

## EFFECTS OF MENOPAUSE AND DIABETES ON THE RAT PAROTID GLANDS: A HISTOPATHOLOGICAL AND STEREOLOGICAL STUDY

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### ABSTRACT

**Background:** The menopause is a physiological process that occurs and diabetes is a metabolic disease that has an increased incidence in advancing age in women.

**Aims & Objective:** The purpose of the study to examine the effect of menopause and/or diabetes that causes pathophysiological status on parotid gland.

**Materials and Methods:** Sprague-dawley 24 adult female rats (12 week old) were randomly, six rats in each group, divided into four groups; non-diabetic healthy (control), Diabetic (DM) group, Ovariectomized (OVX) group, Post Ovariectomy-Diabetes Induced (DM+OVX) group, respectively. To evaluate the results histopathological, histochemical and stereological analyses were performed.

**Results:** Degenerative acini and duct cells with increased cytoplasmic lipid accumulation (vacuolization) in DM groups, polymorph nuclear infiltrations in OVX groups and extensive swollen nucleus with karyorrhexis and increased cytoplasmic lipid accumulation in serous acinus cells in DM+OVX group were detected. Furthermore the changes in the amount and character of secretion especially DM and DM+OVX groups and hypertrophic changes in the acinus epithelium in DM and/or the OVX groups were distinguished.

**Conclusion:** The results revealed the effects of diabetes and ovariectomy on the cellular changes and pathophysiological processes of these changes in parotid glands. These findings may highlight to other pathophysiological processes of the role of the salivary glands associated with the pathogenesis due to diabetes and menopause. Additionally, correlated molecular mechanism of pathophysiological processes of DM and/or OVX on parotid gland, suggested further investigation.

**Key Words:** Diabetes; Menopause; Parotid Gland; Stereology

### Introduction

The menopause is a physiological process that occurs in advancing age in women is characterized by decreasing or terminating of ovarian and uterine cycles due to the alterations in sex hormones.<sup>[1,2]</sup> Steroidal estrogen hormone is a sex hormone has direct and indirect effects on many systems, organs or tissues like cardiovascular system, bone or salivary glands, respectively. Estrogen receptors are induced by hormones which serves as a transcription factor with DNA binding sites that belongs to them. Estrogen in the majority has important roles of mammalian tissues in terms of the continuity of life like cell growth, embryonic development and regulates growing, differentiation, and the reproductive tissue functions. Estrogen deficiency can cause many serious health problems. Oxidative stress which occurs as a result of declining estrogen levels and response to that stress has been researched in many studies.<sup>[3-6]</sup> Oxidative stress triggers production of radioactive oxygen species (ROS) that causes cell or tissue damaging. Increased ROS level that has been displayed for higher risk for some diseases like osteoporosis, cardiovascular disease, or comforts like salivary glands in the body.<sup>[2,7,8]</sup> To reduce damaging of oxidative stress enzymatic and non-

enzymatic antioxidant defence mechanisms play in many tissue metabolisms. Recent years many researches showed that estrogen is very important for regulator of oxidative stress.<sup>[8]</sup>

Diabetes Mellitus (DM), is one of the most common and chronic endocrine and metabolic disorder in the world. It is characterized by hyperglycemia in the blood due to insufficient insulin secretion from  $\beta$  cells in Langerhans islets of pancreas or insulin resistance especially in peripheral tissues. Insulin is the primary stabilizer of carbohydrate level in the blood by affecting carbohydrate metabolism and other metabolisms that related with the metabolism of carbohydrates. Hyperglycemic effects in diabetes mellitus causes oxidative damage which causes cellular damage that disrupts the structure of systems, organs or tissues.<sup>[9]</sup> Due to diabetes mellitus many complications can occur in cardiovascular and nervous systems and organs like heart, kidneys, eyes and salivary glands.<sup>[10-13]</sup> In post-menopausal period DM is frequently encountered situation that causes the oxidative stress-mediated damage on many tissues.<sup>[9,14]</sup> Associated with oxidative stress, free oxygen radicals occur that causes directly or indirectly distressing effects on many tissues.<sup>[15,9]</sup> Recent studies have reported treatment of

estrogen and insulin replacement therapy have shown positive effects on diabetes and menopause created models.<sup>[15]</sup>

Although some studies investigated the effects of diabetes and/or menopause, it was hard to find the sufficient analysis that examined morphological changes, the content of secretion or stereological analysis of acinus epithelium on parotid gland with diabetic or ovariectomized effects. In this study, it was aimed to examine the effects of hyperglycemia and hypo estrogenic situation on parotid gland tissue with histopathological and stereological examine ways in created experimental diabetes and/or menopause models of rats.

## Materials and Methods

### Animals and Experimental Protocol

Animals were kept in facilities attributed by international guidelines, and the experiment was performed and directed in unity with the Institutional Animal Care and Use Committee of Ataturk University. In the study, 24 female Sprague–dawley (12 week old) adult rats were provided and housed in Ataturk University Experimental Animal Laboratory (ATADEM). Six rats per cage were held in groups in controlled environments of regularly recurring temperature/humidity, and they were kept with 12-h light/dark cycle. The rats were allocated 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 were summarized in Table 1.

**Table-1: All details of experimental protocol**

	Groups	Day of Experiment				
		1	90	91	91-122	123
Sprague-Dawley 12 weeks old 24 adult female rats	Control (n:6)	Waiting period	-	-	-	End of Study
	DM (n:6)	Waiting period	-	Diabetes Induction with alloxan	Waiting period	-
	OVX (n:6)	Ovariectomy operation	Waiting period	Waiting period	Waiting period	-
	DM+OVX (n:6)	Ovariectomy operation	Waiting period	Diabetes Induction with alloxan	Waiting period	-

### Experimental Models

**Ovariectomy Procedure:** Bilateral ovariectomy was achieved by making incision (0.5–1 cm) in a longitudinal manner on the lower abdomen midline area of rats, taking out the ovaries and closing the incision.<sup>[16]</sup> As an

analgesic for 25 mg/kg metamizol sodium was given rats for 2 days after ovariectomy. Ovariectomized rats were held alive for 12 weeks. After this period, two groups of rats were induced diabetes (ovariectomized rats and non-ovariectomized rats).

**Alloxan-Induced Diabetes Procedure:** Female Sprague–Dawley rats were induced diabetes by intraperitoneal injection administration of a single dose of aqueous alloxan monohydrate (120 mg/kg for body weight, Sigma–Aldrich Co, Germany) according to defined methods by Halici.<sup>[17]</sup> Alloxan was prepared freshly in solution of 0.9% NaCl and intraperitoneally injected to rats that were unfed for one night. 4–6 h after application of alloxan, depending on high level insulin secretion from the pancreas, lead fatal hypoglycaemia. Intraperitoneally 5 ml glucose solution (20%) was injected to eliminate this adverse effect. Solution of 5% glucose was put into the drinking water for 24 hours and intake of food was allowed. After 72 h of alloxan administration, control fasting blood glucose levels blood samples obtained from the rats' tail vein by a blood glucose monitor (Accu-Chek Active). A diabetic rat was determined as having at least 200 mg/dl of serum glucose level and diabetic rats were held alive for 8 weeks.

### Research Methods

**Histological Examination:** At the end of the study, all rats were sacrificed under high dose of ether anaesthesia, and then each parotid gland was removed and fixed in 10% neutral formalin solution for 72 h. After fixation, dehydrating in increasing alcohol series, clearing with xylene series, immersing in liquid paraffin series and embedding in paraffin wax treatments were made, respectively. 5µm sections were obtained by serial y linterval of 50 µm from paraffin blocks using a microtome (Leica RM2125RT). Then four sections of each rat, after deparaffinizing to water, were stained with Heamatoxylin and Eosin (H&E) for histopathological analysis, Periodic acid Schiff (PAS), Alcian Blue (AB) (pH: 2.5) and PAS/AB (pH: 2.5 and pH: 1) for histochemical analysis, separately. The slides were cover slipped, and photographs were taken using a camera connected light microscope (Nikon Eclipse E600, Japan).

**Semi-quantitative Analysis for Histopathological and Histochemical Alterations:** Histopathological and histochemical evaluations of every rat sections were

scored using light microscope, semi-quantitatively. The 100  $\mu\text{m}^2$  area for each section was selected for evaluation at X10 objective. The secretory unit and connective tissues were calculated in 5 randomly selected microscopic areas under X10 magnification and the arithmetic mean semi-quantitatively was scored. PMNL cell density, lipid accumulation, degenerative cell density, karyorrhexis and changes in the content of secretory granules were scored in histopathological examination. The scoring was decided as follows: none = -, mild = +, moderate = ++, severe = +++.

### Quantitative Analyses

**Stereological Estimation:** Stereo-investigator software version 9 (Microbrightfield, CA, USA) was used for stereological examination. The apparatus was collected of a personal computer, a charge-coupled device digital camera (OptronicsMicroFire), a light microscope (Leica DM4000 B) and motorized computer-controlled specimen stage (Bio- Precision MAC 5000 controller system). The tissue sections were put onto a motor-driven stage connected to the microscope and were reflected, via the camera at 40 objective, onto the monitor. Each serous acinus was determined randomly by systematic sample method, moving the microscope stage left to right, in a stepwise way. Using the "nucleator method" belonging to the software Stereo Investigator (Microbrightfield) system, the mean serous acinus volume was examined according to described by Gundersen.<sup>[18]</sup>

### Statistical Analysis

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

## Results

### Histopathological Results

Control group parotid gland results revealed that it had normally parenchyma and stroma with serous acinus, duct and connective tissue structures. There was more cytoplasmic lipid accumulation seen in the acinus cells and some striated duct cells in DM group than the control group. Besides, in some acinus cells, pyknotic nucleus

and more eosinophilic stained cytoplasm than control group was observed. In addition a few PMNL infiltration in connective tissue was determined. In OVX group sections when compared to DM group the amount of cells that had lipid accumulation were less, but the amount of connective tissue cells and polymorph nuclear cell infiltration (PMNL) in the connective tissue were more found to be increased. Otherwise, in some areas, pyknotic and more eosinophilic cells were seen like DM group but foggy and swollen acinar cells were distinguished different from control and DM group. In DM+OVX group, according to the DM and OVX group, it was detected that extensively increased cytoplasmic lipid accumulation and swollen nucleus with karyorrhexis in acinar cells that was found to be apoptotic. In addition, distinctively, increased amounts of the connective tissue cells, PMNL infiltration and fibrosis were determined in this group connective tissue (Figure1 and Figure 2)

### Histochemical Results

To ensure separation of acinus epithelium secretion content, neutralmuco-polysaccharides stained with periodic acid-schiff (PAS) and acidic muco-polysaccharides stained with alcianblue (AB) staining, histochemical analysis was done. Histochemical examination revealed that in control group of parotid gland, serous acinus cell cytoplasm stained with PAS staining strongly positive, with AB staining weakly positive, and with combined PAS+AB staining, the intensity of PAS was less positive than only PAS staining sections of this group.

When examining DM group, in PAS staining sections it was detected, serous cell cytoplasm were stained more basophilic, and in AB staining sections were darker than the control group. In PAS+AB group sections, PAS densities of cytoplasm were observed weakly positive than PAS staining sections of DM group and PAS+AB staining sections of control group. OVX group section evaluations showed that serous acinus cytoplasm were seen in PAS staining sections slightly more acidophilic than control group, in AB staining slightly darker than control and lighter than DM group. In PAS + AB group sections PAS density was determined weakly positive than only PAS staining sections of OVX group. It was observed these group PAS, AB and PAS + AB stainings were similar to control group than the other experimental groups.

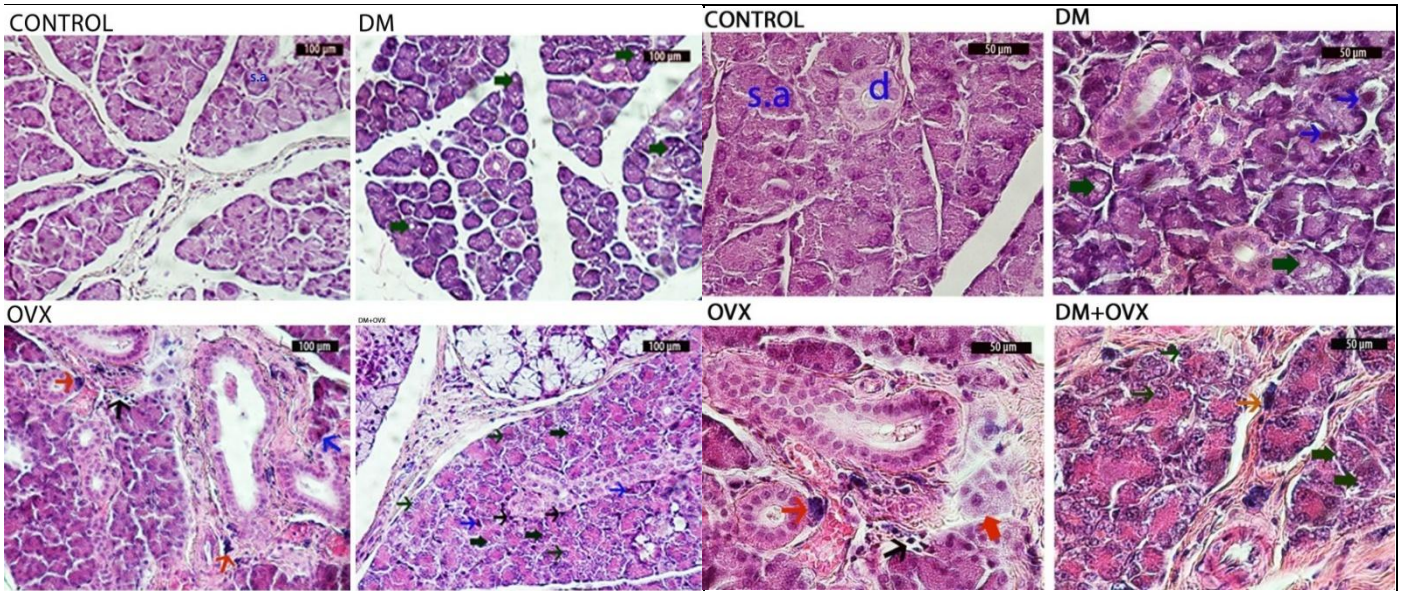


Figure-1: Micrograph of parotid gland in lower magnification for all groups, serous acinus (s.a), degenerative cells (thin blue arrow), lipid accumulation (thick green arrow), swollen nucleus with karyorrhexis (thin green arrow), connective tissue cells (thin black arrow), H&E staining

Figure-2: Micrograph of parotid gland in higher magnification for all groups, serous acinus (s.a), duct (d), Degenerative cells (thin blue arrow), lipid accumulation (thick green arrow), swollen nucleus with karyorrhexis (thin green arrow), PMNL cells (thin orange arrow), foggy swollen acinus cell (thick orange arrow), connective tissue cells (thin black arrow), H&E staining

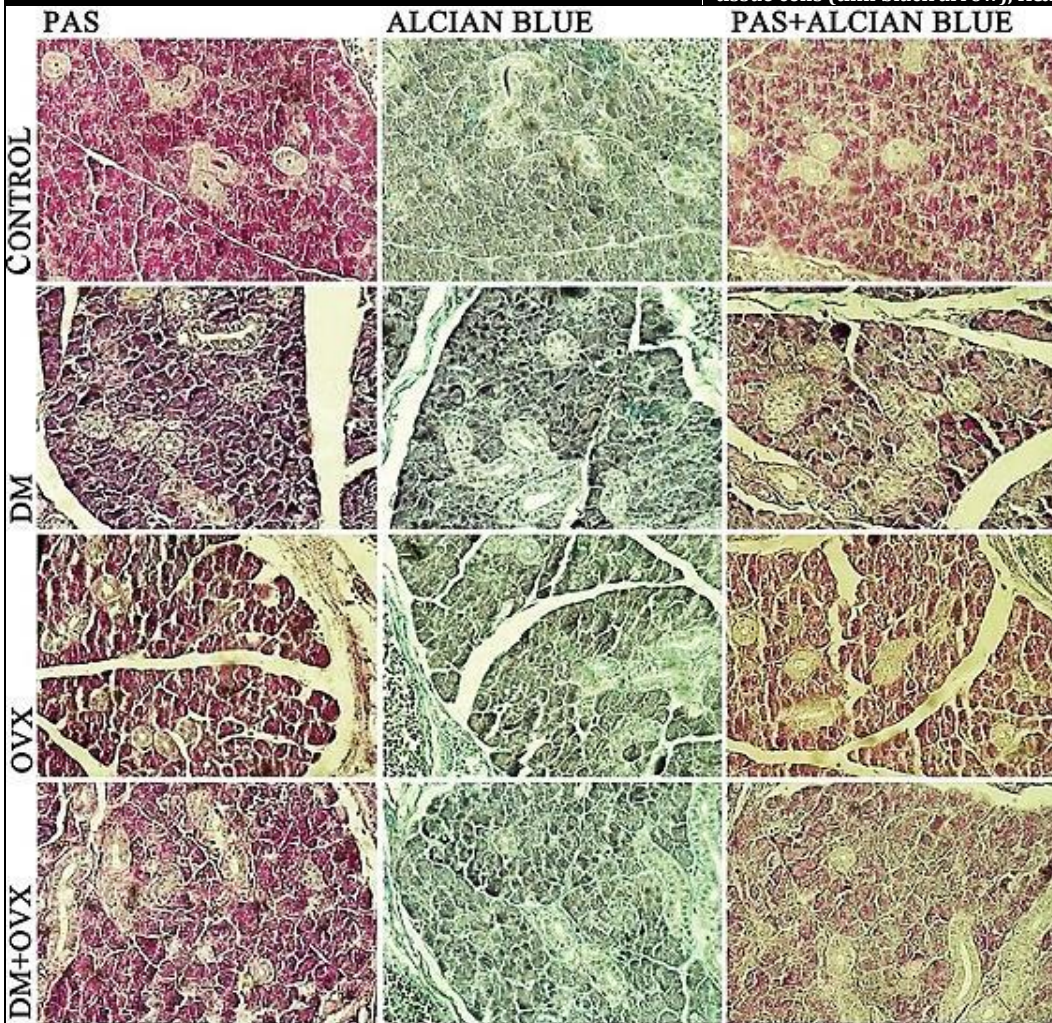


Figure-3: Histological micrograph of control and experimental groups of parotid gland, PAS, AB, PAS+AB staining, X10

DM+OVX group section examinations revealed that acinus cytoplasm of PAS staining sections were more basophilic than control group and less basophilic than DM group. Acinus cytoplasm of AB staining sections were darker than control and lighter than DM groups. In PAS+AB staining sections it was detected PAS density was less positive than PAS staining sections of DM+OVX group and PAS+AB staining of control group but strongly positive than DM group (Figure 3).

### Stereologic Results

Statistical results showed that there were significant differences ( $P < 0.05$ ) between all groups. OVX group mean serous acinus volume was closer and had little increase to control group than the other experimental groups. In DM group sections serous acinus volume higher than control and DM groups. DM+OVX group sections had the most increased serous acinus volume in all groups (Table 3.).

**Table-2: Semi quantitative analysis results of parotid gland**

Groups	PMNL Infiltration	Lipid Accumulation	Degenerative cell density	Karyorrhexis	Changes in the secretory granules
Control	-	-	-	-	-
DM	+	+++	++	+	+++
OVX	++	-	+	+	+
DM+OVX	+++	++	+++	+++	++

**Table-3: Assessments of mean serous acinus volume of all groups**

Groups	Mean serous acinus volume
Control	451,35±0,015*
DM	559,22±0,015*
OVX	458,86±0,015*
DM+OVX	574,70±0,015*

\* Significant differences between groups in the same column

### Discussion

Menopause is characterized by decrease in estrogen production that reduces the quality of life with the increase in the life period of people in the last century that people have to maintain the standard of living. Diabetes is frequently seen in advancing ages in humans, which can affect the whole system that is a life-threatening disease associated with complications together with menopause. Both diseases can lead to many problems despite the pathophysiology of these two cases are not fully understood, yet. As a result of diabetes and menopause, developing metabolic disorders and oxidative stress causes some degeneration in macroscopic salivary glands that was shown in some experimental diabetes and menopause models.<sup>[19,20]</sup> Some studies have reported estrogen that has activating effects on epithelial cell maturation and secretion on the

parotid gland. The reduced secretion in micro flora of oral cavity can cause developing of infectious agents.<sup>[21]</sup> Some studies reported the cellular hypertrophy, atrophy, hyperplasia and DNA in parotid gland of DM.<sup>[22-24]</sup> The products formed as a result of this glycosylation attach to receptors present in cells (endothelium, monocytes, lymphocytes, mesangial cells, and so on.) and thus triggering release of inflammatory cytokines from stimulated immune.<sup>[25]</sup>

In the histological analysis of parotid glands, experimental diabetes and/or menopause, acinus epithelial damage, increased lipid droplets, swollen nucleus with karyorrhexis, eosinophilic stained dense chromatin cells, PMNL cell infiltration and fibrosis in the connective tissue were determined.

Some studies reported that diabetes cause an increase in lipid droplets and some morphological changes in the acinus epithelium of parotid gland.<sup>[26,27]</sup> Mahay et al determined an increase in various diameters of infiltrated lipid droplets in the diabetic parotid tissue and reported that it was related with fat metabolism disorders.<sup>[28]</sup> In the diabetes induced experimental studies, damaged and dysfunctional parotid glands were determined that could be related with oral infection due to the reduced secretion of saliva.<sup>[25]</sup> Estrogen plays an important role in the inflammatory process and estrogen deficiency caused on the parotid gland free radicals mediated apoptosis and some histological alterations.<sup>[29,30]</sup> Syrjanen et al determined that lipid droplets, fibrosis and inflammatory cells in some salivary glands were seen.<sup>[31]</sup>

Considering the findings of our study, the diabetes and/or menopause in metabolic events cause morphological and pathophysiological changes in salivary glands. It was concluded that increase of lipid accumulation cells could be related with carbohydrate and/or fat metabolism and can lead to damage of the acinus epithelium that stimulate migration of inflammatory cells.

PAS, AB, PAS+AB staining of parotid gland analysis revealed noticeable alterations in the content of neutral muco-polysaccharides secretions of acinus epithelium in DM group sections were seen when compared to the control group. The OVX group was closer to the control group and neutral and acidic muco-polysaccharides contents were slightly changed. There was changes in the content of neutral muco-polysaccharides in DM + OVX

group like DM group but less than DM group.

In the diabetes induced parotid glands a reduction in the rate of acyl lipid was seen and reported that diabetes did not alter only the morphology of the parotid gland, at the same time causes the disruption in the secretory function and acyl lipid content of cells of parotid gland.<sup>[28]</sup> Anderson et al detected that in DM induced rats macroscopic salivary glands, a great amounts of clustered of lipid droplets especially in serous acinus and reported that it could be caused by intake of lipid into the cells for using as a resource of energy or reduction in secretory granules or in plasma membrane materials.<sup>[27]</sup> Also, hyperglycemia induces oxidative stress and it was associated with diabetic complications.<sup>[32]</sup>

In the assessment of PAS, PAS+AB and AB staining, it was concluded that alterations in the content of neutral muco-polysaccharides in DM and DM+OVX groups of acinus the cytoplasm could be related with drop in the rate of the acyl lipids or due to the decrease in the synthesis granules or caused by the secretion change in the direction of the hyperglycemia-induced oxidative stress.

Statistical analysis of parotid acinus volume between groups showed significant hypertrophic changes in all experimental groups especially single or combined DM groups and the most in DM+OVX group. Russotto et al reported asymptomatic bilateral parotid gland enlargement in 24% of diabetic patients in their study.<sup>[22]</sup> Also, enlargement in diabetic parotid gland were reported and could be related to fatty infiltration and acinus hypertrophy.<sup>[33]</sup> Sleman et al reported that in the histological examination of the parotid gland in long-term diabetes cases there were hypertrophic changes in acinar cells compared to normal parotid gland.<sup>[34]</sup> Bailey et al determined that bilateral growth of the salivary glands, especially the parotid gland, usually that were seen in obesity that linked to many diseases such as diabetes, hypertension, hyperlipidemia, and menopause.<sup>[35]</sup> Syrjanen et al reported that in post-menopausal process lipid droplets, fibrosis and inflammatory cells and decreased salivary function were detected in their studies.<sup>[34]</sup> In our study, hypertrophic changes in parotid gland acinus in DM and/or OVX groups might be associated with alterations in glucose and lipid mechanisms that could be secondary effects of DM and/or OVX. Deficiency of estrogen can also directly or indirectly affect the glucose and lipid metabolism.

## Conclusion

Finally, it was concluded from the study that diabetic and/or ovariectomy applications in parotid glands can cause metabolic, distinctively significant morphological changes, and alterations in the character and in the amount of secretion, leading to the discomfort of life. Additionally the correlated molecular mechanism of pathophysiological processes of DM and/or OVX on parotid gland, suggested further investigation.

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