

## Effects of diabetes on the post-menopausal rat sublingual glands: A histopathological and stereological examination

Başak Büyük, Seçil Nazife Parlak, Osman Nuri Keleş, Jale Selli, Elif Polat, Bünyami Ünal\*

Department of Histology and Embryology, Faculty of Medicine, Ataturk University, Erzurum, Turkey

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### ABSTRACT

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#### \* Correspondence to:

Bünyami Ünal

Department of Histology and Embryology,

Faculty of Medicine,

Ataturk University,

Erzurum, Turkey

e-mail: bunyamiunal@yahoo.com

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Menopause is a physiological process of ovarian and uterine cycles related with decreasing of steroidal estrogen hormone level that decreases or terminates generally after 45 years old in women. Diabetes Mellitus (DM) is a metabolic disease that is defined high blood glucose level that goes on over a prolonged period due to insufficient insulin secretion. The goal of the study is to observe the effects of diabetes on post-menopausal rat sublingual glands that causes pathophysiological processes because salivary glands are sensitive to estrogen. Adult female 12 weeks old Sprague Dawley rats (n=24) were divided into four groups in a random manner; healthy control group, diabetes induced group, ovariectomized group (OVX), post ovariectomy diabetes induced group (DM+OVX). To evaluate the findings histopathological, histochemical and stereological analysis were achieved. In DM group degenerative serous demilune and duct cells, in OVX group increased polymorphonuclear leukocyte (PMNL) infiltration and in DM+OVX group extensive and increased PMNL infiltration, degenerative serous demilunes and increase in the serous demilune thickness were distinguished. Alterations in the content and amount of neutral mucopolysaccharidosis secretion of serous demilunes in DM or DM+OVX groups and acidic mucopolysaccharidosis secretion of mucous acinus in DM and/or OVX groups were detected. In addition, stereological analysis revealed that hypertrophic changes in DM groups and atrophic changes in OVX groups were occurred in the mucous acinus epithelium. The results suggest that diabetes and/or ovariectomy triggered pathophysiological processes that caused morphological and functional changes at the cellular level in sublingual glands. Molecular mechanism and relation to glucose and lipid metabolisms of pathophysiological processes in the sublingual gland in DM and OVX groups require further investigation.

### 1. Introduction

Menopause is a physiological process that is related with prominent decreasing of steroidal estrogen hormone levels and has direct and indirect effects on tissues like heart, bone or salivary glands in women. Salivary glands and oral mucosa are sensitive to estrogen as they have estrogen receptors that mediate the effects of estrogens by serving as a transcription factor (Valimaa et al., 2004). Estrogen has major roles for cell growth, differentiation, and regulates growing and reproductive tissue functions. Many serious

health problems can occur due to the estrogen deficiency such as cardiovascular disease, osteoporosis, and salivary gland discomforts in the body. Estrogen replacement therapy can be effective to reduce these problems (Mainini et al., 2011).

Diabetes Mellitus (DM) is an endocrine and metabolic disorder characterized by hyperglycemia. Many studies has indicated that hyperglycemia causes damage to tissues and numerous complications in heart, kidneys, liver, and salivary glands (Albayrak et al., 2011; Maekawa et al., 2011; Unal et al., 2011; Perkins et al., 2012). DM is frequently

**Table 1.** All details of experimental protocol

Groups	1 <sup>st</sup> Day of experiment	1-90 <sup>th</sup> Day of experiment	91 <sup>st</sup> Day of experiment	91-122 <sup>nd</sup> Day of experiment	123 <sup>th</sup> Day of experiment
Control (n:6)	Waiting period	Waiting period	Waiting period	Waiting period	End of study
DM (n:6)	Waiting period	Waiting period	Diabetes induction with alloxan	Waiting period	End of study
OVX (n:6)	Ovariectomy operation	Waiting period	Waiting period	Waiting period	End of study
DM+OVX (n:6)	Ovariectomy operation	Waiting period	Diabetes induction with alloxan	Waiting period	End of study

encountered in post-menopausal period that causes damage on many tissues. Recent studies have shown that estrogen and insulin replacement therapies have benefits on experimental menopause and diabetes models (Morris et al., 1992; Maekawa et al., 2011; Yashida et al., 2011).

Although some studies examined the effects of diabetes and/or menopause, it was hard to find the sufficient investigations that examined morphological changes, the secretion content and stereological analysis of sublingual gland in diabetic and/or ovariectomized models Anderson and Garrett, 1986; Kamata et al., 2007; Purushotham et al., 1993). The aim of this study was to investigate the effects of hypo estrogenic situation and hyperglycemia on sublingual gland in experimental menopause and/or diabetes models of rats through histopathological, histochemical and stereological methods.

## 2. Materials and methods

### Animals and experimental protocol

Animals were held in facilities that attributed by international guidelines, and the experiment was achieved and managed in unity with the Institutional Animal Care and Use Committee of Ataturk University. Twelve weeks old 24 female *Sprague-dawley* adult rats were provided from and housed in Ataturk University Experimental Animal Laboratory (ATADEM). The rats were kept with 12 h light/dark cycle and held in regularly repeated temperature/humidity in controlled environment. They were divided into four groups randomly: non-diabetic healthy group (control, n=6), diabetic group (DM; n=6), ovariectomized group, (OVX, n=6), and post ovariectomy diabetes induced group, (DM+OVX, n=6), respectively. All groups are summarized in Table 1.

### Experimental models

#### Ovariectomy procedure

Bilateral ovariectomy was performed by making incision (0.5-1 cm) in a longitudinal manner on the lower abdomen midline area of rats. After incision the ovaries were taken out and then the incision was closed (Albayrak et al., 2011). After ovariectomy, 25 mg/kg metamizol sodium as an analgesic was given rats for 2 days. Ovariectomized rats were kept alive for 12 weeks. Two groups of rats that ovariectomized rats and non-ovariectomized rats were induced diabetes after this period.

#### Alloxan-induced diabetes procedure

120 mg/kg single dose of aqueous alloxan monohydrate (Sigma-Aldrich Co, Germany) was injected intraperitoneally to induce diabetes according to defined methods by Halici (Halici et al., 2009). Alloxan was prepared in solution of 0.9% NaCl freshly and injected to rats unted for one night. 4-6 h after than the application of alloxan, to eliminate fatal hypoglycemia adverse effect due to high insulin secretion from the pancreas, 20% (5 ml) glucose solution was injected intraperitoneally and a 5% glucose solution for 24 h was put into the drinking water and rats were allowed to intake food. Three days after alloxan administration, fasting blood glucose levels of rats were controlled by a blood glucose monitor (Accu-Chek Active, Germany). At least 200 mg/dL of serum glucose level was considered a diabetic rat and diabetic rats were kept alive for 8 weeks.

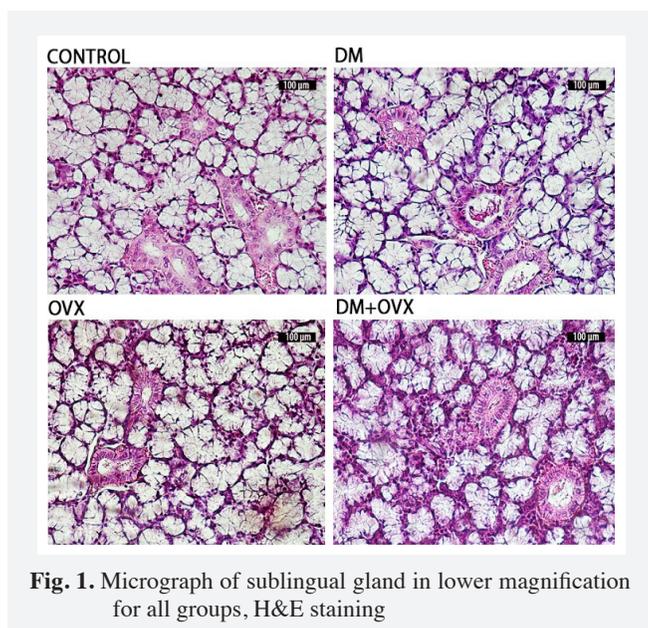
### Research methods

#### Histological analysis

Under a high dose of ether anesthesia all rats were sacrificed at the end of the study. After the sacrifice each sublingual gland was removed and then fixed in 10% neutral formalin solution for 72 h. Later these specimens were dehydrated in increasing alcohol series, cleared with xylene series, immersed in liquid paraffin series and embedded in paraffin wax, respectively. Four 5µm sections were serially obtained from each rat paraffin blocks by interval of 50 µm using a microtome (Leica RM2125RT, Nussloch, Germany). The sections were gotten from deparaffinizing to water and were stained for histopathological analysis with hematoxylin and eosin (H&E) and for histochemical analysis with periodic acid-schiff (PAS), alcian blue (AB) (pH: 2.5) and PAS/AB (pH: 2.5 and pH: 1), separately. Then the cover slipped slides, were photographed with a camera connected light microscope (Nikon Eclipse E600, Japan). Administration of same light settings preserved for photographing, especially for histochemical analysis, to provide unbiased determination.

#### Semi-quantitative analysis

Using a light microscope, histopathological and histochemical examinations of every rat sections were scored, semi-



**Fig. 1.** Micrograph of sublingual gland in lower magnification for all groups, H&E staining

**Table 2.** Assessments of semi-quantitative histopathologic analysis of sublingual glands for hall groups

Groups	PMNL infiltration density	Degenerative cell density	N.M staining density of serous demilunes	A.M staining density of serous demilunes	N.M staining density of mucous acini	A.M staining density of mucous acini
Control	+	+	++	++	++	++
DM	++	++	++++	++++	+++	++++
OVX	+++	+++	+	+	+	+
DM-OVX	++++	++++	+++	+++	+++	+++

The cell and staining densities were scored semi-quantitatively and expressed as weak=+, moderate=++, strong=+++ , or very strong=++++.

quantitatively. For each section, 5 randomly selected microscopic areas (nearly to 100- $\mu\text{m}^2$ ) including the secretory unit and connective tissues were evaluated at X10 objective and the arithmetic mean was scored semi-quantitatively. Polymorphonuclear (PMNL) cell density, degenerative cell density, neutral mucopolysaccharide (NM) staining density of serous demilunes, acidic mucopolysaccharide (AM) staining density of serous demilunes, NM staining density of mucous acini, and AM staining density of mucous acini were scored.

### Quantitative analyses

Stereo-investigator software version 9 (Microbrightfield, CA, USA) was used for stereological evaluation. Stereological analyses were performed for mucous acinus cross-sectional area to distinguish hypertrophic or atrophic alterations in mucous acinus epithelium. It was hard to quantify serous demilunes for that they were not examined stereologically. Although full randomness in the section-sampling process from any organ was necessary for stereological estimating the area or volume of the small objects have been described in the literature (Mattfeldt et al., 1990, Nyengaard and Gundersen, 1992). We did not enhance these requirements completely. 5- $\mu\text{m}$ -thick sections were obtained in the coronal plane according to systematic sampling procedure as described above histological analysis and the “nucleator method” was used to estimate the mean mucous acinus area (Keles et al., 2011).

### Statistical analysis

The statistical analysis was performed using SPSS (IBM SPSS Statistics 18.0, IBM Corporation, Somers, NY, USA). The numerical data of groups were analyzed with using one-way analysis of variance (abbreviated one-way ANOVA) followed by LSD test (P value<0.05 was determined as significant). The values were identified as means  $\pm$  standard deviation.

## 3. Results

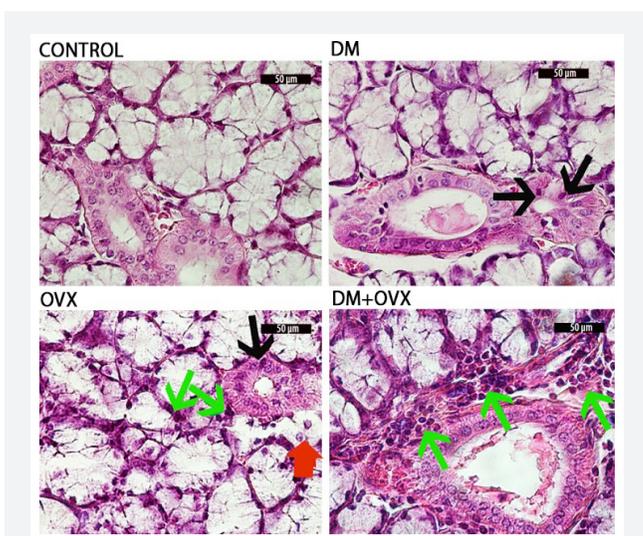
### Histological results

In control group were normal acinus, duct and connective tissue structures. In DM group mucous acinus structures were similar to those in control group but, degenerative striated duct cells and serous demilunes with pyknotic nuclei and acidophilic cytoplasm were distinguished and also serous demilunes were thicker than those in control group. A few PMNL cell infiltrations were noticed in connective tissue, too. In OVX group increased PMNL cell infiltrations, degenerative serous demilunes and striated duct cells were determined and a few swollen mucous acinus cells were detected. DM+OVX group showed the most increased PMNL infiltrations, degenerative serous demilunes and striated duct cells and also the most increased serous demilune thickness (Fig. 1. 2; Table 2).

### Histochemical results

In histochemical analysis sublingual tissues were stained with periodic acid-schiff (PAS) to distinct neutral mucopolysaccharides content and with alcian blue (AB) to distinct acidic mucopolysaccharides content to ensure separation of sublingual epithelium secretion content. Control group sections revealed that serous demilunes' cytoplasm were stained with PAS staining, AB staining, and combined PAS+AB staining as normal. Mucous acinus cytoplasm were stained with these three stainings as normal, too.

In PAS staining sections of DM group, serous demilunes' cytoplasm were stained more basophilic than PAS staining of control group. AB staining sections of this group more were strongly stained than AB stainings sections of control group. In PAS+AB staining sections, PAS densities of serous demilunes' cytoplasm were detected slightly more negative than PAS staining sections of this group and PAS+AB staining sections of control group. In mucous acinus sections it was seen that PAS staining sections of this group were more basophilic than PAS staining of serous demilunes of control group. AB staining sections of this group were more positive than AB staining sections of control group. In PAS+AB sections it was distinguished that AB densities of mucous acini were less positive than AB staining sections of DM group but more positive than PAS+AB staining sections of control group.



**Fig. 2.** Micrograph of sublingual gland in higher magnification for all groups; **Thin green arrow**: Polymorphonuclear cell infiltrations to connective tissue; **Thin black arrow**: Degenerative (more acidophilic cytoplasm with pyknotic nucleus) striated duct cells; **Thick orange arrow**: Swollen mucous acinus cells H&E staining

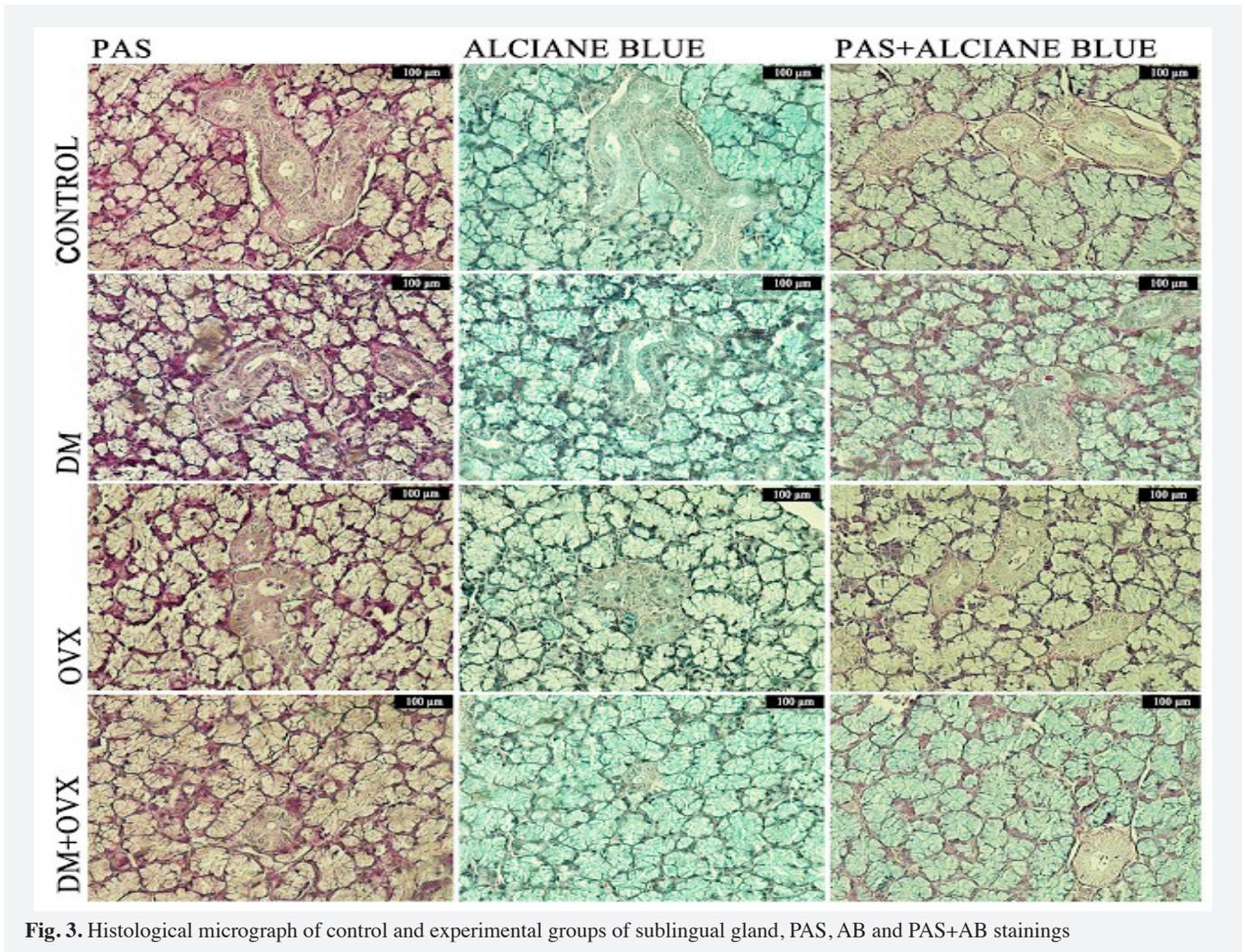


Fig. 3. Histological micrograph of control and experimental groups of sublingual gland, PAS, AB and PAS+AB stainings

OVX group section evaluations showed that serous demilunes' cytoplasm were seen in PAS staining sections of this group slightly less acidophilic than control and more acidophilic than DM group, in AB staining of this group slightly more positive than AB staining of control group and more negative than AB staining sections of DM group. In PAS+AB group sections PAS density was determined, slightly more positive than PAS staining sections of this group. Mucous acinus cytoplasm's PAS, AB and PAS+AB stainings were less positive than control and DM group section stainings.

In DM+OVX group sections it was distinguished that PAS, AB and PAS+AB stainings of serous demilunes and mucous acini were nearly to DM group but also PAS stainings of serous demilunes' were less basophilic and AB stainings of mucous acini were less negative than in DM group (Fig 3 and Table 2).

#### Stereological results

Stereologic analysis of mean mucous acinus area of the sublingual gland revealed that there were significant differences between all groups ( $p < 0.05$ ). It was observed that there was a significant reduction in OVX group ( $p < 0.05$ ) and a significant increase in mucous acinus area of DM group ( $p < 0.05$ ) when compared to control group. DM+OVX had increased mean acinus area ( $p < 0.05$ ) but it was found to be less than that in DM group (Table 3).

#### 4. Discussion

Menopause and diabetes both can lead to many health problems although the pathophysiology of these two diseases are not known, yet. As a result of menopause and diabetes, developing metabolic disorders can cause degeneration on macroscopic salivary glands that have been shown in some experimental menopause and diabetes models (Deniz et al., 2011).

Estrogen plays an important role in the normal flow of inflammatory process and estrogen deficiency syndrome causes structural and functional changes on macroscopic salivary glands. Perimenopausal and postmenopausal symptoms can increase oral mucosa and periodontal discomforts. It has been suggested that metabolic pathways related with estrogen receptors on salivary glands can be researched against decrease in estrogen levels (Iaremenko and Kutukova, 2007; Meurman et al., 2009). Beside this, there are many researchers that indicate antioxidant effects of estrogen on some tissues like heart and bone (Subbiah et

Table 3. Assessments of mean mucous acinus area of all groups

Groups	Mean mucous acinus area
Control	2007.70±0.158 <sup>c</sup>
DM	2145.82±0.072 <sup>a</sup>
OVX	1898.95±0.073 <sup>d</sup>
DM+OVX	2094.66±0.158 <sup>b</sup>

<sup>abc</sup>The footnote letters express between groups the significant differences in the same column.

al., 1993; Mann et al., 2007), or estrogen deficiency causes inflammatory response in some tissues like larynx and skin (Calvin, 2002; Surmeli et al., 2011). Hyperglycemia triggers oxidative stress that is associated with diabetic complications and during diabetes disruptions in the salivary gland tissues occur. As known the modulation of enzymes associated with reactive oxidative stress (ROS) metabolism that triggers oxidative stress is very important. Some studies, showed that in insulin-dependent DM, mucous acinar cells were less damaged than serous demilune cells in sublingual tissue and also there were degenerative alterations in the cellular level on diabetes induced pure serous glands (Kamata et al., 2007; Ibuki et al., 2010; Turner et al., 2011; Parlak et al., 2014). In this study histological analysis revealed that hyperglycemia caused degenerative changes in serous demilunes and striated ducts that can be an inducer for triggering oxidative stress on sublingual tissue. Ovariectomy caused a significant increase in PMNL infiltration that can be a result of the lack of estrogen and can create an inflammatory response in the epithelium of acini and ducts that accompanied with the structural and functional abnormalities. Menopause and diabetes together caused extensive amount of increased PMNL infiltrations and degenerations in serous demilunes and duct cells and also there was a significant increase in the thickness of serous demilunes that can be a result of a synergistic effect of diabetes and menopause together for triggering oxidative stress on sublingual tissue.

Recent studies have shown that male and female sex hormones affect salivary glands and it has been distinguished that estrogen deficiency syndrome cause structural and functional changes in the macroscopic salivary glands (Anderson and Garrett, 1986; Purushotham et al., 1993; Streckfus et al., 1998). Intracellular lipid accumulations, especially in serous cells, have been shown in the majority of diabetic salivary glands and it has been reported that their rapid emergence and reversibility effects can occur on lipid metabolism on salivary glands and also it has been suggested lipid accumulations can be related with a reduction in the synthesis of secretory granules. In some studies it was detected that in diabetic pure serous glands a surprising decline in acyl lipids (neutral polysaccharide) rate occurred that could cause morphological and functional changes in secretion (Anderson and Garrett, 1986; Morris et al., 1992; Mahay et al., 2004; Iaremenko and Kutukova, 2007; Yashida et al., 2011). In this study there were alterations in serous demilune thickness and in the character and amount of neutral mucopolysaccharides of serous demilunes in DM single and combined groups. In mucous acinar cells the amount of acidic mucopolysaccharides was decreased in the OVX group, whereas it was increased DM and DM+OVX group. These results revealed that in serous demilunes due to morphological changes like the alterations in neutral mucopolysaccharide content, although lipid accumulation could not be seen histologically in sections there could be molecular alterations. These molecular alterations can cause changes in the structure and function of secretory glands and glucose and/or lipid metabolisms can be related with alterations in neutral mucopolysaccharide content. The increase in acidic mucopolysaccharide content in mucous acini of DM and DM+OVX groups can be related with glucose and lipid metabolism, too. The reduction in the amount of acidic mucopolysaccharide content of OVX group

can be associated with the estrogen deficiency syndrome from that sublingual gland can be affected negatively.

Purushotham et al. (1993) reported that estrogen could have effects on the salivary glands with a possible mechanism in the signaling of salivary production and its components that could cause salivary glands to be less functional. It was detected a decrease in sublingual and submandibular salivary flow rate of premenopausal, perimenopausal and postmenopausal Caucasian women with increasing age and decreasing estrogen levels (Streckfus et al., 1998). It has also been reported that during diabetes there were disruptions in the structure of the salivary glands (Turner et al., 2011). In this study the statistical results of mean mucous acinus area revealed that there were hypertrophic alterations in DM groups and atrophic changes in OVX group. Our histochemical results support the statistical results in that increased acidic mucopolysaccharide density in DM group and decreased acidic mucopolysaccharide density in OVX group were detected. It has been concluded that hypertrophic changes in diabetic mucous acini can occur due to alterations on secretory granule related with the increase in the function of these cell and, ovariectomy dependent estrogen deficiency may cause decrease in the function of the mucous acinar cells that lead to atrophy of these cells.

In previous studies there were no clear histopathological, histochemical and stereological examinations on the effects of menopause and diabetes on sublingual tissue. In our study it was highlighted that estrogen and/or insulin secretion deficiencies create significant morphological and functional changes on sublingual glands. Increased degenerative parenchyma cells in DM or DM+OVX groups were distinguished that can indicate the oxidative stress effect of hyperglycemia. A significant increase in PMNL infiltrations in the OVX and DM+OVX combined groups were determined that showed that estrogen suppressed cellular degeneration or had antioxidant effects enhancing cellular defense on sublingual glands. The most excessive amount of degenerative parenchyma cells and PMNL infiltrations in DM+OVX group can be a result of the oxidative stress effect of hyperglycemia and inhibited antioxidant effect of estrogen together. The alterations of neutral mucopolysaccharide content of serous demilunes and acidic mucopolysaccharide content of mucous acinar cells and also the increase in the serous demilune thickness were observed, too. These findings can be related with the pathways that cause the protein variation, change in the direction of secretion, hypertrophic and atrophic changes. It was concluded that glucose and lipid metabolisms may be related to these pathways.

In conclusion, it was determined that the effects of diabetic and/or ovariectomy applications on sublingual glands can create major metabolic, morphological changes, and particularly alterations in the amount and character of secretion, leading impair to the comfort of life. Sublingual gland is one of the major glands that participate in the formation of saliva. The amount and content of saliva is very important for protecting oral cavity flora and alterations in salivary component cause disruptions in chemical, physiological and morphological structure of oral cavity. Due to these alterations the levels of IgA, minerals, digestive enzymes or the environments of taste buds are affected. Beside, increase in bacteria causes tooth decay and oral discomforts.

If it is not treated earlier it can lead to tooth loss, sepsis or carcinogenesis. It was requested further examination for the molecular mechanisms related with oxidative stress, glucose

and lipid metabolisms to define the pathophysiological processes of DM and/or OVX on sublingual gland.

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