Histomorphometric and Immunohistochemical Study Comparing the Effect of Diabetes Mellitus on the Acini of the Sublingual and Submandibular Salivary Glands of Albino Rats

Sarah Yasser1*, Ahmed Atef Shon2

1Department of Oral Biology, Faculty of Dentistry, Tanta University, Algeish Street, Tanta City, Al Gharbeyah, Egypt; 2Department of Prosthodontics, Faculty of Dentistry, Gulf Medical University, Ajman, United Arab Emirates

Abstract

AIM: This study was designed to compare the effect of diabetes on the mucous and seromucous acini of the sublingual (SLG) and the submandibular (SMG) salivary glands of albino rats, respectively.

METHODS: Twenty male albino rats were assigned into two groups: control and diabetic. Three months following the induction of diabetes mellitus (DM), both the SMG and the SLG glands were removed, randomly sectioned and stained with hematoxylin and eosin to estimate the volume-weighted mean volume of the acini of both glands together with examining their morphology. Furthermore, immunohistochemistry was done to examine the expression of the proliferating cell nuclear antigen (PCNA) in both of them.

RESULTS: We found that, unlike the SMG acinar cells, diabetes appeared not to affect both the morphology and the volume of the SLG acini. Interestingly, PCNA expression in diabetic SMG glands acini was significantly higher to the control and diabetic groups in both glands.

CONCLUSION: We concluded that the mucous acini of the SLG glands are less affected by the oxidative damage induced by DM.

Introduction

Salivary glands have a key role in maintaining oral health, as they are exocrine glands that secrete saliva which is an enriched milieu composed mainly of water, electrolytes, and biologically active proteins, including growth factors and cytokines [1]. They have important functions including epithelial hydration, mastication facilitation, taste, swallowing, and speech. Furthermore, they have a buffering action with bicarbonate ions that prevent enamel decalcification while promotes remineralization.

The major salivary glands consist of paired submandibular (SMG), sublingual (SLG), and parotid glands that work simultaneously with other minor salivary glands scattered all over the oral cavity. Each one of these major salivary glands consists of a specific combination of both mucous and serous acinar cells, which are responsible for synthesizing protein components of saliva and transporting water and electrolytes [2]. A total of 1.5 L of saliva are produced by the major salivary glands: Parotid: 20–25%, SMG: 70–75%, and SLG: 5% and the rest by the minor salivary glands. Most of the resting or unstimulated saliva is from the SMG glands, while parotids contribute only to the stimulated secretions [3], [4].

Diabetes mellitus (DM) is a chronic metabolic disease that results from the failure of pancreas to produce insulin or the body cells to use this insulin. Defects in either insulin secretion or its cellular receptors cause sustained hyperglycemia, which is considered the main feature of DM and its destructive cause [5], [6].

The previous studies have focused on the physiological, pathological, and morphometrical alternations of the parotid and the SMG caused by DM [7]. However, studies on the SLG are sparse and their results are conflicting [8], [9]. This may be explained by the fact that the SLG contribution to total saliva volume is the least among all three major salivary glands. However, the SLG is still one of the major salivary glands that should undergo more detailed histological and morphometrical examination to assess DM effect. In this study, we compared the effect of DM on the acinar part of both the rat SLG and the SMG;
this was done using stereological methods, histological, and histochemical examination.

Materials and Methods

Animals

This study was performed on 20 adult male rats with an average body weight of 200–250 g. All animals were housed in temperature-controlled cages on a 12 h alternating light-dark cycle, for 3 months.

The protocol is designed in accordance with the guidelines for the responsible use of animals in research as a part of scientific research ethics recommendation of Research Ethics Committee, Faculty of Dentistry, Tanta University. The animals were divided into two equal groups; each group consists of 10 rats. The groups were divided into a control and a diabetic untreated group.

For DM induction, a single intraperitoneal injection of 150 mg/kg monohydrated alloxan dissolved in sterile 0.9% saline was used [10]. On the other hand, the control group was injected with sterile saline to control the effect of injection stress.

Tissue preparations

At the end of the experimental period, rats were euthanized by cervical dislocation under general anesthesia. The SMG and the SLG of each animal were cut into small portions (3 × 3 × 3 mm). About 4% paraformaldehyde was used as a tissue fixative. To achieve random orientation of the specimens, the tissues were embedded randomly in paraffin wax [11]. The sections were cut at 5 μm intervals and stained for histological and histochemical examination.

Stereo logical study

On each cut section, five fields were selected randomly. This was done by moving the microscope’s stage in horizontal and vertical directions (Leica, Germany). We examined a total number of 95–100 acini/animal at × 400. A grid system (ImageJ) was used to estimate volume-weighted mean volume of both SMG and SLG acini using point sampled intercepts method [12], [13]. The intercept (l0) was measured at each point of the grid which hits an acinus by calculating the length of the line passing through this point from one side of the acinus to the opposite side in an isotropic direction. Then, the volume-weighted mean volume (Vv) of the acini was calculated using the next equation.

\[ V^v = \frac{\pi}{3} l_0^3\times M. \]

M is a correction factor calculated as \( \left( \frac{1}{Mag} \right)^3 \), where Mag is the microscope magnification used to examine the slides (Table 1) [14].

\( (A) x \) is the class number with a total number of classes \( n = 11 \). (B) The formula for calculation of the upper limit length \( l^3 \) of a class number \( x \) is \( \left( \frac{l_3}{10^{(x-1)}} \right) -1 \). (C) The upper limit length of class \( x \) on a normal scale, the ruler length here = 25 mm. (D) The class width length \( l_3 \) is calculated using column B, for example, the class width length of a class \( x = \) upper limit length \( l^3 \) of class \( x \) minus the upper limit length \( l^3 \) of class \( (x-1) \). (E) Class midpoint of a class \( x = \frac{1}{2} \) class width length \( l^3 \) of class \( x + \) upper limit length \( l^3 \) of class \( (x-1) \). (F) The number of intercepts recorded in each class for acini of the control group. (G) The result of multiplying the midpoint lengths \( l^3 \) by the number of intercepts recorded in each class. Column G calculates the sum of \( \sum l^3 \) so the calculation of volume-weighted mean volume (Vv) of the control acini was done using the following equation:

\[ V^v = \frac{\pi}{3} l_0^3\times M=105\times\frac{40.83\times10^{14}}{94}\times\frac{1}{400^3} = 7.114 \times 10^5 \mu m^3. \]

Immunohistochemistry

Sections were incubated with anti-PCNA antibody (mouse monoclonal antibody, clone PC10, Thermo Scientific Lab Vision) followed by incubation with secondary antibody (Biotinylated Goat Anti-polyvalent; Thermo Scientific Lab Vision). Following

Table 1: The estimation of the volume-weighted mean volume (Vv) of SMG acini of the control group using the \( l_0 \) ruler

| A | B | C | D | E | F | G |
|---|---|---|---|---|---|---|
| Class no. X | Upper limit length (µm × 10^-6) | Upper limit length (µm × 10^-3) | Class midpoint (µm × 10^-3) | Class midpoint (µm × 10^-3) | Observed number per class | E+F (µm × 10^-3) |
| 1 | 0.35 | 0.7 | 0.35 | 0.18 | 0 | 0 |
| 2 | 0.79 | 0.92 | 0.44 | 0.57 | 0 | 0 |
| 3 | 1.34 | 1.1 | 0.55 | 1.07 | 4 | 4.28 |
| 4 | 2.04 | 1.27 | 0.7 | 1.69 | 15 | 25.35 |
| 5 | 2.91 | 1.43 | 0.87 | 2.48 | 8 | 19.84 |
| 6 | 4.02 | 1.59 | 1.11 | 3.47 | 24 | 83.28 |
| 7 | 5.41 | 1.76 | 1.39 | 4.72 | 21 | 99.12 |
| 8 | 7.16 | 1.93 | 1.75 | 6.29 | 9 | 56.61 |
| 9 | 9.36 | 2.11 | 2.2 | 8.26 | 8 | 69.08 |
| 10 | 12.13 | 2.3 | 2.77 | 10.75 | 5 | 53.75 |
| 11 | 15.62 | 2.5 | 3.49 | 13.88 | 0 | 0 |

SMG: Submandibular, SLG: Sublingual

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that incubation with the appropriate percentage of DAB substrate with DAB chromogen was done until desired reaction was achieved. Finally, the slides were counterstained with hematoxylin. The results were examined using light microscope [15].

**Statistical analysis**

Data were analyzed using unpaired Student’s t-test. Results are expressed as means ± standard deviation and $p < 0.05$ was considered statistically significant.

**Results**

**Light microscope examination**

Unlike the control SMG that showed normal structures, the seromucous acini of diabetic SMG were atypical and depicted severe cytoplasmic vacuolization (Figure 1).

However, the mucous acini of SLG were similar to those of the control group with no obvious signs of degenerative changes (Figure 2).

**Stereological study**

In Table 2, we found that, 3 months after DM induction, the volume-weighted mean volume of the seromucous SMG acini showed a significant increase.

**Immunohistochemical study**

We found in Table 3 that PCNA expression pattern did not differ significantly between control in both SMG and SLG acini. Interestingly, PCNA expression of diabetic SMG was significantly higher than diabetic SLG acini.

Furthermore, we found that the expression pattern of PCNA was significantly higher between the control and diabetic groups in both glands (Figures 4-6).

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**Table 2: Changes in volume-weighted mean volume ($\mu m^3$) measurements of SLG and SMG acini (values are mean ± SD)**

|                      | SMG          | SLG          |
|----------------------|--------------|--------------|
| Control groups       | 7.11×10^5 ± 26200 | 12.38×10^5 ± 48700 |
| Diabetic groups      | 10.30×10^5 ± 22400  | 11.97×10^5 ± 47500  |

*Control versus diabetic SMG ($p<0.0001$), control versus diabetic SLG ($p=0.204$). SMG: Submandibular, SLG: Sublingual, SD: Standard deviation.

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**Table 3: Changes in PCNA expression levels of SLG and SMG acini (values are mean±SD)**

|                      | SMG          | SLG          |
|----------------------|--------------|--------------|
| Control groups       | 12.19±4.9    | 11.35±3.17   |
| Diabetic groups      | 24.64±7.02   | 15.23±1.06   |

*Control versus diabetic SMG ($p=0.017$), control SMG versus control SLG ($p=0.756$), control versus diabetic SLG ($p=0.32$), diabetic SMG versus diabetic SLG ($p=0.018$). SMG: Submandibular, PCNA: Proliferating cell nuclear antigen, SLG: Sublingual.

On the other hand, the mucous SLG acini of the diabetic group showed no significant increase from the control counterpart (Figure 3).

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**Figure 1:** Light micrograph illustrates (a) the seromucous acini of control submandibular, (b) the seromucous acini of diabetic group shows sever vacuolization of the acinar cells (black arrows) (hematoxylin and eosin stain, original magnification ×400)

**Figure 2:** Light micrograph illustrates (a) the mucous acini of control sublingual, (b) the mucous acini of diabetic group shows almost no change in their morphology (hematoxylin and eosin stain, original magnification ×400)

**Figure 3:** Changes in volume-weighted mean volume ($\mu m^3$) between sublingual and submandibular acini

**Figure 4:** Changes in proliferating cell nuclear antigen expression levels of sublingual and submandibular acini (values are mean ± standard deviation)
The exact mechanism of oxidative stresses induced cell injury can be explained through indirect and direct pathways. Indirect pathways include influx of calcium from their intracellular stores and across the plasma membrane.

Increased cytosolic Ca ions activate a number of enzymes responsible for cellular damage including phospholipases, proteases, endonucleases, and adenosine triphosphatases (ATP), thereby hastening ATP depletion. This ATP depletion causes structural disruption of the protein synthetic apparatus. Furthermore, ATP depletion reduces the activity of the plasma membrane energy-dependent sodium pump, resulting in intracellular accumulation of Na⁺ and efflux of K⁺, causing cell swelling and dilation of the ER [25]. Moreover, hyperglycemia causes impairment of sodium-glucose cotransporters, aquaporins, or both affecting cellular water content [26], [27], [28], [29]. This can explain the significant increase in the volume-weighted mean volume of the SMG seromucous acini of diabetic rats.

On the other hand, direct pathways include lipid peroxidation of the polyunsaturated membrane lipids that are vulnerable to attack by oxygen-derived free radicals. The lipid radical interactions yield peroxides, which are reactive, unstable, and endanger the integrity of cellular and nuclear membranes. This allows oxygen radicals to attack chromatin, causing fragmentation of DNA. Furthermore, free radicals react with thymine in the nuclear and mitochondrial DNA, causing single-strand breaks. This DNA damage results in cell aging and death [30].

To eradicate this DNA damage, base excision repair (BER) is activated to repair single-strand breaks and oxidized bases [31]. PCNA was found to be essential for gap filling in BER, this makes PCNA important to repair DNA lesions caused by oxidative stresses [32], this can explain the elevated level of PCNA expression in the seromucous acini of diabetic SMG, compared to the control, in an attempt to bypass the damage induced by oxidative stresses.

On the other hand, similar to earlier studies, we observed almost no changes in the cell architecture of the mucous acini of diabetic SLG; they retained their normal shape and morphology [8], [33], [34], [35]. Furthermore, we found no significant changes in the volume-weighted mean volume of mucous acini of diabetic SLG compared to the control [21]. However, PCNA expression level in the mucous acini of diabetic SLG was significantly higher than that of the control. This indicated that there are still degenerative changes caused by oxidative stresses that need to be repaired.

Interestingly, PCNA expression was higher in the diabetic compared to the control groups in both glands. However, PCNA expression was still significantly higher in seromucous acini of diabetic SMG in comparison with mucous acini of diabetic SLG. This indicated that DM adversely affected the seromucous acinar cells of SMG much more than the mucous acinar cells of SLG.

This was explained in early studies by the fact that serous acini contain secretory granules rich in proteolytic enzymes, while mucous cells do not [36], [37], [38]. Moreover, in recent studies, secretory granules of the serous acini were found to be rich in heavy metals so they are more vulnerable to metal-catalyzed attack by...
free radicals and hydrogen peroxide prompted from the accumulation of AGES [39]. Although hydrogen peroxide is not a free radical, it has the ability cross the cell membranes into different cellular compartments. Hydrogen peroxide that is not neutralized has a great affinity to react with heavy metals and forms the highly reactive hydroxyl radical (OH) which augments lipid peroxidation [40], [41].

Furthermore, the difference can be explained by the rapid intracellular lipid accumulation in serous acini, unlike the mucous acini, of diabetic salivary glands [21]. It has been suggested that lipid accumulations can be related to the reduction in the synthesis of secretory granules [30], [42], [43], [44]. This increase in free fatty acids causes increase in the production of monocyte chemoattractant protein-1 that attracts monocytes in adipose tissue and transforms them into tissue-resident inflammatory M1 phenotype macrophage [45]. This exaggerates the inflammation, resulting in a further increase in the free radicals production with subsequent weakening of the antioxidant barrier and oxidative damage to the organ [46]. In addition, Cecchini et al. (2009) found that Clara cell secretory protein (CC10), that has a protective effect against inflammatory response and oxidative stresses, was found exclusively in the SLGs [16].

Conclusion

We concluded that the SMGs are much more sensitive to the deleterious effect of DM than SLGs, which are far more resistant to these damaging effects. This is mainly contributed to the nature of the acinar cells represented by the seromucous and the mucous cellular phenotype in SMGs and SLGs, respectively. The seromucous acini is more affected by the damaging effect of oxidative stresses accumulation compared to the mucous acini.

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Histology