Hypoglycemic effects of an aqueous extract of Bauhinia forficata on the salivary glands of diabetic mice

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Abstract: The objective of this study was to evaluate the salivary glands in diabetic mice, analyzing alterations in the secretory epithelium and interactions with the stromal compartment acquired during a prolonged period of treatment with Bauhinia forficata extract. Female mice were divided into two groups: Nonobese diabetic (NOD) mice treated with Bauhinia forficata (I), and NOD mice not treated with the hypoglycemic agent (II). After treatment, the salivary glands were collected for analysis by transmitted and polarized light microscopy, complemented by three-dimensional analysis of these tissues. The results showed weight loss in animals of group II and weight recovery in treated animals. Glucose levels were elevated in group II, but declined in group I. In the two groups, the salivary glands were characterized by involution of the secretory epithelium, presence of an inflammatory infiltrate and an increase of extracellular fibrillar components. It can be concluded that treatment with Bauhinia forficata reduced glucose levels and contributed to weight recovery in treated animals. However, the observation of tissue destructuring and compromised epithelial-stromal interactions, with consequent impairment of glandular function, demonstrates that Bauhinia forficata exerts an effect on the recovery of body metabolism but this improvement does not influence in the tissue recovery.

Keywords: Treatment; hypoglycemic effect; diabetes mellitus; salivary glands.

INTRODUCTION

Diabetes mellitus is a chronic metabolic condition, generally characterized by hyperglycemia, which results from the inability of the organism to produce or respond to insulin. The number of patients with this disease is increasing every year. In Brazil the estimated mean incidence of diabetes in the adult population is 5.2%. According to these data, in some years there will be approximately 438 million people with diabetes in the world, with more prevalence in developed countries such as the United States or in developing countries such as Brazil (Gan, 2003; Unwin N et al., 2010; Kumar et al., 2010; Ministério da Saúde do Brasil, 2010). These data are important since diabetes continues to be an irreversible disease that is associated with various complications, including impairment of salivary gland function.

The importance of the salivary glands and their secretions for the maintenance of oral and general health of the organism has been documented in the literature (Abiko and Saitoh, 2007; Miletich, 2010). These glands possess an epithelial secretory structure and stroma that give support to this parenchyma, presenting blood vessels, lymphatic vessels and nerves that supply these organs, with these compartments being important for glandular structure and function (Barcellos and Andrade, 2005). As a consequence, changes in the structure and function of these and other organs may indicate an abnormal glycaemic condition and its relationship with other diseases. In this respect, studies have shown an association between hyperglycemia and neoplastic alterations (Caldeira et al., 2005; Caldeira and Cagnon, 2008; Simões et al., 2009; Nicolucci, 2010; Rossi et al., 2010). Thus, to understand the mechanism of diseases it is fundamental to determine their effects on tissues, including in the extracellular matrix (Suba and Ujpál, 2007; Felix et al., 2010).

In view of the consequences of diabetes and the difficulty in establishing truly effective treatments due to the diverse tissue responses to this disease, new or alternative therapies are an important tool to restore the damage caused by this hyperglycemic condition as demonstrated by Singh and Gupta (2007). However, in some cases these therapies may not promote effective tissue recovery as observed experimentally in salivary glands (Murakami et al., 2009), demonstrating the complexity of treatment and cell recovery. Therefore, the objective of the present study was to evaluate the cellular architecture of the salivary glands of diabetic mice, analyzing possible alterations in the secretory epithelium and interactions with the stromal compartment acquired during a prolonged period of alternative treatment with Bauhinia forficata extract.

MATERIALS AND METHODS

Animals, tissue and extract preparation
Twenty 15-week-old NOD mice, weighing on average...
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20g, were divided into two groups: 10 diabetic NOD mice (group I) and 10 also diabetic NOD mice (group II). The animals were obtained from the Animal House of Universidade Estadual de Campinas (CEMIB-UNICAMP) and were kept under standard conditions of housing, feeding and treatment at the Sector of Laboratory Animal Experimentation (SEA), Department of Morphology and Basic Pathology, Faculty of Medicine of Jundiaí.

Blood glucose (mg/dL) was measured weekly in all animals with a blood glucose meter (Accu-Chek Performa, Roche, Switzerland). After characterization of the diabetic condition, animals of group I that presented weekly glucose levels higher than 300 mg/dL (Shirai et al., 1998) were treated with fractions of an aqueous extract (800 mg/kg) of Bauhinia forficata leaves (Silva et al., 2002) for a period of 20 days. The aqueous leaf extract was prepared using a standard method, which consists of ultra-freezing and trituration of the specimen. The powder obtained was then diluted in 70% alcohol. Excess alcohol was evaporated at 40°C and, the plant extract was thus obtained (Oliveira et al., 2005). The extract was lyophilized and added to the drinking water of the animals. To simulate the experimental conditions of the treated group, animals of group II were manipulated in the same way and received pelleted diet and water ad libitum but no hypoglycemic agent. After treatment, the animals were anesthetized with a mixture (1:1) of ketamine hydrochloride (Ketalar, Parke-Davis) and xylazine hydrochloride (Rompun, Bayer Animal Health) at a dose of 0.02 g/ml and salivary gland samples were collected for analysis by transmitted and polarized light microscopy. All procedures were performed according to the ethical guidelines established by the Brazilian College of Animal Experimentation (COBEA).

Samples of the parotid and submandibular salivary glands were fixed in Bouin’s solution (picric acid solution), embedded in plastic resin (Paraplast Plus, Oxford Lab, USA), and stained with hematoxylin-eosin (H.E.). Parts of these samples were also stained with Picrosirius Red (saturated aqueous solution of picric acid supplemented with 0.1 g Sirius red F3b; Bayer) for analysis of extracellular matrix fibrillar components by polarized light microscopy.

Stereology-Three-dimensional analysis of tissues

The nuclear and cytoplasmic volumes of acinar cells of the parotid and submandibular glands were determined in H.E.-stained histological sections by transmitted light microscopy. For this purpose, 40 cells were analyzed per animal, for a total of 400 acini per experimental group, by the point counting method described by Weibel (1979). Only intact cells and circular or ellipsoid nuclei with defined limits were considered for this study. In addition, collagen fibers (types I, II and III) and the spatial volume density of these components were analyzed under polarized light and calculated as the mean of four regions in each Picrosirius-Red-stained histological section by the point-counting method (Weibel, 1979; Mandarim de Lacerda et al., 1995). All analyses were performed with a Nikon Eclipse microscope using 40x and 100x planachromatic objectives for transmitted light microscopy and birefringent lenses for polarized light microscopy. The microscope was coupled to the SD-3.3 CCD image acquisition system of the Department of Morphology and Basic Pathology, Faculty of Medicine of Jundiaí.

**STATISTICAL ANALYSIS**

The results are reported as the median and mean ± SD. The variation in body weight (g), glucose level (mg/dL), nuclear and cytoplasmic volume of acinar cells of the parotid and submandibular glands (µm³) and volume density of collagen fibers (µm³) were compared by analysis of variance (ANOVA), complemented by the Kruskal-Wallis nonparametric test involving all pairs of groups (Norman and Streiner, 1994). The level of significance was set at 5% for all tests.

**RESULTS**

**Body weight**

All animals presented weight loss after establishment of the diabetic condition. However, at the end of the experimental period mice treated with Bauhinia forficata extract had gained weight when compared to untreated diabetic animals (table 1).

**Table 1:** Mean body weight variation (initial weight – final weight) and glucose levels of animals treated with Bauhinia forficata aqueous extract (group I) and untreated animals (group II) over the experimental period.

<table>
<thead>
<tr>
<th></th>
<th>Group I</th>
<th>Group II</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial weight (g)</td>
<td>21.0±1.00</td>
<td>20.6±1.14</td>
</tr>
<tr>
<td>Final weight (g)</td>
<td>30.8±1.92*</td>
<td>19.0±1.0*</td>
</tr>
<tr>
<td>Initial glucose level (mg/dL)</td>
<td>364.2±27.43</td>
<td>363.2±28.32</td>
</tr>
<tr>
<td>Final glucose level (mg/dL)</td>
<td>235.4±20.58*</td>
<td>362.6±25.64*</td>
</tr>
</tbody>
</table>

Mean ± SD.

*Difference at the 5% level of significance.

**Proteinuria and urine pH**

In animals of group I, urine pH ranged from 7.0 to 7.5 and no proteins were detected in urine. In contrast, animals of group II presented a urine pH of 8.5 to 9.0 and proteinuria, findings indicating an uncontrolled diabetic state.
**Glucose levels**

Animals of group II presented elevated blood glucose levels, thus maintaining the diabetic state throughout the experimental period. In contrast, a significant reduction of glucose levels was observed in animals of group I treated with the aqueous extract of *Bauhinia forficata* (table 1).

**Transmitted and Polarized Light Microscopy**

**Parotid Glands**

Pleomorphic serous acini characterized by a reduced spatial area occupied by secretory epithelium, as well as an intense inflammatory process, were observed in the parotid glands of the two groups (fig. 1 and table 2). The basophilic cytoplasm and nucleus were also present. Some of these nuclei showed characteristics of pyknosis accompanied by intense hyperchromatism, findings characterizing the chromatin condensation typically observed during apoptotic processes (fig. 1). Neutrophils containing a polylobulated nucleus attached by a nuclear filament, large macrophages and lymphocytes containing a round nucleus, condensed chromatin and poorly basophilic cytoplasm were noted among the acini (fig. 1). The stroma was found to be enlarged in both groups, with a higher volume density of collagen type I, followed by type III and type II (fig. 1 and table 3).

**Submandibular Glands**

In the submandibular glands, atypical and involuted seromucous acini were also observed in the two groups studied (fig. 2). At higher magnifications, the cytoplasmic components of the secretory epithelial cells were poorly defined and there was a significant reduction in

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**Fig. 1**: Photomicrographs of the parotid glands. A: Atypical serous acini containing hyperchromatic nuclei (arrow) were observed in animals of group I, in addition to an inflammatory infiltrate consisting of lymphocytes (l), neutrophils (n) and macrophages (m). H.E. B: Stromal space containing collagen type I (I), type II (II), and type III (III). Picrosirius Red. C: Similar features were observed in animals of group II, including atypical serous acini with pleomorphic and hyperchromatic nuclei (arrow), in addition to an inflammatory infiltrate mainly consisting of macrophages (m) and lymphocytes (l). H.E. D: Stromal space containing collagen type I (I), type II (II), and type III (III). Picrosirius Red.

**Table 2**: Median nuclear and cytoplasmic volume (µm³) of acinar cells of the parotid and submandibular glands of animals treated with *Bauhinia forficata* aqueous extract (group I) and untreated animals (group II).

<table>
<thead>
<tr>
<th>Group</th>
<th>Parotid gland</th>
<th>Submandibular gland</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Nuclear volume</td>
<td>Cytoplasmic volume</td>
</tr>
<tr>
<td>I</td>
<td>17.03a</td>
<td>31.90b</td>
</tr>
<tr>
<td>II</td>
<td>16.61a</td>
<td>30.68b</td>
</tr>
<tr>
<td></td>
<td>0.2332</td>
<td>0.2482</td>
</tr>
</tbody>
</table>

Medians followed by the same superscript letter did not differ at the 5% level.
cytoplasmic volume (table 2). The nuclei were pyknotic and hyperchromatic, findings also characterizing the chromatin condensation typically observed during apoptotic processes. Enlargement of the interacinar space was similar in the two groups studied. Extracellular matrix alterations were observed in the stroma, with the observation of an increase in the connective tissue component, mainly collagen type I followed by type III and type II (fig. 2 and table 3).

DISCUSSION

Diabetic animals of group II, which were not treated with Bauhinia forficata extract, consumed higher amounts of food and fluid than treated animals. These animals also presented lesser weight throughout the experimental period, whereas animals of group I treated with Bauhinia forficata extract recovered weight. Diabetes mellitus causes metabolic disorders in various organ systems, weight loss and destructuring of different tissues (Cagnon et al., 2000; Caldeira et al., 2004; Caldeira et al., 2005). However, body weight recovery and gain during the experimental period were observed in a study on diabetic rats receiving hypoglycemic treatment for 7 days (Anderson, 1983). These findings demonstrate that diabetes alters overall metabolism, causing weight loss in animals, and that some treatments may reverse this damage, as observed in the present study in which animals treated with Bauhinia forficata extract showed body weight recovery and gain.

With respect to glucose levels, animals of group II presented elevated levels throughout the experimental period, whereas a significant reduction of glucose levels was observed in diabetic animals of group I. In a study using insulin replacement therapy, a proven hypoglycemic treatment, Hu et al. (1992) showed that normal glucose levels in healthy animals are close to 180

**Table 3:** Median variation in the volume density (µm$^3$) of collagen in the parotid and submandibular gland of animals treated with Bauhinia forficata aqueous extract (group I) and untreated animals (group II).

<table>
<thead>
<tr>
<th>Parotid gland*</th>
<th>Collagen type I</th>
<th>Collagen type II</th>
<th>Collagen type III</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group I</td>
<td>26.77</td>
<td>0.56</td>
<td>3.21</td>
</tr>
<tr>
<td>Group II</td>
<td>27.47</td>
<td>0.48</td>
<td>3.45</td>
</tr>
<tr>
<td>Submandibular gland*</td>
<td>Collagen type I</td>
<td>Collagen type II</td>
<td>Collagen type III</td>
</tr>
<tr>
<td>Group I</td>
<td>37.17</td>
<td>4.72</td>
<td>11.98</td>
</tr>
<tr>
<td>Group II</td>
<td>39.36</td>
<td>5.77</td>
<td>12.65</td>
</tr>
</tbody>
</table>

*Difference at the 5% level of significance.

Fig. 2: Photomicrographs of the submandibular glands. A: Involved seromucous acini with hyperchromatic nuclei (arrow) were observed in animals of group I. H.E. B: Stromal space containing collagen type I (I), type II (II), and type III (III). Picrosirius Red. C: Similar features were observed in animals of group II, including atypical seromucous acini and hyperchromatic nuclei (arrow). H.E. D: In these animals the stromal space contained collagen type I (I), type II (II), and type III (III). Picrosirius Red.
obese and diabetic animals (Gaamoussi et al., 2010). For example, therapeutic administration of Ginkgo biloba improved glucose metabolism in rats with chemically induced diabetes. The authors suggested that the mechanism of action of this plant may be related to stimulation of GLUT4 messenger RNA expression, a protein that plays an important role in glucose transport and insulin secretion, thus promoting glycemic control (Li et al., 2010). Similarly, Chamaerops humilis (L.) (dwarf fan palm) was found to control glucose levels in obese and diabetic animals (Gaamoussi et al., 2010). In this respect, Bauhinia forficata, another plant phytotherapeutic, has been shown to be effective in different applications, especially the control of hyperglycemia. Previous studies have shown that the aqueous extract of Bauhinia forficata leaves reduces problems related to carbohydrate and protein metabolism, which are common in hyperglycemic conditions, suggesting a potential clinical application of this plant for the treatment of diabetes (Lino et al., 2004; Pepato et al., 2004). However, this plant was not effective in reducing glucose levels in other studies. In this respect, Volpato et al. (2008), treating diabetic rats with Bauhinia forficata extract, observed no hypoglycemic effect in these animals.

Nevertheless, the results of this study permit to conclude that the animals presented an effective diabetic condition in terms of glucose levels and that treatment with Bauhinia forficata leaf extract played an important role in the reduction of hyperglycemia. However, glucose levels did not return to normal. This finding might be related to the time of treatment or to the complexity of glycaemic control as demonstrated in the literature.

With respect to glandular tissues, similar changes in salivary gland architecture were observed in both treated and untreated animals, with the cells responsible for the secretion of saliva being involuted and atypical. There was also a similar increase in extracellular matrix, especially a higher density of type I collagen fibers, followed by type III and type II, in addition to the presence of an inflammatory infiltrate in parotid glands. However, in general should be noted that the submandibular glands were more intensely affected than the parotid glands.

The effects of diabetes on the salivary glands have been documented in the literature. Simões et al. (2009) observed the accumulation of lipid droplets in the glands of hyperglycemic rats, elements characteristic of processes of tissue damage. Alterations in saliva components were also observed in the salivary glands of diabetic animals and the tissue responses to this hyperglycemic condition differed when compared to mucous and serous glands (Kamata et al., 2007; Medneiks et al., 2009). Other investigators also emphasized that, although similar epithelial alterations occur in different salivary tissues, the submandibular glands undergo more significant modifications than the parotid glands (Caldeira et al., 2005).

In addition to effects on the epithelium, the importance of connective tissue for health maintenance or for the development of diseases in different organs has been recognized (Merne and Syrjänen, 2003; Rozario and DeSimone, 2010). In this respect, Li et al. (2010) demonstrated that an increase of type I collagen promoted invasion and metastasis of neoplastic cells, probably because of an increased enzymatic activity in the region. Similarly, Félix et al. (2002) observed an increase of type I collagen, followed by type III, in salivary gland carcinoma, especially in more invasive cases. In addition to type I and III collagen, alterations in type II collagen may also be associated with cell carcinogenesis, as well as with inflammatory processes that induce an increase in these extracellular fibers, promoting an irreversible cycle and signaling the formation of these tumors (Landini, 1991; Kaden et al., 2005).

This interaction between the stromal microenvironment and neoplastic cells requires triggers similar to those observed in the present study, in which diabetes caused alterations in glandular tissues. Within this context, alternative treatments may also act on the recovery of these tissues. In animal models for Sjögren syndrome, in which the animals show signs and symptoms similar to those observed in diabetes, treatment with Ophiopogon japonicus, a plant used in Chinese medicine, was found to improve the salivary flow of these animals (Wang et al., 2007). Mei et al. (2009), treating rats with Sjögren syndrome with a polysaccharide extract obtained from Liriope, also observed significant improvement in salivary flow of the submandibular glands. In contrast, Lim et al. (2006), studying the effect of Bauhinia forficata on cell cultures, found no tissue recovery. Instead, the authors observed apoptosis and reduced cell proliferation. This result demonstrates that the mechanisms underlying diabetes overlap with currently used treatments, in agreement with the present study and literature reports (Silva et al., 2009). Taken together, these findings indicate that the discovery of a truly effective therapy for the damage caused by diabetes is complex and may still be far.

In conclusion, either the connective tissue alterations triggered by the diabetic condition in this study provide a favorable environment for the destructuring of glandular cells, or the structural modifications in the salivary glands trigger alterations in the stromal microenvironment.
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Within this context, in the present study the parotid glands were found to be less affected by the hyperglycemic condition, a finding that might be related to a better mechanism of homeostasis of this organ or better recovery from the effects of diabetes. Thus, Bauhinia forficata exerts an effect on the recovery of body metabolism but this improvement does not influence in the tissue recovery.

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