. 2 – Deposition of type I collagen fibre in rat parotid glands. Panels A, C, E, and G show collagen staining, and panels B, D, F, and H show H&E staining. Compared with the NDM group, the intensity of type I collagen staining was increased around
possible effect of acarbose affecting functional aspects of the parotid gland.

Our findings for oxidative stress biomarker levels were in agreement with previous studies that reported a correlation between the diabetic state and antioxidant levels in rat salivary glands (i.e., TBARS detection reflects the effects of damage in cells). A significant increase of MDA was observed in the parotid glands of the rats in the diabetic group compared with the other groups. Similar to previous studies, we found alterations of the MDA index in the salivary glands of diabetic animals, which indicates that oxidative stress may have affected the activity of SOD, CAT and GPX. Interestingly, acarbose treatment diminished the oxidative damage caused by diabetes mellitus.

We observed an elevation of the antioxidant activity of both SOD and GPx in diabetic rats. The decreased activity of these enzymes in acarbose-treated animals was probably a consequence of a protective effect of acarbose treatment against oxidative damage in parotid glands. Our data show a positive correlation between the results for SOD, GPx and MDA, and all of these results were consistent with an increase in antioxidant activity in diabetic individuals as a result of increased reactive oxygen species production. The level of SOD activity in the diabetic group was significantly higher than the other groups. In the enzymatic salivary antioxidant system, however, peroxidase is the most important enzyme. An increase in SOD activity in diabetic animals may be due to increased dismutation of superoxide anions to molecular oxygen and hydrogen peroxide as an adaptive response to increased oxidative stress. Our study found that there was higher specific activity of GPx in parotid glands from diabetic rats, which has also been shown by Qujeq et al. and Nogueira et al. Interestingly, in diabetic conditions, GPx activity has been shown to be lower in liver, kidney, and muscle tissue compared with non-diabetic conditions; however, no differences were observed in heart or brain tissue. In the present study, we did not find any differences in our TAS analysis. Our hypothesis was that the parotid gland presents aerobic metabolism, and can therefore be naturally prepared to prevent the oxidative damage caused by reactive oxygen species.

Although previous studies have shown differences in CAT activity in the kidneys of diabetic rats compared with non-diabetic rats, we did not observe any differences in the CAT activity of the parotid glands of diabetic rats. Interestingly, CAT activity levels under diabetic conditions have been found to be variable amongst different diabetic tissues. Compared with non-diabetic rats CAT activity has been reported to be higher in the muscle, heart and brain, and lower in the liver. Recently, in a temporal study (7–60 days) of rats with STZ-induced diabetes, Ibuki et al. reported that CAT activity was maintained at increased levels in the parotid gland of diabetic rats. The differences observed in the parotid gland may be due to treatment and animal conditions, such as STZ dose and the observation that the rats in the present study had lost weight at the end of the experimental period.

In the present study, typical serum biochemical parameters, such as alkaline phosphatase activity, were found to be elevated in diabetic rats. The activity of this enzyme is often tested to evaluate whether the liver is damaged or diseased, which occurs in subjects with type 1 diabetes. Our data also show that there was increased activity of ALT after the onset of diabetes mellitus. Acarbose treatment in diabetic rats, however, caused a partial reduction in the plasma activity of these enzymes compared with non-treated diabetic rats. Scavenging mechanisms or neutralization of free radicals suggest interactions with the oxidative cascade, quenching of oxygen, inhibition of oxidative enzymes and peroxidation of membrane lipids, which results in maintenance of cell membrane integrity and functions. An increase in urea levels in diabetic subjects was also observed by Kaleem et al. and Garg et al. However, urea levels have been found to be slightly reduced in acarbose-treated diabetic individuals, which indicates that drug administration has some beneficial effects on kidney function. High levels of plasma urea in DM animals have been shown to be associated with renal damage due to abnormal glucose regulation, including elevated glucose and glycosylated protein tissue levels, haemodynamic changes within the kidney tissue, and increased oxidative stress.

In the present study, light microscopy studies show marked differences in the macrostructural morphology of parotid glands between diabetic and treated diabetic rats. Indeed, H&E-stained sections of diabetic rat parotid glands were extensively infiltrated with lipid droplets of various magnitudes. In contrast, non-diabetic glands display normal macrostructural properties with little or no distribution of lipid droplets. In the present investigation, picrosirus red staining shows that the content of type I collagen increased in the parotid glands of diabetic rats, which was in agreement with the findings for the heart and kidney in STZ-diabetic rats.
In the present study, acarbose treatment in diabetic rats reduced the amount of collagen to a similar level as the non-diabetic group. Information about the collagen type I distribution and staining pattern in diabetic rat parotid glands is scarce. The changes observed in diabetic rats may affect the structure and function of acini, which would compromise gland function. The findings of Anderson, have also identified clear granule-like structures, which appeared to be lipid droplets, in parotid gland sections from diabetic rats. These findings suggest that parotid gland cells become dysfunctional during insulin-dependent diabetes mellitus. In the present study, acarbose treatment was able to reduce some of the damage caused by DM, which led to a reduction in the size and number of lipid droplets and a partial reversal of the observed biochemical changes. Our data show that there was a positive correlation between the amount of type I collagen and oxidative parameters, which was also suggested by Baynes. These researchers reported increased levels of glycoxidation products in collagen, which can be attributed to increases in glycation and oxidative stress. We observed that morphological changes in the rat parotid glands were correlated with biochemical parameters, which reinforced the relevance of acarbose treatment for attenuating the severity of alterations caused by this disease.

Amylase may impact antioxidant levels and collagen deposition in diabetic animals, and studies have shown reductions in amylase expression in the parotid glands of STZ-induced DM rats. We must also mention that DM patients can have an increase in overall salivary antioxidants those results from a state of systemic oxidative stress. Indeed, systemic oxidative stress induces a general increase in serum antioxidants, and serum composition tends to reflect saliva composition. For instance, Reznick et al. reported that amylase activity in uncontrolled DM patients was very high, and they suggested that the overall changes in salivary content were a consequence of oxidative stress in the parotid gland as well as the whole organism. Thus, amylase levels may correlate with the oxidative conditions of the gland rather than playing an active role in increasing oxidative stress. In conclusion, acarbose treatment exerted an antioxidant effect in the parotid glands of diabetic rats. The picrosirius red staining technique was used to assess collagen type I deposition in the parotid gland of diabetic rats, and it was also useful for comparing antioxidant treatments. Acarbose treatment is effective in the reduction of oxidative stress in the parotid gland, and the observed positive correlation between type I collagen and oxidative stress is also important. Further studies are necessary to evaluate collagen type I deposition after different treatment periods.

Conflict of interest statement

There are no conflicts of interest with regard to our manuscript.

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Ethical approval

All animal procedures were approved by the Ethics Committee in Animal Research of the Federal University of Uberlândia, Brazil (CEUA/UFU 051/08).

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