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Effects of Diabetes on Post-Menopausal Rat Submandibular Glands: A Histopathological and Stereological Examination

Diyabetin Post-Menopozal Sıçan Submandibular Bezlerinin üzerine Etkileri: Histopatolojik ve Stereolojik İncelenmesi

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Abstract

Objective: The menopause in elderly women is a physiological process where ovarian and uterine cycles end. Diabetes means higher blood glucose level that is a metabolic disease and has an increased incidence. The aim of the study was to examine the single or combined effects of menopause and diabetes that causes pathophysiological processes on submandibular gland on ovariectomy and diabetes induced rat models.

Materials and Methods: Sprague Dawley twelve weeks old female (n=24) rats were divided randomly into four groups; Healthy control group (n=6), diabetic group (DM, n=6), ovariectomized group (OVX, n=6), post ovariectomy diabetes induced group (DM+OVX, n=6) individually. Histopathological, histochemical and stereological analyses were done in these groups.

Results: Significant neutrophil cell infiltrations and myoepithelial cell proliferations, granular duct and seromucous acini damages and changes in the content of especially seromucous acini secretion in DM and/or OVX groups and distinctive interstitial and striated duct damages in post ovariectomy diabetes induced group were detected. Alterations ingranular ducts hypertrophic and in seromucous acini atrophic were determined in DM and/or OVX groups.

Conclusion: The results revealed the pathophysiological processes that lead to morphological and functional alterations on the cellular level in submandibular glands. The molecular mechanisms related with pathogenesis of diabetes and menopause need further investigation.

Keywords: Diabetes, gland, menopause, rat, submandibular

Özet

Amaç: İlerleyen yaş ile birlikte, menopoz, kadınlarda gözlenen overial ve menstrual sikluslarda azalma veya bitiş ile karakterize fizyolojik bir süreç ve diyabet ise insidansı artan yüksek kan şekeri seviyesi ile karakterize metabolik hastalıktır. Overektomi ve diyabet indüklenen sıçan modelleri submandibular bezi üzerinde menopozun ve diyabetin tek veya kombine etkilerinin neden olduğu patofizyolojik süreçleri incelemek amacı ile çalışma tasarlandı.

Gereç ve Yöntem: Sprague Dawley on iki haftalık dişi (n=24) sıçanlar dört gruba randomize bir şekilde rastgele ayrıldı; Sağlıklı Kontrol Grubu (n=6), Diyabetik grup (n=6), Overektomize grup (n=6), Post Overektomize Diyabet İndüklenen Grup (n=6). Sonuçlar histopatolojik, histokimyasal ve stereolojik analizler ile değerlendirildi.

Bulgular: Anlamlı nötrofil infiltrasyonları ve myoepitel hücre proliferasyonları, granüler kanal ve seromuköz hücre dejenerasyonları ve özellikle seromuköz asinusların salgısı içeriğindeki değişimler DM ve/veya OVX gruplarında, belirgin intersistiyel ödem ve çizgili kanal dejenerasyonları diyabet indüklenen post overektomize grup submandibular dokularında izlendi. Granular kanallarda hipertrofik değişiklikler, seromuköz asinuslarda atrofik değişiklikler DM ve/veya OVX gruplarında tespit edildi.

Sonuç: Diyabet ve overektominin submandibular bezlerinde yol açtığı patofizyolojik süreçlere bağlı hücresel düzeyde morfolojik ve fonksiyonel değişiklikler gösterildi. Diyabet ve menopozun patogenezi ile ilişkili moleküler mekanizmalara yönelik submandibular bez üzerinde daha fazla araştırma yapılabilir.

Anahtar Kelimeler: Diyabet, menopoz, sıçan, submandibular, tükürük bezi

Introduction

Menopause is a physiological condition generally seen after age 45 in women. This physiological condition occurs due to deficiencies in estrogenic secretion, which cause the reduction or cessation of ovarian and menstrual function. Oestrogen is a sex hormone that has direct and indirect effects on many systems. Oestrogen receptors are found in the cells of many tissues belonging to the reproductive system organs, brain, kidney, heart, bone, and salivary glands [1-3]. These receptors serve as transcription sites for the hormones that function in the area of DNA binding sites.

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Oestrogen performs important functions in many mammalian tissues, including roles in cell division, proliferation and growth, and has prominent effects on embryonic development and the continuity of life. A deficiency of oestrogen can cause health problems [4], such as cardiovascular disease, osteoporosis, and discomfort in the salivary glands [5].

Diabetes mellitus (DM) is a metabolic disorder characterized by hyperglycaemia that occurs due to inadequate insulin secretion from the pancreas. Insulin is the primary stabilizer of the carbohydrate metabolism and has significant effects on and associations with other metabolic activities. Hyperglycaemia can cause cellular damage that creates losses in the structure and function of systems, organs, and tissues, such as the nervous system, kidney, and salivary glands [6-8].

The effects of menopause and diabetes on the salivary glands have been investigated in many studies, but studies on submandibular glands are limited, and histopathological, histochemical and stereological analyses have been found to be insufficient. This study aimed at investigating the effects of hypoestrogenic and hyperglycaemic situations on rats' submandibular glands through an experimental model of menopause and diabetes.

Materials and Methods

Animals and experimental protocol

In the study, totally 24 female Sprague-Dawley rats, 12 weeks old, were supplied from Atatürk University Medical Experimental Application and Research Centre (ATADEM). Under a regular 12/12 light/dark cycle room temperature (20±2°C) and humidity (50-60%) in controlled environments the rats were kept constant. They were given ad libitum access to food and water. 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.

Experimental procedure

Atatürk University Experimental Animal Practice Lab was used to perform experimental animal models and applications in the study. Ovariectomy procedure was applied on OVX and DM+OVX groups of rats (n=12). 90 days after the ovariectomy procedure in 91 day alloxan-induced diabetes procedure was applied with alloxan inducing on DM and DM+OVX groups of rats (n=12). No experimental procedure was applied to control group of rats, they left in the same conditions. 123 days after the start of the study, all groups of rats were sacrificed by perfusion fixation process.

Experimental models

Ovariectomy procedure

Two groups of rats (OVX and DM+OVX) were ovariectomized. They were anesthetized with 20 mg/kg dose of sodium thiopental (Pentothal). After applying by a 2-3 cm incision from the lateral portion of longitudinal lower abdomen midline, the abdomen was opened, and passing through the peritoneum and abdominal muscles the ovaries were reached. The ovaries were removed by bilateral excision [9]. After the incision was closed, rats were allowed to live in the appropriate environment and 25 mg/kg dose of metamizole sodium was given to rats as an analgesic two times a day for the first two days. Wound dressing was applied to prevent the risk of infection for a week every day. The ovariectomized rats were fed with water and pellets for 12 weeks, and the required time to survive was created for them.

Alloxan-induced diabetes procedure

Two groups of rats (DM and DM+OVX) were induced diabetes according to defined methods by Halici [10]. 150 mg/kg single dose of alloxan monohydrate solution (Sigma-Aldrich Co, Germany) was applied intraperitoneally to rats. It was allowed to settle pancreatic beta cell damage to induce diabetes. After an overnight fast, the rats were intraperitoneally injected with alloxan that was freshly prepared with 0.9% NaCl solution. Besides, non-diabetic groups of rats were injected intraperitoneally with pure 0.9% NaCl solution. 4-6 h after alloxan administration of about 5 ml of 20% glucose solution was injected intraperitoneally to the rats to prevent foetal hypoglycaemia depending upon the discharge of insulin that stored in the pancreas. Moreover, to prevent hypoglycaemia, 5% glucose solution added to drinking water of the rats for 24 hours was given. 72 hours later, fasting blood sample was taken from the tail vein of each and blood glucose was measured with blood glucose monitor (Accu-Chek Active, Germany). At the end of the third day, rats are considered as diabetic with at least 200 mg/dL serum glucose levels. Diabetic rats were kept alive for 2 months.

Research Methods

Histological analysis

In all groups, the submandibular gland tissues of rats were given code numbers and put into bottles containing 10% neutral formaldehyde. After 72 hours, the following tissue process was applied for these tissues: washing overnight, dehydration in an increased alcohol series, clearing through a xylene series, immersion in liquid paraffin, and embedding in paraffin blocks. From the paraffin blocks of each rat, four 5-µm serial sections with intervals of 50 µm were taken using a microtome (Leica RM2125RT, Nussloch, Germany).

The obtained sections were brought from deparaffinization to the water and stained with haematoxylin and eosin (H&E) for histopathological examination and with Periodic acid-Schiff (PAS), Alcian Blue (AB) (pH 2.5), and PAS/AB (pH 2.5 and 1) for histochemical analysis. Later the cover slipped sections were photographed with a camera attached light microscope (Nikon Eclipse E600, Japan). Management of the same light settings was performed for photographing, especially in the histochemical analysis, in order to permit unbiased evaluation.

Semi-quantitative analysis

Histopathological and histochemical investigations were done using a light microscope. Every rat section was semi-quantitatively scored. For each section, 5 microscopic areas, nearly 100 μm2, were selected randomly. Neutrophil infiltration density, myoepithelial cell density in the degeneration area, degenerative granular duct cell density, degenerative seromucous acinus cell density, and changes in the content of the secretory granules of seromucous acini and granular ducts of the parenchyma and stroma were calculated at X10 objective. The arithmetic mean of the histopathological evaluation was scored semi-quantitatively. The scoring was labelled as follows: none= –, mild= +, moderate= ++, severe= +++.

Quantitative Analysis

Stereological analysis

In this study, the mean granular duct and seromucous acinus areas were calculated using the nucleator method, one of the stereological methods with an unbiased counting frame. The Stereo Investigator (MicroBrightField 9.0, Colchester, VT, CA, USA) software system was used. This system consists of a camera attached to a light microscope, a motorized system that carries a microscope tray, and a computer with a software system. H&E-stained sections were put on the microscope tray, and their sectional boundaries were determined using this program. After determining the area, frames separated from each other were determined by systematic random sampling of the sections, according to the rules of space fragmentation with the step interval of the x and y-axis. Then, in 20 different selected areas, the mean areas of the seromucous acini and granular ducts of all groups were measured following the method described by Gundersen [11].

Statistical Analysis

Statistical analysis of the mean area of the seromucous acini and granular ducts of all groups was performed using SPSS (IBM SPSS Statistics 18.0, IBM Corporation, Somers, NY, USA). Because the data showed a normal distribution with the coefficient variables which were more than 20%, differences between the groups were tested using one-way analysis of variance (ANOVA) followed by an Least Significant Difference LSD test, the numerical data of groups were analysed (a P value <0.05 was selected as significant). The values were determined as means±standard deviation.

Results

Histological results

In the submandibular gland tissues of the control group, the structure of the seromucous acini, ducts, and connective tissue were observed to be normal. In the DM group, some seromucous acini and granular duct cells with more eosinophilic cytoplasm and hyperchromatic nucleus were detected. Disrupted membrane structures and autophagic structures were also identified in granular duct cells. Moreover, neutrophil infiltrations were detected and, interestingly, cells were thought to be myoepithelial in the degeneration areas, when compared to the control group. In the OVX group, there were more disruptions in the granular duct cell membranes and separations between the basal lamina and reticular lamina in cells thought to be apoptosis. Distinctively, increased neutrophil cells were observed in this group, too. In the DM+OVX group, the most degenerated seromucous and granular duct cells, increased neutrophil infiltrations, and cells such as myoepithelial cells were detected. Increased degenerative striated duct cells and interstitial oedema were also seen in this group (Figure 1, Table 1).

Table 1. Semi quantitative analysis results of submandibular gland

Groups	Neutrophil infiltration density	Myoepithelial cell in degeneration area	Degenerative granular duct cell density	Degenerative seromucous acinus cell density	Changes in the content of secretory granules	
					Seromucous acinus	Granular duct
Control	-	-	-	-	-	-
DM	+	++	+	+	+++	+++
OVX	+++	+	++	+	+	+
DM+OVX	+++	+++	+++	++	++	++

Control: non-diabetic healthy group; DM: diabetic group; OVX: ovariectomized group; DM+OVX: post ovariectomy diabetes induced group.

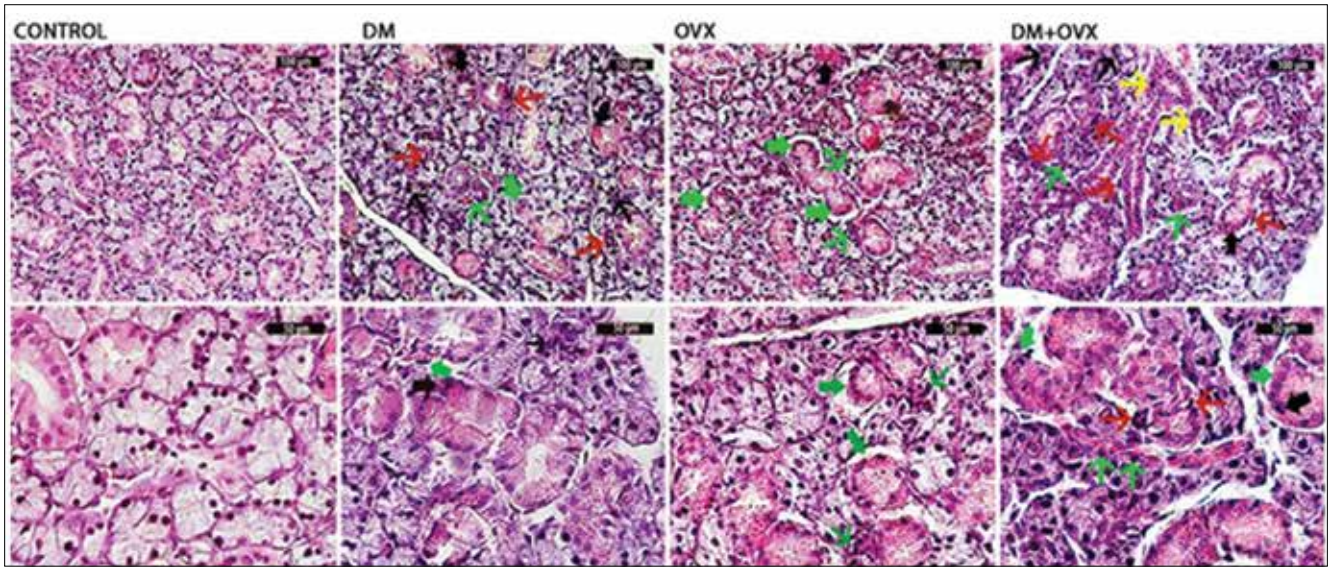

Figure 1. *Micrograph of sublingual gland in the lower and higher magnification for all groups, thick black arrow; degenerative (more acidophilic cytoplasm with pyknotic nucleus) granular duct cells, thick green arrow; granular cells being apoptosis, thin black arrow; degenerative seromucous cells, thin green arrow; neutrophil cell infiltrations to connective tissue, thin yellow arrow; degenerated striated duct, thin orange arrow; cells were thought myoepithelial cells, H&E staining.*

Histochemical results

To ensure the separation of submandibular gland epithelium secretion in histochemical examination, tissues were stained with PAS to show the content of neutral mucopolysaccharides and with AB to show the content of acidic mucopolysaccharides. The granular duct cells of the submandibular glands of the control group were observed to be normal with pure PAS staining, pure AB staining, and PAS+AB staining. In the DM group, the granular duct cells had more staining than the pure PAS staining and the nearly pure AB staining of the control group. In the PAS+AB staining, the PAS density was found to be a little less than the pure PAS staining of the DM group and more acidophilic than the PAS+AB staining of the control group. In the OVX group, all three stainings produced granular ducts similar to those of the control group. In the DM+OVX group, the PAS density was close to that of the poor pure PAS staining of the DM group, while the AB density was similar to that of the pure AB staining of the other three groups. In the PAS+AB staining, the PAS density was close to that of the poor PAS+AB staining of the DM group (Figure 2, Table 1).

The seromucous acinus cells of the submandibular gland of the control group were observed to be normal with pure PAS staining, pure AB staining, and PAS + AB staining. In the DM group, seromucous acinus cells had a more acidophilic stain than the pure PAS staining and close to the acidophilic level of the pure AB staining of the control group. In the PAS+AB staining of the DM group, the PAS staining was more basophilic than the pure PAS staining of the DM group and the PAS+AB staining of the control group. In the OVX group, the PAS and AB staining of seromucous acinus was similar to that of the DM group. In DM+OVX group, the PAS density was close to that of the poor pure PAS staining of the DM group, and the AB density was close to that of the pure AB staining of the DM and OVX groups. In the PAS+AB staining, the PAS density was close to that of the poor PAS+AB staining of the DM group (Figure 2, Table 1).

Stereological Results

In the analysis of the mean area of the granular ducts of the submandibular gland, there were significant differences among all groups ($p<0.05$). A significant increase was observed in the OVX group's mean acinus area, but it was close to that of the control group in all experimental groups. The mean acinus area of the DM group was slightly larger than that of the OVX group. The greatest increase in the mean acinus area of all groups was observed in the DM+OVX group ($p<0.05$) (Table 2).

There were significant differences observed between all groups in the analysis of the mean seromucous acinus area of the submandibular glands ($p<0.05$). A significant reduction in the mean seromucous acinus area in the experimental groups was found compared to the control group. The DM group had an acinus area closer to that of the control group than the other experimental groups. The DM+OVX group had a greater reduction than the DM group. The OVX group had the largest reduction in mean seromucous acinus area among all groups (Table 2).

Table 2. Assessments of mean seromucous acinus area and mean granular duct area of all groups

Groups	Mean seromucous acinus area	Mean granular duct area
Control	953.57±0.155[a]	1617.44±0.323[d]
DM	927.73±0.325[b]	1787.13±0.074[b]
OVX	872.09±0.730[c]	1775.35±0.223[c]
DM+OVX	877.93±0.615[d]	1997.59±0.013[a]

[abc] The footnote letters expresses the significant differences between groups in the same column.
Control: non-diabetic healthy group; DM: diabetic group; OVX: ovariectomized group; DM+OVX: post ovariectomy diabetes induced group.

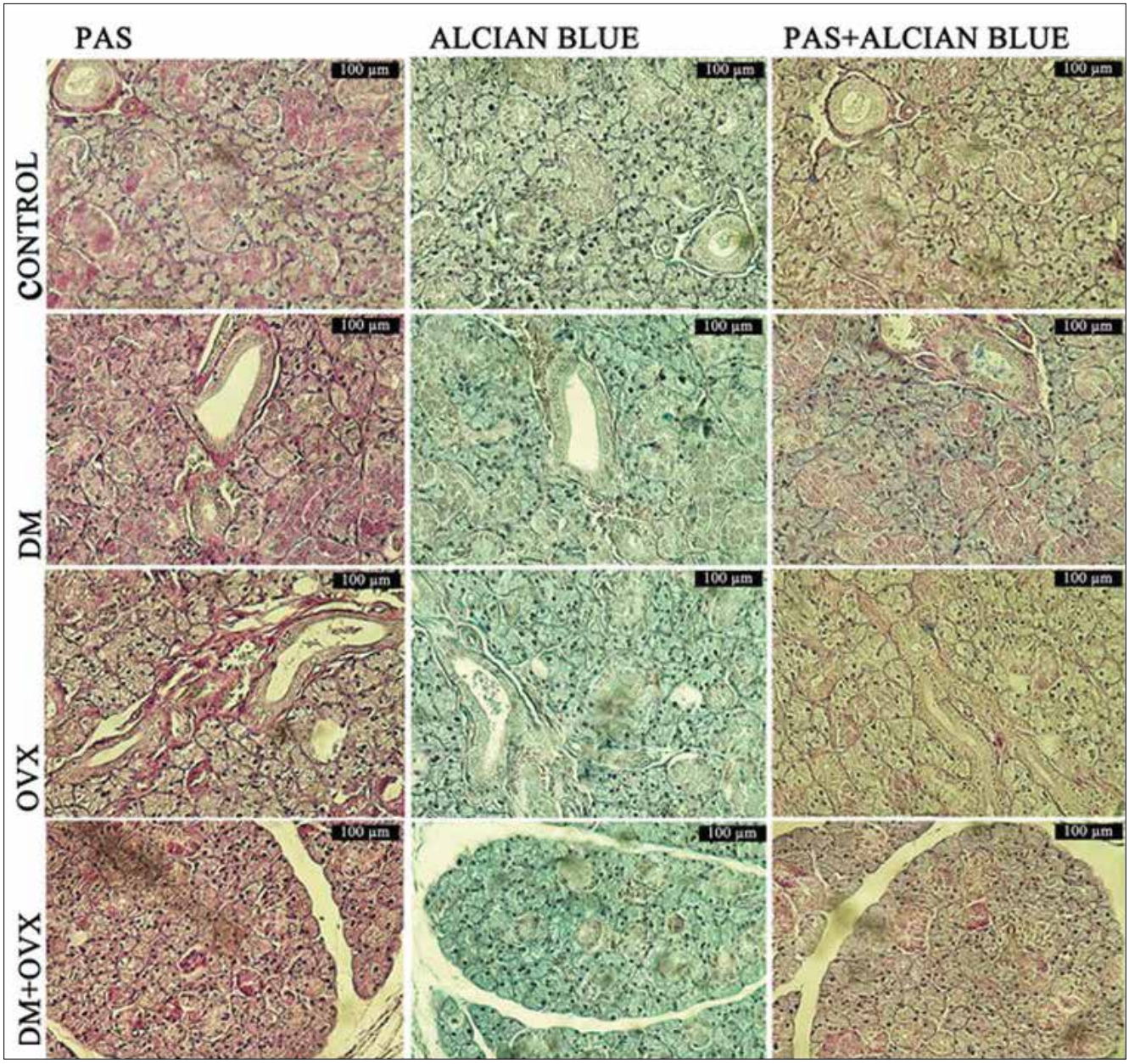

Figure 2. *Histological micrograph of control and experimental groups of submandibular gland, PAS, and AB, PAS+AB stainings.*

Discussion

Menopause and climacterium are physiological processes that reduce the quality of women's lives. Diabetes is a disease that is associated with life-threatening complications that can affect many systems like nerve system and urinary system in humans. The incidence of this disease is increasing in the world. Both conditions lead to many problems that reduce the quality of life, although their pathophysiology has not been fully explained yet.

Many studies have investigated the oxidative stress and response to oxidative stress that occur during menopause as a result of declining oestrogen levels [12, 13]. Various causes induce oxidative stress, which triggers reactive oxygen spices (ROS) production in tissues. Both enzymatic and non-enzymatic antioxidant defence mechanisms are activated in many tissues' metabolisms in order to reduce damage [14, 15]. Studies on diabetes have shown that it causes damage to major salivary glands, including DNA damage and cellular hypertrophy, atrophy, and hyperplasia [16-19]. Due to hyperglycaemia, non-enzymatic glycosylation of proteins occurs in blood during the early stages of DM and in interstitial tissue during the later stages of DM. As a result, glycosylation products attach to cells' receptors, triggering inflammation that stimulates immune cells to release cytokines [20]. Several experimental menopause and diabetes models have shown that the development of metabolic disorders causes some degeneration in the salivary glands [21, 22]. Hypoestrogenic and hyperglycaemic effects cause the loss of the balance in the body's defence system that induces oxidative stress. In addition, oestrogen supports salivary gland cell maturation and has activating effects on secretion [3]. Diabetes can cause dehydration and inadequate blood glucose control, resulting in the hypofunction of the salivary glands. Both of these conditions decrease oral cavity resistance to infective agents, which is a basis for developing inflammation [20].

In this study's histopathological evaluation of the submandibular gland of all groups, degenerative seromucous and granular duct cells were detected in the DM and OVX groups. Autophagic structures in granular duct cells were also noted in these groups. In the single and combined DM groups, myoepithelial cells were seen in the degenerative acini areas. In the single and combined OVX groups, increased neutrophil infiltrations and granular duct cells that were apoptosis were observed. In these groups, basal laminas belonging to the granular duct cells were apoptosis and separated from their reticular laminas. In addition, significantly increased interstitial oedema and degenerative striated duct cells were observed in the DM+OVX group.

Related studies [23-27] showed that, in diabetes-induced rat submandibular glands, the secretion material accumulate in acinar cells' cytoplasm, resulting in degenerative changes followed by cell death. In late-phase diabetes, an increase in the number of autophagic structures occurs in granular duct cells. The histological findings from non-obese diabetic mice's submandibular gland tissues also indicate damage accompanied by massive cell infiltration [28-31]. Some studies have shown that oestrogen and progesterone regulate the structure and function of the submandibular glands. Additionally, estradiol deficiency might cause changes in the histological structure of granular duct epithelial cells, which could affect the secretion content through changes in protein synthesis. As well, the lack of oestrogen in the submandibular glands of mice caused the severe development of destructive autoimmune lesions, which were removed by oestrogen management. Oestrogen deficiency in rat submandibular serous epithelial cells caused selective apoptosis. Parlak et al. [32] found significantly increased polymorphonuclear infiltrations in single and combined ovariectomy applied groups of female rat parotid glands. Takahashi et al. [33] found that, in atrophic rat submandibular glands, apoptosis and proliferation of myoepithelial cells occurred.

The findings of the present study have showed that, depending on changes in the metabolic processes of diabetes and menopause, secretion accumulation in the granular duct cells and seromucous cells occurs and can cause cellular degeneration. Autophagic structures in these cells result in increased apoptosis, and to compensate for these degenerations, there can be an increase in the number of neutrophil and myoepithelial cells in the degeneration area.

In the histochemical analysis of the submandibular gland of all groups, the amount of neutral mucopolysaccharides was significantly increased in granular duct cells, especially in the seromucous glands of the DM and DM+OVX groups, and the amount of acidic mucopolysaccharides was slightly increased in all experimental groups, compared to control group.

Alves et al. [34] reported that, depending on the inflammatory response to the metabolic and energy disorders, there were significant protein variations in the submandibular glands of female rats induced with diabetes. Turner et al. [35] detected structural failure in the salivary glands during diabetes and reported the importance of the management of oral pathology during oxidative stress with a ROS metabolism related to enzyme modulation. Carvalho et al. [36] reported that ovariectomized rats' submandibular gland acini and ducts are affected by oestrogen deficiencies, and the secondary effects of ovariectomy are associated with decreased secretory function, which can reduce the quality of acini and ducts. Flynn et al. [37] determined that estradiol-caused changes in the cytology of the ducts could be related to changes in protein synthesis.

In this study, the increased neutral mucopolysaccharides content of granular ducts and seromucous acini in the single

and combined DM groups, and the higher acidic mucopolysaccharides content of the experimental groups can be related to oxidative stress. This is because the ROS metabolism, which is related to enzyme modulation and alterations in the cytology of the granular ducts and seromucous acini, can be associated with the changes in protein synthesis that affect secretory function.

Stereological analysis of the submandibular granular duct area was intensified in the experimental groups, especially in the DM+OVX groups. Anderson et al. [24] reported that, in diabetes-induced rat submandibular gland granular duct cells, a reduced area of secretory granules density emerged, causing hypertrophic changes in these cells. Uyanikgil et al. [38] detected in the submandibular glands of ovariectomized rats an increase in the diameter of acinar cells, accompanied by decreased numbers and percentages of acini.

The present study's findings showed that, in submandibular diabetic granular duct cells, diabetes- and/or ovariectomy-triggered hypertrophic changes can result from a decreased area of secretory granule densities and an increased diameter of acinar cells in contrast to the decreased number and percentage of acini. The most hypertrophic changes in the DM+OVX group can be associated with the synergetic effects of diabetes and ovariectomy.

Stereological assessment of the submandibular seromucous acinus area showed a significant reduction ($p<0.05$) in the DM and OVX groups, compared to the control group. The largest decrease in the acinus area was observed in the OVX group.

Cutler et al. [39] reported that changes characterized by the accumulation of secretion materials in the cytoplasm of diabetic submandibular serous acinar cells caused degenerative changes, leading to apoptosis. An histomorphological analysis of the submandibular glands of ovariectomized rats performed by Carvalho et al. [36] showed a lack of effects from oestrogen secretion and reduced acini and ducts associated with submandibular gland function.

The present study's findings revealed that diabetes and/or ovariectomy can lead to degenerative changes in the submandibular seromucous acini and that ovariectomy can reduce the amount and function of the secretion of acini. Therefore, it was determined that the hypoestrogenic and hypoglycaemic conditions that cause significant morphological and functional changes in submandibular glands have significant effects on metabolism. The changes of the neutral mucopolysaccharides content and the acidic mucopolysaccharides content of granular ducts, and especially seromucous acinar cells can be related to the metabolic pathways that lead to protein variations, atrophic and hypertrophic changes, and alterations in the course of secretion. It has been found that the glucose and lipid metabolisms might be associated with these pathways. The existence of increased neutrophil infiltrations in the single and combined OVX groups revealed that oestrogen inhibited cellular degeneration and displayed antioxidant effects that enhanced the cellular defence in submandibular glands. The greatest neutrophil infiltrations and apoptosis of submandibular epithelial cells in the DM+OVX group could be due to synergetic effects of the oxidative stress effect of hyperglycaemia and the suppressed antioxidant effect of oestrogen. In degenerative seromucous acini and granular ducts, the presence of increased myoepithelial cells was very noticeable. Researching whether myoepithelial cells in the degenerative epithelial area have a function, like a phagocytic or stem cell, is suggested.

In conclusion, it was determined that menopause and diabetes can cause important metabolic and morphological alterations and can affect the amount and characteristics of secretions in submandibular glands, possibly lessening women's quality of life. The molecular mechanisms related to oxidative stress and the glucose and lipid metabolisms that can cause degeneration and apoptosis in submandibular stromal cells should be researched. In particular, proteins that bind basal laminas to reticular laminas should be examined, along with their related oestrogen mechanisms. In addition, investigations should target molecular mechanisms that activate neutrophil and myoepithelial cells and the function of myoepithelial cells, if they have a function like phagocytic or stem cells. The pathophysiological processes of menopause and diabetes in the submandibular gland warrant further research.

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