Morphometric, Histological and Immunohistochemical Study of Tongue Epithelium in Diabetic Rats

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Abstract: Background: Diabetes Mellitus (DM) is one of the most common endocrine metabolic disorders in the world. It is associated with several tissue changes which result in many systemic and oral complications. **Aim of study**: Investigate the tissue alterations as a result of hyperglycemia on rats tongue epithelium. **Material and Methods**: Twelve, adult male Sprague–Dawley rats were allocated equally into two groups: **Group I** (Control) and **Group II** (DM). Diabetes was induced by a single intraperitoneal injection of aqueous alloxan monohydrate (ALX) After 6 weeks, animals were euthanized, and samples were processed for routine histological Hematoxylin and Eosin (H & E) and immunohistochemical staining for P53 and Ki-67. Student t-test was used to compare P53 and Ki-67 expressions between groups. **Results**: Morphometric comparison revealed significant reduction in tongue epithelium height in diabetic rats. Histologically, tongue mucosa in diabetic rats showed abnormal epithelial morphology with atypical epithelial ridges. Epithelial cells showed signs of degeneration such as pyknotic nuclei, karyolysis, binucleated cells, Karyorrhexis and hyperchromatic nuclei. Immunohistochemical studies showed significant reduction in Ki-67 expression with a significant increase in P53 expression in diabetic group when compared with the control one. **Conclusion:** DM affects the oral epithelium in a way that affects its integrity and accordingly makes it more vulnerable to variable disease and infections.
[Mona A. Abd-Elmotelb. **Morphometric, Histological and Immunohistochemical Study of Tongue Epithelium in Diabetic Rats.** *Life Sci J* 2018;15(11):1-6]. ISSN: 1097-8135 (Print) / ISSN: 2372-613X (Online). http://www.lifesciencesite.com. 1. doi:10.7537/marslsj151118.01.

Key words: Diabetes mellitus, Tongue, Oral epithelium, Ki-67, P53

1. Introduction

The oral cavity is lined everywhere with stratified squamous epithelium. In areas subject to mechanical forces associated with mastication (i.e., the gingiva and hard palate) there is a keratinizing epithelium. Whereas, in the floor of the mouth and buccal regions which require flexibility to accommodate chewing, speech are covered with a nonkeratinizing epithelium. The dorsum of the tongue is covered by a specialized epithelium, which can be represented as a mosaic of keratinized and nonkeratinized epithelium.

The primary function of oral mucosa, including the epithelium and the underlying connective tissue, is to protect the underlying structures [1]. Changes indicative of disease are seen as alterations in the oral mucosa lining the mouth, which can reveal systemic conditions, such vitamin deficiency or diabetes DM represents a metabolic syndrome that have hyperglycemia as a common feature. Which is the result of poor glucose use because of insufficient insulin secretion and hepatic gluconeogenesis [2, 3]. There are three types in DM: Type I diabetes (Insulin dependent) is due to immune mediated beta-cells destruction, leading to insulin deficiency. If there was no known etiologies, Type I is known then as Idiopathic diabetes and is strongly inherited. Type II diabetes (Non-Insulin dependent) is due to insulin secretory defect and insulin resistance. And the last type is gestational diabetes mellitus is any form of intolerance to glucose with onset or first recognition of pregnancy.

Hyperglycemia has profound and severe effect on different body systems as well as oral tissues. The main complications associated with DM are retinopathy, neuropathy, nephropathy, and macro/ microangiopathy. An association between DM and alterations in the oral mucosa has been observed in experimental studies and clinical cases. DM damages tissue repair processes and causes dysfunction of oral mucosa which in turn my compromise tissue function to favor the occurrence of oral infections and oral metaplasia [4]. Several studies suggest a higher prevalence and severity of some pathologies in the oral tissues of patients with DM like gingivitis, periodontitis, dental caries, candidiasis, and other oral manifestations such as alteration of salivary flow and oral burning sensation.

Aim of the Work

Aim of this work is to investigate the morphometric, histological and immunohistochemical discrepancies in diabetic rats tongue epithelium that might be the roots for various diseases.

2. Material and Methods

Animals

Twelve, adult male Sprague–Dawley rats weighting on average 150-200 gm were allocated

equally into two groups: **Group I** (Control) and Group **II** (DM). Animals were housed - at faculty of Medicine-Zagazig University- in individual cages and received a standard diet for rodents and tap water *ad libitum*. Room temperature and humidity were maintained at 23° C and 60%, respectively. All animal experiments were carried out in accordance with the guidelines of the National Institutes of Health (NIH) for the care and use of laboratory animals (NIH Publication, Number 85-23, Revised 1985).

Induction of diabetes

DM was induced by a single intraperitoneal injection of aqueous ALX (120 mg/kg for body weight, Sigma–Aldrich Co, Germany) according to defined methods by Halici *et al*. [5]. ALX was dissolved in 0.9% NaCl solution, freshly prepared, and injected intraperitoneally to rats that were fasted for one night. After ALX application, the pancreas secretes insulin at high levels. As a consequence, fatal hypoglycemia can occur. To prevent this adverse effect, 5 ml 20% glucose solution were injected intraperitoneally 4–6 h after ALX treatment. We then added a 5% glucose solution to the rats' drinking water for 24 h to prevent possible hypoglycemia. After 72 h, fasting blood samples were collected via the tail vein. The samples were then used to measure blood glucose levels using the Accu-Check Active blood glucose monitor (Accu-Chek Active, Roche, Shanghai, China). A rat was accepted as diabetic with at least 200 mg/dl of serum glucose level.

Animal euthanasia and samples collections

At 6 weeks, after the confirmation of hyperglycemic conditions, the experiment was ended. Treatment and food were stopped 12 hours before sacrificing the rats. Animals were then anesthetized with ketamine at a dose of 50 mg/kg body weight and samples of parotid salivary glands were collected and fixed with 4% buffered formalin solution. The animals were then scarified with overdose of anaesthetic, according to the ethical guidelines, confirmed with cervical dislocation.

Tissue preparation

Hematoxylin and Eosin staining and epithelial measurement

After fixation, the specimens were dehydrated by a graded ethanol series. Tongue samples were cut into two halves each and were embedded in paraffin. 4µ sections were cut by rotary microtome and representative sections were stained with H & E for conventional histological assessment using light microscope (Leica ICC50 HD). Epithelium thickness was determined as the mean height of four regions in each mouse and was measured in (mm) using Photoshop CS4 software with a scale bar 110mm=200µm.

Immunohistochemistry

Avidin–Biotin–Complex (ABC) method was used for immunohistochemical labeling. Representative sections were deparaffinized in xylene and re-hydrated through a descending series of ethanol concentrations. The sections were washed with TBS (20 mM Tris- HCl, 150 mM NaCl, pH 7.4). Then they were incubated in 0.3% H2O2 in dH2O at room temperature (30 min) to inhibit endogenous peroxidase. Antigen retrieval was performed according to the manufacture instructions. For increasing permeability, slides were placed in 0.1-0.5% triton X100 in PBS for (10-20 min). After that, slides were placed in 100 µl blocking solution (Abcam), for 30 minutes at room temperature. Then incubated with two ThermoFisher, USA primary antibodies; (p53 Mouse Monoclonal Antibody, Clone: PAb 122, Cat. No: MA5-12453, at dilution 1:80)/ Ki-67 Rabbit Monoclonal Antibody, Clone: SP6, Cat. No: MA5-14520 at dilution 1:200) at 4ºC overnight. Sections were washed in 1X Phosphate buffered saline (PBS) and then incubated with the appropriate secondary antibody (Ready to use HRP EnVision Kit, DAKO Co.) 20 min at room temperature in a humidified chamber. For peroxidase visualization; color reaction was developed by adding DAB solution (0.5 mg/ml DAB and 0.1% H2O) onto the sections. When color reaction was acceptable, it was stopped by rinsing with H2O for 5-10 minutes, and then sections were counterstained with hematoxylin for 2 minutes. Sections were gradually dehydrated and mounted with coverslips. Immunohistochemical staining photos and data were obtained by using Full HD microscopic camera operated by Leica application module for tissue sections analysis (Leica Microsystems GmbH, Wetzlar, Germany).

Statistical Analysis

Expression areas percentage of P53 and Ki-67 were obtained from 5 random non-overlapping fields per tissue section. For statistical analysis, measurement data were presented as mean ± standard deviation. Statistical analyses were performed using unpaired student t-test. Values of $P < 0.05$ indicated a statistically significant difference. Prism Pad Graph (V 6.01) was used for data analysis.

3. Results

Morphometric and Histological Results

Measurements showed that, epithelial thickness in diabetic group was significantly thinner (107.3 ± 1.315) than the control one (118.8 ± 3.146), (P value =0.0150). (Figure 1).

Histologically, the tongue dorsal surface in the control group, was normal. It showed numerous, thin and long finger- like projections of filiform papillae covered by a keratinized epithelium; having the typical form of the basal, spinous, granular and cornified

epithelial cells beside the normal shape of epithelial ridges (Figure 1, A and A'').

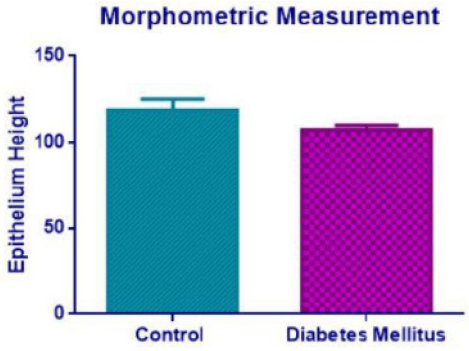

Figure 1: **Shows rat dorsal tongue epithelium height:** Statistical analysis using student unpaired t-test shows significant difference between the control group epithelium height and the diabetic one with a P value = 0.0150. Error bar=Standard deviation.

Whereas in the diabetic rats, aberrant epithelial cells morphology with atypical epithelial ridges were noticed in diabetic group (Figure 1, B). With higher magnification, signs of degeneration were evident. Cells showed Pyknotic nuclei (Black arrows), karyolysis (white arrows), binucleated cells (Red arrowhead) Karyorrhexis (white arrowheads) and hyperchromatic nuclei (black arrowheads) (Figure 1, B'').

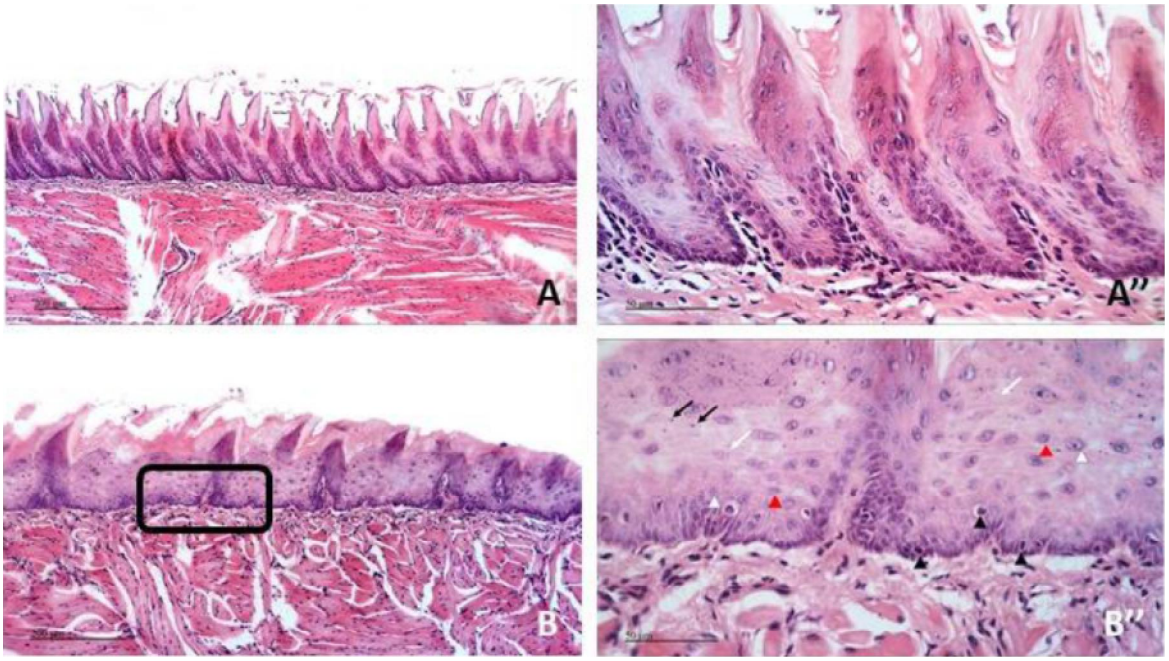

Figure 2: H & E stained sections showing rat dorsal tongue epithelium: (A and A'') Control group tongue mucosa showing numerous, thin and long finger- like projections of filiform papillae covered by a keratinized epithelium; having the typical form of the basal, spinous, granular and cornified epithelial cells. In diabetic group, tongue epithelium shows atypical epithelial ridges **(B)**. **(B'')** Higher magnification of outlined area in (B) shows signs of degeneration as Pyknotic nuclei (Black arrows), karyolysis (white arrows), Karyorrhexis (white arrowheads). In addition to binucleated cells (Red arrowhead) and hyperchromatic nuclei (black arrowheads). Scale bar: A and B= 200µm, A'' and B''= 50µm.

Immunohistochemical Results

P53 expression increased in the diabetic group when compared to the control one. In normal rats, P53 expression was limited to the basal and para basal cell layers (Figure 3, A). However, in diabetic group, P53 expression increased in intensity and extended to include more cells in the prickle and spinous layers in addition to the basal and para basal ones (Figure 3, B).

On the other hand, Ki-67 immunoreactivity decreased with diabetes. Normal rats showed ki-67 expression mainly in basal, para-basal layers with few prickle and spinous cells (Figure 3, C). In the diabetic group, even though same layers were involved,

expression was less intense and limited to fewer cells (Figure 3, D).

In both, P53 and Ki-67 immunoreactivity, there was a significant difference between the control and diabetic groups (Figure 4).

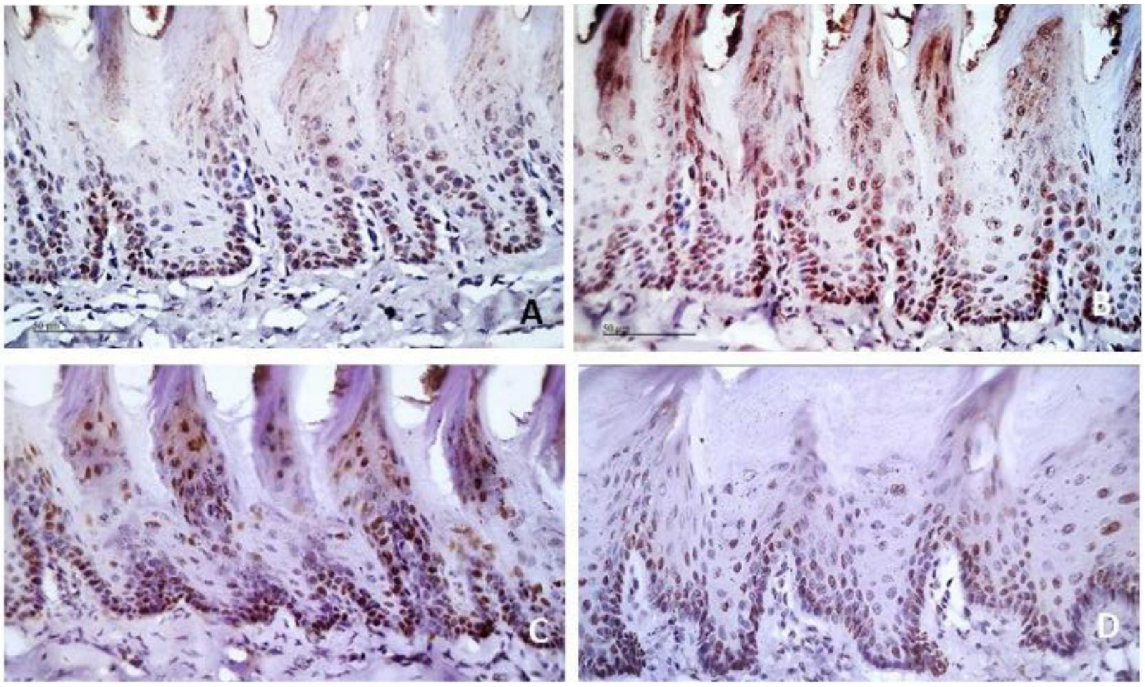

Figure 3: Immunodetection of P53 and Ki67 in rat tongue dorsal epithelium. (A) Shows p53 immunoreactivity in tongue epithelium of the control group. Its expression is limited to the basal and parabasal cell layers. **(B)** Diabetic group shows higher p53 expression in some prickle and spinous cells in addition to the basal and parabasal cell layers. **(C)** Demonstrates Ki-67 expression in the control group. It is expressed mainly in basal and para basal cell layers. Yet, few prickle and spinous cells are positively stained. **(D)** Ki-67 expression is less in intensity and is shown in the same layers as in the control group. However, fewer cells are expressing it. Scale bar=50µm.

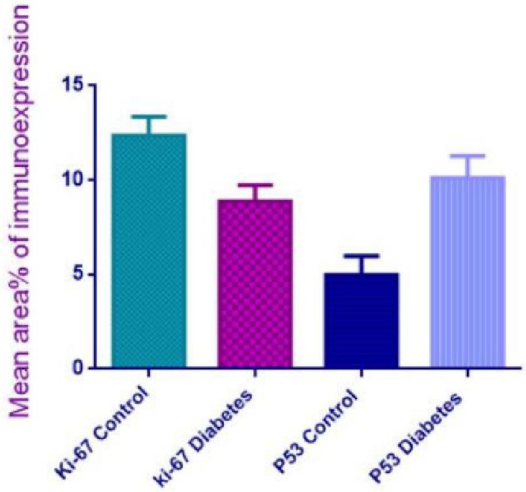

Figure 4: Ki-67 and P53 area % of immunoexpression in rats tongue epithelium: Statistical analysis using Student t-test revealed a significant difference between the control and diabetic groups with each protein ($p<0.0001$), Error bar=Standard deviation.

4. Discussion

The present study aimed to evaluate the effect of DM on the oral epithelium. This evaluation included the morphological and histological changes in rats tongue dorsal surface epithelium. In addition, the expression of P53 and Ki-67 proteins had been assessed as an indication of proliferation and apoptosis activity.

In the current study, microscopic examination of the diabetic group revealed an epithelium thickness that was significantly thinner than the control one. This finding agrees with Caldeira *et al*., who investigated the morphometry and the ultrastructure of check mucosa epithelium in autoimmune non-obese diabetic (NOD) mice. They found that, epithelium thickness in diabetic autoimmune NOD mice was significantly less than that in control BALB/c and non-diabetic autoimmune NOD mice [6]. Also, in a study of the histological changes in diabetic rats under denture base without mechanical pressure. Shirai *et al*. reported that, in diabetes-non-denture-wearing group, there was slight and gradual decrease in the thickness of the epithelium from 4-20-week stage because of

prickle cells decreasing number [7]. On the other hand, Hamilton and blackwood reported no differences were detected between normal and diabetic animals in respect of either the thickness of the epithelium or the staining prosperities of the tissue after 3-4 weeks of diabetes induction [8]. This reduction in height could the result of decreased epithelial cells proliferation or increased cells degeneration.

Histological findings in this study match with the diabetic status. Epithelium showed peculiar ridges and the cells showed signs of degenerations such as Pyknotic nuclei, karyolysis, binucleated cells, Karyorrhexis and hyperchromatic nuclei. These findings agree with the histological changes in the oral mucosa of diabetic autoimmune NOD mice. Authors faced hard identification of the different cell layers. Also, the basal layer had pleomorphic cells with a marked reduction in cytoplasmic and nuclear volume. In addition, Spinous and granular cells were apparently involuted, and the corneum layer showed minor desquamation [6]. Similarly, cytological smears studies reported the presence of Karyolysis, karorrhexis, hyperchromatic nuclei and binucleated cells in diabetic samples [4, 9, 10]. In contrast, another study could not notice any histopathological evidence for the causative factor when examined the palatal mucosa of diabetic autoimmune NOD mice [7]. Cellular changes found in the current study were like to those found in aging cells. Although not significant, morphological alterations seen in aging as irregular and abnormally lobed nuclei and pleomorphism had been noticed in diabetic patients [9]. This preponderance of old cells could be the result of limited production of young ones since cellular turnover in the epithelium is reduced [10].

The p53 protein is a regulative factor of many processes necessary for the proper functioning of cells, and it corresponds to several processes associated with its life and death. The p53 protein regulates the repair of cellular DNA and induces apoptosis when the damage of the gene is too serious, and it is impossible to repair. It has also been demonstrated that the p53 protein, as a result of stress factors, crosses into the mitochondria and activates the expression of pro-apoptotic genes, such as Puma, Bax, Apaf-1, Noxa, as well as inhibits the expression of anti-apoptotic genes, such as those of the family Bcl-2 (Bcl-2, Bcl-X, Bcl-in, Mcl-1) [11]. In this study P53 protein showed expression in both groups; the control and the diabetic. However, its expression is diabetic group was broader and more intense than the control one. This increase in p53 expression is in agreement with Heidari *et al*., they detected a significant increase in P53 expression in oral mucosal exfoliated cells in diabetic patients compared to the healthy ones [12]. Also, in a study of P53 and c-myc expression in an experimental model of chemically induced carcinogenesis in normal and diabetic rats. They noticed that, despite similar expression pattern, P53 expression in diabetic rats oral mucosa was higher than the normal rats. This was described as a diabetic effect leading to increased accumulation of mutations in the p53 gene, which contribute to greater prevalence of oral lesions. In contrast, Dincer *et al*. showed that, the serum level of p53 was lower in the patients with type 2 diabetes than in controls or in subjects with impaired glucose tolerance [13]. This increase in P53 intensity could be related to increased oxidative stress caused by the diabetes which leads to the upregulation of P53 expression [12]. Also, in a review study done by Kung and Murphy, they concluded that, P53 protein is induced and activated in many cell types in different diabetic models. This activation might suggest a key role for P53 in diabetes as it could affect several genes that are specifically induced in diabetic tissues [13].

Ki-67, is a non-histone nuclear protein and is an important marker for proliferating cells. It can be used as a marker to estimate tissue growth [14]. In this study, diabetic rats showed less Ki-67 protein expression when compared to the control group. Thisfinding agrees with Hamilton and blackwood who reported reduced cell proliferation after almost one month of chemically induced diabetes in rats [6]. Similarly, in two separate studies about the effect of chemically induced diabetes in rats, with/without mechanical pressure on the oral mucosa. They reported reduced rate of cell proliferation [7, 8]. In addition, Akiya *et al*. reported that, BrdU labeling index among the basal cells was reduced in diabetic rats as a result of decreasing the number of S phase cells which explained a possible retardation of their epithelial-tissue activity [15]. In contrast, Açikgoz *et al*., found no significant difference in manner of keratinocyte proliferation between diabetic and healthy group. They added that diabetes did not have an additional effect on the mitotic activity of gingival keratinocytes [16]. Also, Kranti *et al*., observed that Ki-67 staining was found to be negative in gingival biopsy samples from periodontally healthy patients whereas mild staining was noticed in periodontitis and periodontitis with type II DM. However, the difference between these two later groups were not significant which excludes the effect of diabetes on the increased proliferation [17].

Decreased proliferation in this study could be due to sustained hyperglycemia which causes greater accumulation of advanced glycation end products by abnormal glycation of proteins, lipids, and nucleic acids in the walls of large blood vessels as well as in the basement membrane of the microvasculature. The progressive narrowing of the vessel lumen leads to

decreased perfusion of the affected tissue and consequently decreases cell turnover [4, 6, 10].

Conclusion

In conclusion, these structural and, most importantly, molecular alterations detected here clarified the harmful effects of diabetes. These effects alter the oral epithelium homeostasis and hinder its healing and regeneration. This explains why patients with DM showed a higher prevalence of oral lesions. However, as a chronic disease, these affects are expected to progress with time. So, a timed detailed molecular study of the oral epithelium is recommended.

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11/10/2018