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Proliferative Activity of Myoepithelial Cells in Normal and Diabetic Parotid Glands Based on Double Immunostaining Labeling

Sherif Hassan[1,2]*, Ibraheem Bamaga[1]

[1]*Department of Basic and Clinical Oral Sciences, Oral Biology Division, Faculty of Dentistry, Umm Al-Qura University, Makkah, KSA;* [2]*Department of Oral Biology and Dental Anatomy, Faculty of Dentistry, Al-Azhar University, Assiut, Egypt*

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***Correspondence:** Sherif Hassan, Department of Basic and Clinical Oral Sciences, Oral Biology Division, Faculty of Dentistry, Umm Al-Qura University, Makkah, KSA. E-mail: drsherifhassan@hotmail.com
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Abstract

AIM: This study aimed to examine the effect of diabetes mellitus on the histology of parotid glands and to give a scientific overview of the distribution and the proliferative activity of myoepithelial cells (MECs) encircling both ducts and acini in parotid gland of both normal and diabetic mongrel dogs.

MATERIALS AND METHODS: Twelve male mongrel dogs were used in the experiment and divided into two equal groups, group I, control group, group II, dogs with alloxan-induced diabetes. The dogs of the group II were injected by fresh preparation of a single dose of 100 mg/kg body weight of alloxan monohydrate dissolved in physiological saline. Ten days later, blood glucose level was determined using enzymatic colorimetric test; dogs presented a glucose level at or above 200 mg/dL were included in the diabetic group of the experiment. Three months later, dogs were sacrificed and the parotid glands from all groups were dissected and prepared for histological examination and double immunohistochemical expression of both actin and proliferating cell nuclear antigen (PCNA).

RESULTS: Histological findings using H and E staining confirmed that the parotid gland parenchyma of the diabetic group had glandular atrophy characterized by loss of normal gland structure, acinar degeneration, and dilatation of the duct system with the presence of duct like structure. Moreover, there was a predominance of the fibrous component with the presence of fat cells within the gland compartments. Immunohistochemical findings of parotid gland of control group revealed positive scattered actin staining of weak to mild quantity in cells embracing some acini and intralobular ducts. Expression of PCNA in actin-positive cells revealed few scattered reactions embracing some acini and small ducts. Parotid gland of diabetic dogs revealed positive actin staining of mild-to-moderate quantity in the cells encircling the acini, intralobular, and some interlobular ducts. Expression of PCNA in actin-positive cells revealed mild-to-moderate positive reaction more concentrated in the cells surrounding both acini and intercalated ducts.

CONCLUSION: Routine histological findings of diabetic dogs in our findings showed abundant pathological changes in parenchymal tissue elements, including acinar, ductal, and MECs that had a significant impact on saliva production and secretion resulting in dry mouth. The proliferative activity of MECs in the control group indicated a routine regeneration process, whereas the abundant proliferative activity in the diabetic group might indicate pathological transformation rather than regeneration, especially because no remedial measures were taken during this investigation.

Introduction

Salivary glands are exocrine merocrine glands that deliver saliva to the oral cavity and play an important role in digestion of food and maintaining the oral health [1]. The four major salivary glands in dogs include the parotid, mandibular, sublingual, and zygomatic glands, while minor glands are spread throughout the oral cavity [2]. The parotid glands are large encapsulated salivary glands of mixed type consisting of predominant spherical serous acini with a few scattered mucous acini [3]. Saliva is secreted mainly from submandibular gland (65%), the parotid gland (23%), the sublingual glands (4%), and the remaining minor glands (8%). Saliva has many major functions in the oral cavity including buffering, lubrication, antimicrobials [4], digestion, hormone regulation [5], and the sense of taste [6]. Saliva of major gland is digestive and protective in nature, while minor glands serve to lubricate the walls of the oral cavity [7]. Hypofunction of the salivary glands is a significant problem that leads to severe adverse health outcomes including tooth decay, gum disease [8], swallowing difficulties [9], and taste disturbance [10].

Diabetes mellitus (DM) is a growing disease that impacts the global health expenditure [7]. DM is a metabolic disease in which the long-term prognosis of patients depends on the consistency of residual fasting plasma glucose levels >126 mg/dL [11]. According to the World Health Organization, the Kingdom of Saudi Arabia ranked second in terms of the incidence of diabetes in the countries of the Middle East [12], where the number of people with diabetes reached nearly one-fifth of its citizens [13]. DM is one of the most common diseases that cause salivary gland dysfunction, resulting in histological damage and alteration of the salivary

secretion mechanism [14]. Sreebny *et al*. (1992) reported that 43% of individuals had dry mouth syndrome, at the onset of diabetes [15]. In general, diabetic animals showed various salivary gland disorders including decreased acinar volume, growth retardation, and weight loss in both the parotid and sublingual glands [16]. Several authors recorded that the biological level of amylase in the parotid gland saliva of diabetic animals was significantly lower than that in the control group [17], [18].

Myoepithelial cells (MECs) are star-shaped contractile cells that are observed in many exocrine glands including salivary glands and embracing both acini and intercalated ducts [19], [20]. MECs show all characteristics of the epithelium in that they are located between the secretory cells and basal lamina; they also contain intermediate filaments [21]. MECs have many cytoplasmic processes containing actin and myosin filaments like those found in smooth muscle fiber [22]. In well-developed glands, MECs were considered to have very low mitotic activity because they are terminally differentiated cells [23]. On the basis of their structure and location in acini and ducts, MECs are considered to play some roles in both secretion and excretion of saliva [24]. In addition, MECs play an important role in promoting epithelial cell differentiation by synthesizing and secreting extracellular matrix, basement membranes and act as tumor suppressor cells [25]. On the other hand, salivary gland MECs have received attention in recent years due to their potential involvement in the pathogenesis of various tumors. MECs have been speculated to play an important role in the pathogenesis of some salivary gland tumors, such as pleomorphic adenoma, adenoid cystic carcinoma, and myoepithelioma [26].

Proliferating cell nuclear antigen (PCNA) is a non-histone nuclear protein that functions as a cofactor for DNA polymerase-delta and plays a role in the initiation of cell proliferation [27]. Background PCNA has been found in the nuclei of all animal cells that undergo cell division, suggesting a function in cell cycle regulation and/or DNA replication. It subsequently became clear that PCNA also played a role in other processes involving the cell genome. However, besides DNA replication, PCNA showed to be associated with other vital cellular processes such as chromatin remodeling, DNA repair, sister-chromatid cohesion, and cell cycle control [28]. Several authors examine the major salivary glands of rats during atrophy and/or regeneration using double immunohistochemistry for actin and PCNA and note many double-positive cells surrounding both acini and intercalating ducts [29], [30].

Materials and Methods

Animal groups and diabetes induction

Twelve healthy adult male mongrel dogs (8–12 kg weight) were used in the experiment. Dogs were fed on boiled horse meat, bread, and water ad libitum. Dogs were kept under observation for approximately 14 days before the start of the experiment to ensure that no rabies infection. The dogs were divided into two equal groups, control group I and diabetic group II (6 dogs in each). The dogs of the group II were prevented from eating for 12 h, then injected intravenously with fresh preparation of a single dose of 100 mg/kg body weight of alloxan monohydrate (Sigma Chemical Company) dissolved in physiological saline (0.9% NaCl). Six hours after alloxan injection, the blood glucose level was measured every 4 h until resolution of the hypoglycemia. If the blood glucose levels were too low, a glucose solution (5–10%) was given intravenously [31].

Determination of blood glucose levels

Ten days later, blood glucose concentration was measured using enzymatic colorimetric test; dogs presented a glucose level at or above 200 mg/dL were included in the diabetic group of the experiment. To ensure that the diabetes persists, the glucose level was checked at different intervals every 2 weeks (3 times interval) to ensure that the diabetes persists.

Tissue preparation

Dogs were sacrificed 90 days after induction of diabetes [32], and the parotid glands of animals (Figure 1) were extracted and cut into small cubes (4 × 4 × 4 mm) and fixed in Bouin's fixative for 3 days. Fixed tissues were washed and then dried with ascending degrees of alcohol and infiltrated with molten paraffin wax to build a block. Serial tissue sections 5 μm thick were mounted on a glass slide to be stained by H and E for routine histological examination.

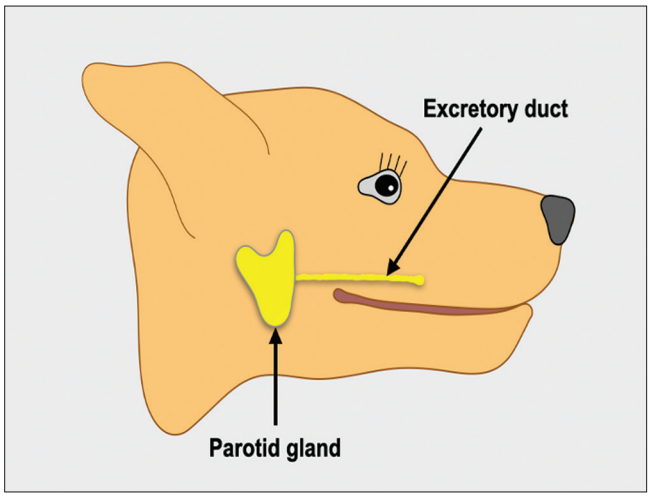

Figure 1: Anatomy of the dog parotid gland

Processes of Immunohistochemistry

Paraffin sections of 5 μm thickness were immersed in 0.3% H_2O–/methanol for 30 min to prevent

endogenous peroxidase activity, rinsed with phosphate-buffered saline (PBS). In PCNA immunostaining, sections were incubated with anti-PCNA mouse monoclonal antibody as a marker for proliferating cells, biotinylated anti-mouse rabbit polyclonal antibody (Dako: N1529 clone PC10), and streptavidin-biotin horseradish peroxidase (HRP) complex (SABC); then, the immunoreaction of PCNA was visualized with 3-3diaminobenzidine (DAB). For immunostaining with actin filaments, slides were incubated for 1 h in a monoclonal anti-actin antibody (Dako: N1584 clone 1A4) a marker of MECs and rinsed further with PBS then incubated for 10 min with biotinylated anti-mouse IgG, IgA, and IgM. The streptavidin-biotin HRP method was applied using DAB followed by counterstaining with Mayer's hematoxylin. The reaction of PCNA was expressed as brown nuclei, whereas the actin reaction was expressed as a purple staining filament. Counting was done by two researchers separately and a scoring system for both actin immunostaining and PCNA-positive cells was calculated and percentage of cells in relation to the normal was calculated as follows: Normal expression up to 10%, (1) weak, up to 20%, (2) mild, up to 40%, (3) moderate, up to 60%, and (4) strong, >60%. Statistical Package for the Social Sciences, version 23 (IBM, US) was used for analysis of data. The descriptive data for both groups were presented as mean, standard deviation, and using Levine's test to evaluate equality of variances for the computed variable data. The differences between two independent groups with quantitative data and a parametric distribution were made using an independent t-test. p-value was considered significant at level ≤0.05.

Results

Histopathological examination

Histological examination of the parotid gland of non-diabetic dogs showed the presence of lobules of parenchymal tissue filled with integrated serous acini interspersed with isolated mucous secretory units and including intercalated, striated, and exocrine ducts with all characteristic structures for each. These parenchymal elements were supported by a fibrous stroma that divides the gland into lobes and lobules (Figure 2). On the other hand, the parotid glands of diabetic dogs showed a variable extent of pathological changes ranging from abnormal glandular structure with a decrease in acinar size to severe atrophy of the gland without definite acinar arrangement. Atrophic changes were characterized by a decrease in parenchymal elements accompanied by an increase in the amount of fibrous tissue stroma. The remaining viable acinar tissues were smaller, interspersed, and scattered with a non-specific cellular arrangement. The lobules appeared to have many structures resembling ducts (duct-like structure) that were indistinguishable from the original ducts. The duct system was dilated and surrounded by remnants of acinar cells (Figures 3 and 4).

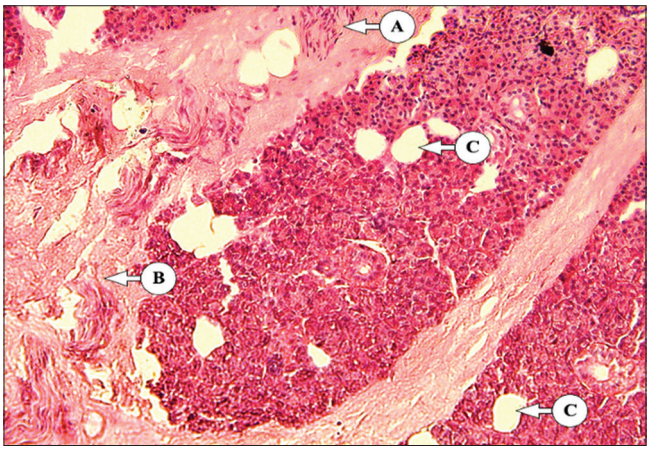

Figure 3: Diabetic parotid gland showing, degenerated acini (A), fibrosis (B), and fatty degeneration (C) (H and E ×100)

Immunohistochemical examination

Parotid gland of control group revealed positive scattered actin staining of weak to mild quantity in cells embracing some acini and intralobular ducts.

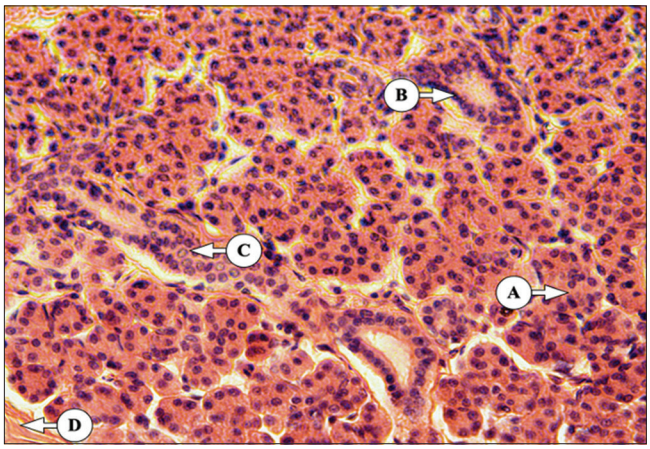

Figure 2: Control parotid gland showing: Serous acini (A), intercalated duct (B), striated duct (C), and fibrous stroma (D) (H and E ×200)

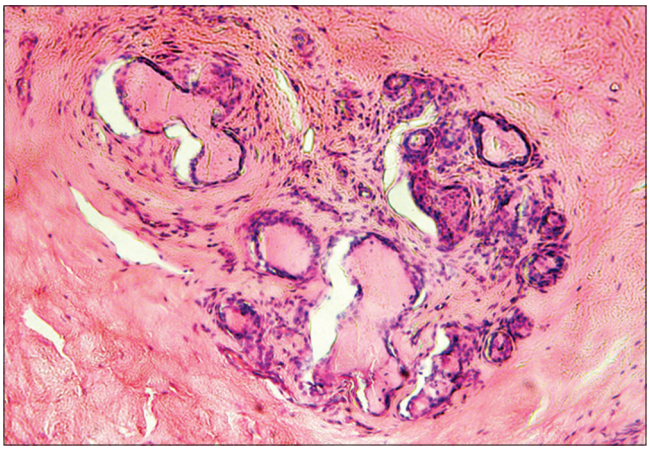

Figure 4: Diabetic parotid gland showing, complete degeneration of gland lobule (H and E ×200)

Immunostaining of PCNA in actin-positive cells (MECs) revealed few scattered reactions embracing some acini and small ducts. However, there were negative reactions of both markers around both striated and excretory ducts (Figure 5).

Parotid gland of diabetic dogs revealed statistically significant (Tables 1-3) positive actin staining of mild-to-moderate quantity in spindle cells encircling the acini, intralobular and interlobular ducts. Immunostaining expression of PCNA in the observed MECs revealed mild-to-moderate positive reaction more concentrated in the cells surrounding both acini and intercalated ducts (Figures 6 and 7). On the other hand, both atrophic and persisted acini as well as duct system including larger one showed mild reaction of PCNA staining diffused throughout the gland parenchyma.

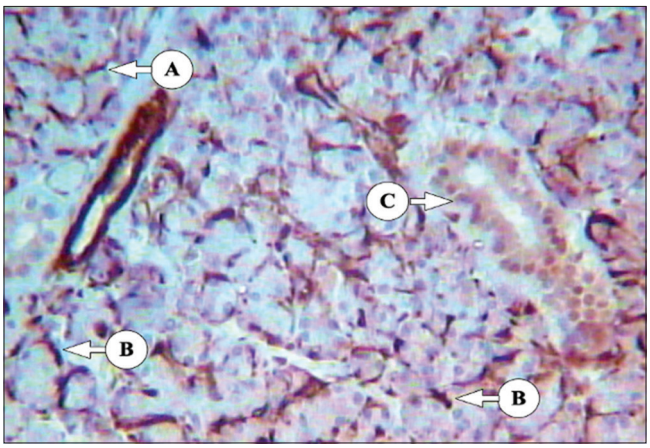

Figure 5: Double expression of both actin and proliferating cell nuclear antigen of control group, actin-positive myoepithelial cells (MECs) of acini (A), weak double-positive MECs of acini (B), and negative expression in ducts (C) (×400)

Discussion

Parotid gland atrophy can be considered either physiological (age change), pathological caused by several diseases such as DM, or induced in laboratory experiments by duct ligation [33]. DM is one of the most common diseases affecting humans and leads to pathological changes in the salivary glands, and scientific researchers are still raising questions about these changes. Based on the present study, diabetes provokes structural changes in the parotid gland characterized by a decrease in parenchymal elements accompanied by an increase in the amount of fibrous tissue stroma, this leading to insufficiencies in tissue maintenance and regeneration processes. Caldeira *et al*. (2005) noted that the morphological changes in salivary glands were detected in both controlled and uncontrolled diabetes [14]. The reduction of some acinar cells can be considered as a sophisticated defense mechanism that protects the acinar cells during the period of potential damage so that the acinar cells return to a resting state with reduced secretory capacity until the pathological attack subsides. The presence of a marked number of viable diminished acini may indicate that the gland still has the ability to secrete, but to a lesser extent. Mata *et al*. (2004) reported that the persistent acini found in the gland tissue have been suggested to be involved in its ability to regenerate [34]. The present investigation showed a significant increase in the mucous acini than was observed in the control group, which may be due to the resistance of the mucous acini to any atrophic changes, indicating that there are clear differences in nature of the changes of the different parenchymal elements of the parotid gland. Many authors pointed that the predominated mucous cells are related to the increased proliferative activity over the serous one [29], [30]. The results of our study were unable to make any attempt to distinguish between duct-like structures, new ducts, are they normal structures or pathological interaction. However, Takahashi *et al*. (2004) noted that duct-like structures appear to be increased by the proliferative activity of duct system in the way of regeneration [23]. In reverse to the atrophic changes of the parenchymal elements, the connective tissue stroma reacts by a proliferative activity, which illustrate the differences of tissue reaction of both epithelial and connective tissues. Furthermore, inflammatory changes within connective tissue have been recorded by Dawson *et al*. (2006), this in a contrast with the absence of any inflammatory cells in our study, which may be related to the elongated time elapsed after induction of diabetes [35].

Dual immunohistochemical staining is an advanced technique that can be used to assess the presentation of two different antigens in the same cell within a tissue, the first is to confirm the existence of the cell, and the second is to confirm; how many cells are in the proliferation phase? Examination of the control parotid gland using double immunostaining of actin and PCNA in our study revealed little positive reactivity for actin in both serous acini and intralobular ducts of about <2% with extremely low or absent proliferative activity.

Table 1: Distribution of double immunostaining actin and PCNA in the different groups

Animals	Actin-positive staining		PCNA staining in actin-positive cells	
	Control group	Control group	Control group	Diabetic group
1	0	3	-	2
2	1	2	0	3
3	1	2	1	1
4	0	1	-	1
5	1	2	1	3
6	1	1	1	2

PCNA: Proliferating cell nuclear antigen.

Table 2: Group statistics of all groups

Group	n	Mean	Standard deviation	Standard error mean
Actin-positive				
Control	6	0.6667	0.51640	0.21082
Diabetic	6	1.8333	0.75277	0.30732
PCNA in Actin-positive				
Control	4	0.7500	0.50000	0.25000
Diabetic	6	2.0000	0.89443	0.36515

n: number of animals, PCNA: Proliferating cell nuclear antigen.

Table 3: Independent samples t-test for immunohistochemical expression of both actin and PCNA in actin-positive cells of all groups

Labeling of actin and PCNA positive cells	Levene's test for equality of variances		t-test for equality of means						
	F	Sig.	t	df	Sig. (2-tailed)	Mean difference	Standard error difference	95% confidence interval of the difference	
								Lower	Upper
Actin-positive cells									
Equal variances assumed	0.328	0.580	−3.130	10	0.011	−1.16667	0.37268	−1.99704	−0.33629
Equal variances not assumed			−3.130	8.853	0.012	−1.16667	0.37268	−2.01187	−0.32147
PCNA in actin-positive cells									
Equal variances assumed	1.074	0.330	−2.513	8	0.036	−1.25000	0.49739	−2.39698	−0.10302
Equal variances not assumed			−2.825	7.895	0.023	−1.25000	0.44253	−2.27285	−0.22715

F: f-test of variances, Sig: significances, t: t-test distribution, df: Degrees of freedom, PCNA: Proliferating cell nuclear antigen.

These results confirmed that MECs encapsulate a small proportion of both serous acini and intercalating ducts, as they require no further pressure to draw aqueous saliva into the duct system. These findings paralleled the concern of Alali and Kochaji (2018) which reported that the proliferative activity of MECs in normal salivary glands is approximately 1.6% [19]. Many authors report that there is a small percentage of proliferative activity of the cells of myoepithelium in normal salivary glands and interpret this as all salivary gland cells are capable to proliferate under certain conditions to maintain the gland in a stable state by regeneration of both acini and intralobular duct [6], [36], [37]. Redman (1994) also reported that MECs in the parotid gland are evident around both intercalating ducts and acini from postnatal days 1–14, but those surrounding the acini are usually lost by day 18 [24].

Examination of parotid gland in diabetic group revealed statistically significant actin-positive spindle cells with many short processes embracing several acini and small ducts. This finding suggests that MECs in of diabetic dogs have a high resistance to atrophy. The previous studies indicated the presence of atrophic acini completely covered by MECs that were observed to undergo proliferative changes [38]. These actin-positive cells in our study revealed significant proliferative activity in the form of brownish PCNA staining reaction which may have a high risk for initiation of salivary gland tumor. This result comes in agreement with Kodama *et al*. (2010) who stated that hyperglycemia in diabetic patient's increase the risk of developing neoplasms as insulin is a growth factor with mitogenic effects [21]. On the contrary, other literature have indicated that the MECs have been implicated in inhibiting the development and progression of salivary gland tumors [39]. Specifically, MECs accelerate the differentiation of epithelial cells and participate in the formation of basement membrane by means of a paracrine effect. They are also known to suppress tumor invasion by secreting serine protease inhibitors and matrix metalloproteinase inhibitors. Finally, there are still opinions about whether this proliferative growth of MECs in diabetic parotid glands is the beginning of cancerous pathogenesis or part of the gland's rehabilitation against atrophy.

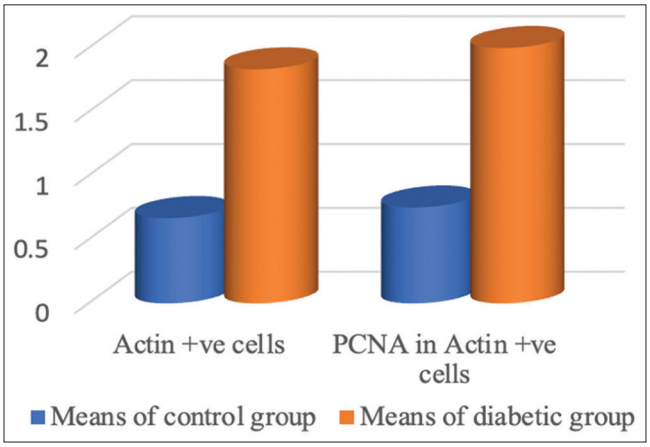

Figure 7: Chart of the mean values of both control and diabetic group

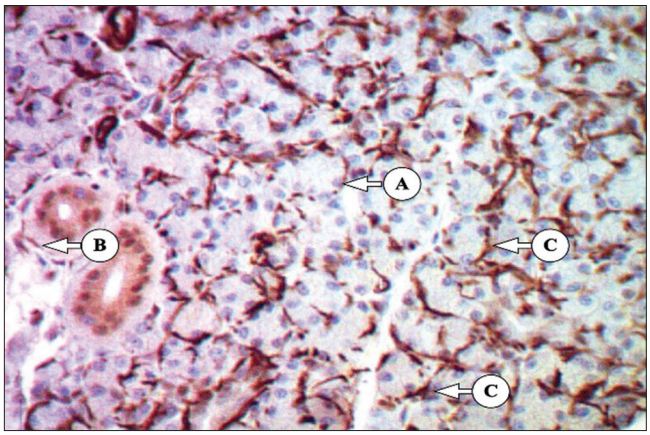

Figure 6: Double expression of both actin and proliferating cell nuclear antigen of control group, actin-positive myoepithelial cells of acini (A), and ducts (b), moderate double-positive myoepithelial cells of acini (C) ×400

Conclusion

Routine histological findings of diabetic dogs showed abundant pathological changes in parenchymal tissue elements, including acinar, ductal, and MECs that had a significant impact on saliva production and secretion resulting in dry mouth.

Ethical Approval

Dogs were kept in an animal health-care facility under the supervision of the local ethical committee

in a laboratory animal colony, Faculty of Veterinary Medicine, Cairo University, Egypt.

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