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Expression of cytokeratin 17 in normal and diabetic submandibular salivary gland (histological and immunohistochemical study)

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Background
Diabetes mellitus is a generalized notorious chronic disease characterized by hyperglycemia. Salivary glands play an important role in the oral health maintenance by production of saliva. One of the most common diseases that compromise salivary gland function is diabetes mellitus leading to xerostomia.

Aim
The aim of this study was to determine the histological findings and immunohistochemical expression of cytokeratin 17 (CK17) in normal and diabetic submandibular gland to give an explication about the pathological effects of diabetes mellitus on the intracellular structures of the gland parenchyma.

Materials and methods
Twelve healthy male albino rats were utilized in the experiment and divided into two equal groups (normal and diabetic). Forty-five days postinduction of diabetes, submandibular gland was dissected out and prepared for both histological and immunohistochemical studies.

Results
Histological results confirmed that, submandibular gland of diabetic rats undergoes rapid, progressive and severe atrophy of the parenchymal elements accompanied by increases in the fibrous component and presence of fatty degeneration. The atrophied gland was characterized by loss of the gland architecture with the presence of degenerated acini, dilated duct system and formation of duct-like structure. Expression of CK17 using immunoperoxidase technique revealed a mild to moderate cytoplasmic expression in all duct cells and some acinar cells as well as the granular convoluted tubules of normal gland either diffuses or concentrated lateral and basal to the nucleus compared to strong expression of diabetic glands that concentrated at apical part of cells.

Conclusion
The intensity and diffusion of CK17 expression in our results foretells the pathological influence of diabetes mellitus to the intracellular filaments of salivary gland parenchyma that interfered with production and/or secretion of saliva leading to xerostomia.

Keywords:
cytokeratin 17, diabetes mellitus, submandibular gland

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Introduction

Diabetes mellitus is a generalized chronic disease characterized by hyperglycemia with abnormalities in carbohydrate metabolism resulting from insulin insufficiency in action and/or secretion [1]. Diabetes mellitus is a widespread disease that is associated with high morbidity [2]. The complications of hyperglycemia affecting the body organs including neuropathy, nephropathy, retinopathy, cardiomyopathy, microangiopathy, atherosclerosis, and foot ulcers [3]. Because of the high incidence of diabetes mellitus in humans, induction of diabetes in animal models has been occurred to study this disease. Single injection of streptozotocin or alloxan to an animal produce low insulin plasma level causing insulin-dependent diabetic syndrome [4].

Salivary glands play an important role in the oral health maintenance [5,6]. Saliva have many functions, moisten the oral mucous membrane, facilitate speech [7,8] and also secretes antimicrobial agents as IgA, lysozymes, and lactoferrin [9]. Submandibular gland is a mixed major gland that plays the largest role in saliva production, secreting 60% of the total salivary output [10]. One of the most common diseases that compromises the salivary glands is diabetes mellitus which, altering their structure leading to hyposalivation [11,12]. Salivary gland hypofunction is a major problem leading to severe adverse oral health outcome including difficulties in swallowing [13,14], inability to eat, taste disorder [15], dental caries, and periodontal diseases [16,17]. Generally, diabetic animals have demonstrated many histological salivary

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gland disorders including reduction in the acinar volume, growth retardation, and weight loss of both parotid and submandibular glands [18].

Cytokeratin intermediate filaments are a family of related proteins coded by different genes and expressed in various epithelial cells [19]. Cytokeratin constitute an important biological marker as they are stable, relatively resist degradation, formalin fixed and paraffin embedded. Also, cytokeratin intermediate filaments showing great fidelity of expression and are very antigenic [20].

Distribution of cytokeratin 17 (CK17) of parotid gland are related to intercalated, striated, and excretory duct cells, while acinar cells have little or no cytokeratin in their cytoplasm [21,22].

Our study aimed to determine the immunohistochemcal effect of diabetes mellitus to the intracellular structure of submandibular gland acini to provide more information about salivary gland pathology of diabetes mellitus.

Materials and methods

Grouping

Twenty-four healthy mature male albino rats were utilized in this work. Their age was about 5 months and their body weight were ranged from 160 to 180 g. Rats were housed in polycarbonate cages (four rats/cage) under 8–16 dark–light cycles. A mixture of hard and soft foods was given *ad libitum* with unrestricted access to water. Rats were maintained in animal health care facility under the local ethical committee at Laboratory Animals Colony, Faculty of Veterinary Medicine, Cairo University, Egypt. Rats were divided into two equal groups: control group I and diabetic group II.

Induction of diabetes mellitus

Rats of group II (12 h fasted before) were intraperitoneal injected by single dose of 150 mg/kg body weight of alloxan tetrahydrate (Sigma Chemical Co., St Louis, Missouri, USA) according to Jelodar *et al*. [23] dissolved in physiologic saline (0.9% NaCl) for induction of diabetes mellitus by the known cytotoxic action on ß cells of islets of Langerhans [24]. Ten days thereafter, animals presented a glucose level at or above 200 mg/dl were included in the diabetic group of the experiment in addition to the observation of polydipsia, polyuria, and polyphagia. On the other hand, blood samples were collected from both groups at three intervals per 15 days to ensure the diabetic free group I and the persistence of diabetes mellitus in group II. The diabetic rats did not maintain under neither dietary nor pharmacological regimen and feeds as the control animals.

Histological tissue preparation

At 45th day post to the induction of diabetes, the rats of both groups were anesthetized with diethyl ether to be sacrificed and the salivary gland complex of each animal was extracted and fixed in Bouin's fixative for 3 days. The fixed tissues were washed then dehydrated in ascending grades of alcohol, cleared in xylol and infiltrated by molten paraffin wax for block building. Serial tissue sections of 4 μm thickness were mounted on glass slide to be stained by hematoxylin and eosin for routine histological examination.

Immunohistochemistry

Tissue section for immunohistochemistry must be mounted on poly-L lysine coated slide recommended for staining procedures requiring target retrieval solution. Paraffin sections (4 μm thick) were immersed in 0.3% H_2O_2/methanol for 30 min to prevent endogenous peroxidase activity, rinsed with phosphate-buffered saline. Sections were incubated with monoclonal anti-CK17 E3 antibody (Sigma Chemical Co.) using labeled streptavidin biotin (Sigma Chemical Co.) method and hematoxylin counter stain. The positive staining reaction appeared in the form of brownish staining which reflect the intracellular distribution of intermediate filaments CK17 within the tissue compartments. The intensity of the staining was assessed semiquantitatively and scored as follow: negative staining (0), trace or weak (1), mild (2), moderate (3), and strong staining (4).

Statistical analysis

Data were collected, revised, coded and entered to the statistical package for social science (IBM SPSS), version 23 (IBM Inc., Chicago, Illinois, USA). The quantitative data were presented as mean, SD, and ranges when their distribution found parametric by Kolmogorov–Smirnov test of normality. The comparison between two independent groups with quantitative data and parametric distribution was done by using independent *t* test while the comparison between two paired groups regarding quantitative data with parametric distribution was done by using paired *t* test. The confidence interval was set to 95% and the margin of error accepted was set to 5%. So, the *P* value was considered significant at the level of less than or equal to 0.05.

Results

Histopathological examination

Histological examination of submandibular gland of control slides using hematoxylin and eosin staining revealed a closely packed in both serous and mucous acini arranged in lobular pattern. The lobules contain both intercalated, striated ducts, and granular convoluted tubules (Fig. 1).

During surgery, there is a great reduction in salivary gland size of diabetic group, in which it become difficult to dissect out from the surrounding tissue. The glandular element of diabetic group showed severe atrophic changes characterized by reduction in the parenchymal elements accompanied by increase in the quantity of the fibrous tissue stroma. The atrophied acini showed decrease in the acinar cell size with loss of normal gland architecture and consists of a collection of smaller cells with invisible lumen. In many fields, the acini were replaced by fat cells rather than fibrous one (Figs. 2 and 3). There was an increase in the mucous secreting cells among the persisted serous acini. The common finding of many specimens of diabetic group is the presence of duct-like structure with dilated lumen and flat cells.

Immunohistochemical examination

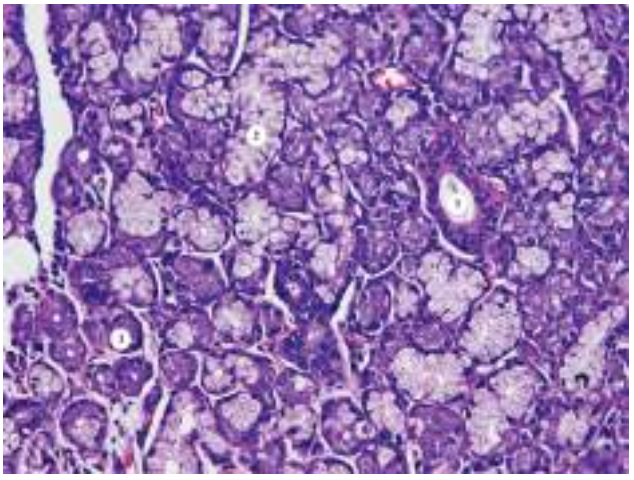
Immunohistochemical finding of the intensity of CK17 at both acinar and ductal cells was done to a serial section (1, 11, 21, 31) for each animal through two different members in a blind manner, the results were collected together for statistical analysis (Tables 1 and 2).

Examination of submandibular gland of control sections incubated with anti-cytokeratin E3 antibody for CK17, using immune-peroxidase technique (color was developed by DAB chromogen) revealed that, all intercalated, striated duct, and granular convoluted

Table 1 Average of cytokeratin intensity upon groups

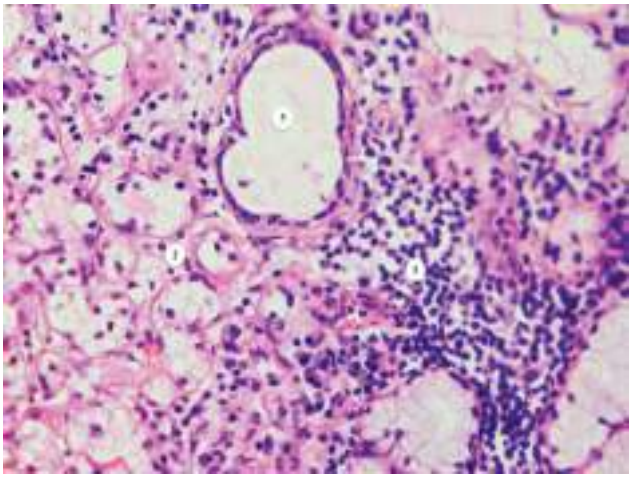
Rats	Control group	Diabetic group	Control group	Diabetic group
	Acinar cells		Duct cells	
1	0.50	2.00	1.25	2.75
2	0.75	2.25	1.50	3
3	1.25	2.75	1.25	2.75
4	1	1.5	1.50	3.25
5	0.75	1.75	1.00	2.75
6	1.25	2.00	1.25	2.5
7	1.75	2.25	1.50	3
8	1.25	2.75	1.25	2.75
9	1	1.5	1.50	3.25
10	0.75	1.75	1.00	2.75
11	0.75	2.00	1.25	2.5
12	1	1.5	1.00	2.75

Figure 1



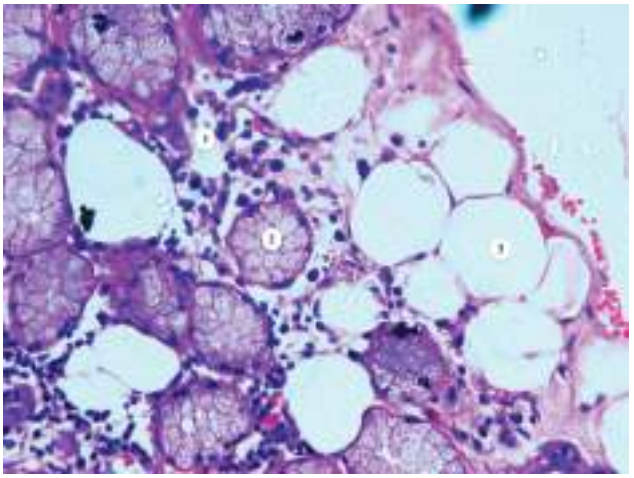
Control submandibular gland: serous acini 1, mucous acini 2, and striated duct 3. Hematoxylin and eosin, ×100.

Figure 2



Diabetic submandibular gland: loss of gland architecture 1, degenerated acini 2, and duct-like structure 3. Hematoxylin and eosin, ×200.

Figure 3



Diabetic submandibular gland: loss of gland architecture 1, decrease in the acing size 2, fat spaces 3. Hematoxylin and eosin, ×200.

tubular cells displayed weak to mild cytoplasmic expression of CK17 in a diffuse pattern (Fig. 4). In many sections, both intercalated and striated ducts showed weak expression at the apical part of cells with moderate expression at the basal part. Also, some serous acinar cells showed weak to mild expression of CK17, whereas, the mucous acini were negatively stained.

All duct cells of diabetic rat displayed moderate to strong cytoplasmic expression of CK17 of two patterns of expression. The first predominating one, the expression of cytokeratin was uniform and diffuses (Fig. 5).

In the second pattern, the duct cells displayed strong expression at their apical (luminal) part of duct cells with mild to moderate expression at their basal part. Many serous acinar cells displayed moderate to strong diffuse cytokeratin expression which appeared more prevalent than that found in control group. On the other hand, granular convoluted tubular cells showed mild to moderate staining reaction of diffused pattern (Fig. 6).

Statistical analysis revealed that there was a significant positive statistical correlation of the intensity of CK17 expression in both acinar and duct cells between both diabetic and control groups ($P < 0.05$) (Tables 3 and 4).

Discussion

In general, damage to submandibular glands is a well-known sequela of diabetes mellitus in both human and experimental animals. The results of the present study reported that, diabetes provoke structural alterations ranged from reduction in the acinar size to severe gland atrophy which replaced by either fibrous or fatty tissues with proliferation of duct-like structures. This result explains the occurrence of xerostomia with failure of performing the secretory activity. The fibrous tissue replacing the degenerated gland components appeared very extensive proposing the permanent changes with inability of the glands to regenerate at latter time. In many diabetic specimens, the presence of a number of both normal and shrinked acini suggested that the gland still to perform their secretory ability but with minimal degree. Moreover, Mata *et al.* [11] reported that the persistent acini found in the gland tissue have been suggested to participate in

Table 2 Comparison between control group and diabetic group regarding cytokeratin intensity in acinar cells

Acinar cells	Control group (*n*=12)	Diabetic group (*n*=12)	Test value	*P* value	Significance
Mean±SD	1.00±0.34	2.00±0.44	6.254	<0.001*	HS
Range	0.5-1.75	1.5-2.75			

HS, highly significant.*Significant at *P* value less than or equal to 0.05.

Table 3 Comparison between control group and diabetic group regarding cytokeratin intensity in duct cells

Duct cells	Control group (*n*=12)	Diabetic group (*n*=12)	Test value	*P* value	Significance
Mean±SD	1.27±0.20	2.83±0.25	17.12	<0.001*	HS
Range	1-1.5	2.5-3.25			

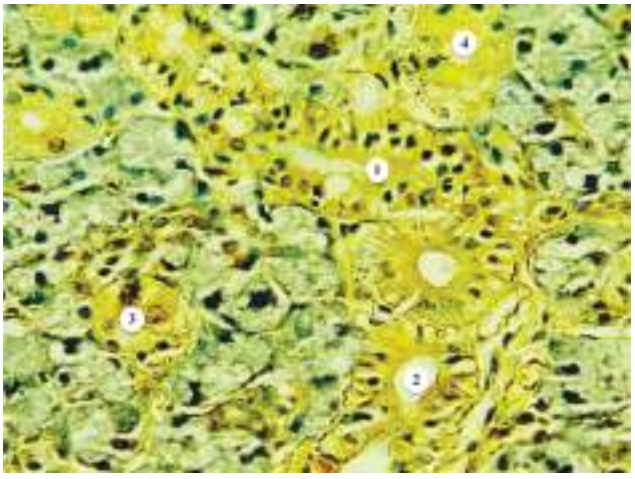
HS, highly significant.*Significant at *P* value less than or equal to 0.05.

Table 4 Comparison between acinar cells and duct cells regarding cytokeratin intensity in diabetic rats

Diabetic group	Acinar cells (*n*=12)	Duct cells (*n*=12)	Paired *t* test		
			t	*P* value	Significance
Mean±SD	2.00±0.44	2.83±0.25	5.087	<0.001*	HS
Range	1.5-2.75	2.5-3.25			

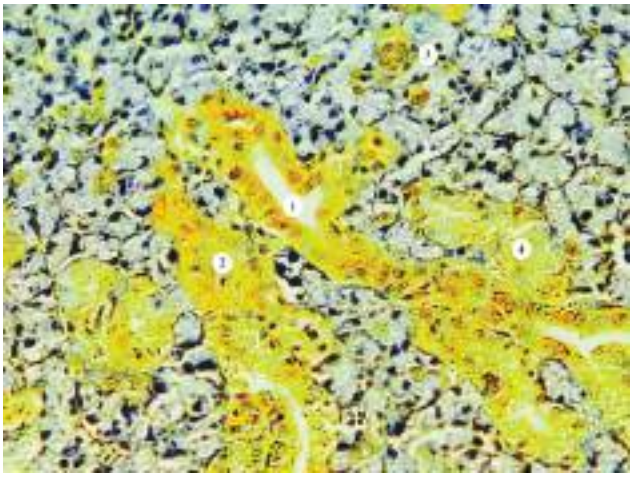
HS, highly significant.*Significant at $P \leq 0.05$.

Figure 4



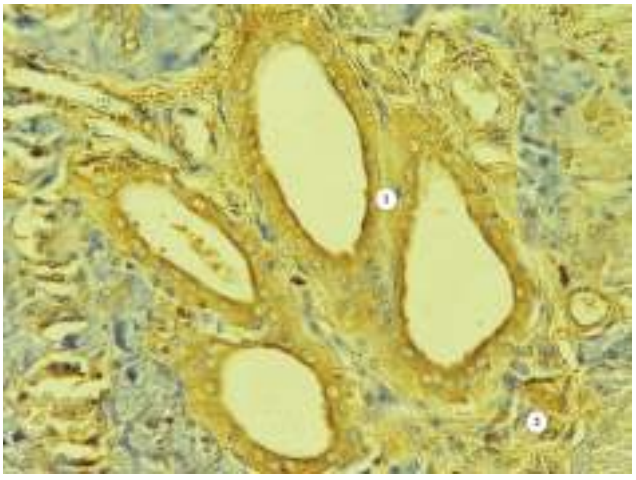
Expression of CK17 in control group: mild in striated duct 1, intercalated duct 2, and serous acini 3, weak in GCT 4. ×200. CK17, cytokeratin 17; GCT, granular convoluted tubular cells.

Figure 5



Expression of CK17 in diabetic group: moderate in striated 1, and intercalated ducts 2, and mild in serous acini 3, and GCT 4. ×200. CK17, cytokeratin 17; GCT, granular convoluted tubular cells.

Figure 6



Expression of CK17 in diabetic submandibular gland: strong in striated duct 1, and moderate in serous acini 2. ×400. CK17, cytokeratin 17.

the ability of the gland to regenerate. The results of our study are unable to make any attempt to distinguish between duct-like structures and pre-existing one that may be present in the diabetic group. This finding was supported by Takahshi *et al.* [25], which reported that duct-like structures seem to be increased due to the proliferative activity of duct system cells. 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.

Expression of CK17 in duct system of control submandibular gland not only existing in larger duct but also in the smallest one. This observation might support the accuracy of this technique for detection of the intermediate filament. The staining pattern of both acinar and duct cells was either diffuses or concentrated lateral and basal to the nucleus. These patterns that explain the distribution of cytokeratin within the cells thought to be related to the functional activity of the gland in which the diffuse pattern referred to the resting phase, whereas, the basal concentration of cytokeratin to the active secretory state leaving the area for exocytosis. According to the statistical data in the present study, both serous and mucous acinar cells of control sections revealed an expression of CK17 ranged from weak to mild staining while expression increases in the duct system. The stronger staining reaction of duct system upon acini indicated that the acini is the most differentiated components of the gland in which the expression occurred more in the early branching system. Diabetic submandibular gland displayed an intensive expression of CK17 in all gland parenchymal cells (duct and acini) ranged from moderate to strong. Statistical results indicate that the relationship between expression of cytokeratin in duct and acini of diabetic group increased significantly than that in the control group but with the persistence of maintaining the difference of expression ratio (Tables 1–3 and diagram 1). These results indicated that, diabetes affect both duct and acini with the same manner. The diffuse pattern of cytokeratin expression might indicate the damaging effect which interfere with the functional activity of the gland. On the other hand, it thought to be that the diffuse distribution pattern of CK17 represent an early stage of gland damage, whereas the luminal pattern was the advanced one.

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Conflicts of interest
There are no conflicts of interest.

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