Ultra structural evaluation for oral keratinocytes of diabetic albino rats treated with Epidermal Growth Factor

Original Article

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ABSTRACT

Introduction: Diabetes had harmful effects on different tissues of the body, oral mucosa one of them. The purpose of the study was to estimate the effect of epidermal growth factor on oral keratinocytes of diabetic albino rats (Ultrastructure evaluation).
Materials and Methods: Select 18 adult male albino rats which divided into three groups. Group I (control rats group) received no drugs. Rats received only normal saline. (6 rats), while group II (diabetic rats group) received a single dose of alloxan) ALX monohydrate, Sigma Aldrich pharmaceutical company) intraperitoneal injection and without any treatment. (6 rats). Moreover, rats of group III diabetic rats treated with EGF at the dose of 1.25 µg of EGF (sigma)\kg. (Intra peritoneal injection) (Using an insulin injector, a 1.25 microgram/kg dose of EGF containing the saline solution (6 rats) for 2 months. Buccal mucosa was dissected from all rats and processed for transmission electron microscopy evaluation.
Results: Ultra-structural examination by (TEM) of group I showed normal histological features of the epithelium of buccal mucosa that consist of flattened granular cell layer with flat nuclei revealed electron dense keratohyline granules, which covered by cornified (keratin) layer. Ultrastructural examination (TEM) of buccal mucosa specimens of group II (diabetic G) revealed there was atrophy of epithelial cell layers and loss of its normal arrangement. The overlying layer of keratin appeared in wavy and irregular pattern. Ultrastructural examination (TEM) of buccal mucosa specimens of group III showed slight similarity with histological features of the control rats.
Conclusion: The structural alterations detected in this study confirmed the harmful effects of diabetes showed in oral keratinocytes of buccal mucosa. EGF cause relatively improvement of the damaged epithelium.

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Key Words: Albino rats, buccal mucosa, diabetes mellitus, EGF.

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INTRODUCTION

A group of systemic and irreversible chronic metabolic diseases is called diabetes mellitus (DM) (abnormalities of carbohydrate, protein and fat metabolism) which include chronic high level of glucose in blood which result from failures in production of insulin, action of insulin, or a combination of the two[1]. Based on etiology and clinical history, diabetes mellitus can be classified as type 1(the devastation of beta cells of pancreas leading to ultimate or near-absolute defect of insulin). Type 2 (progressive insulin secretory defect arising from insulin resistance); gestational occurs during the pregnancy (second or third trimester); hybrid form (diabetic adults of slowly developing immune-mediated and diabetes of ketosis-prone type 2) as well as other types as monogenic defects in function of β-cell and in action of insulin occasioned by (genetic mutations)[2,3].

It is predicted that the diabetes involve more than 100 million people worldwide to reach 366 million by 2030[4]. Egypt has the highest prevalence of diabetes, at 10.9%. If concerted action is not taken, there are 34.6 million people with diabetes in the Middle East and North Africa, by 2035a number that will almost double to 67.9 million[5].

Hyperglycemia of chronic action has several complications in many organs of the body, one of them is the oral mucosa, and so the control of glucose blood is very important. Defect in neutrophil function, high collagenase activity, and decrease in synthesis of collagen, retinopathy and neuropathy are mechanisms that corelated to oral proplems of diabetes[6].

Moreover, diabetes is also associated with many oral complications, such as delayed wound healing and inflammation of soft tissue, including inflammation of gingiva and periodontal tissue[7]. The most important side effects of diabetes mellitus are decrease salivary secretion that leads to xerostomia, periodontal disease gingivitis, dental caries, and increase the rate of infection of the oral cavity or even tooth loss[8].

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Diabetic Patients present improper function of neutrophils (adhesion of leukocyte, chemotaxis, and impaired phagocytic action), defect of bactericidal activity, abnormal react to antigens, and changing the function of T lymphocytes[9].

Also, the negative effect of DM on salivary flow and immune function, thus mucosal lesions involve the mucosal atrophy, lichen planus or lichenoid mucositis and candidiasis (thrush), are more in diabetic patients[10,11]. For that, patients with diabetes mellitus have oral soft-tissue disease more frequently than in non-diabetic patients[12].

In the previous study that was made by Saini *et al*. to relate between diabetes mellitus and the lesions of oral mucosa, which showed that diabetic patients have a high percentage of oral mucosal lesions along with oral premalignant lesions than healthy individuals. This is due to defects of immunological system arising from disorganiztion of the endocrine system in the diabetic patients[11].

The chemical that induces loss of pancreatic cells and causing severe hyperglycemic condition is known as Alloxan (AL), thereby causing diabetes mellitus in animals[13].

Circulating platelets, macrophages and mononuclear cells are secreting EGF. EGF connect to its corresponding receptor on epidermal cell surface membranes and fibroblast cell to build collagenous tissue, and hasten the generation of wound healing and epithelial tissues, which hasten the wound healing process[14]. Also A pivotal factor in the healing mechanism is EGF which acting on fibroblasts and epithelial cells and enhancing recovery of damaged epithelium. Repaired cells in diabetic mice, can enhance blood flow by improving new vessel formation, which helps in the healing process[15].

Diabetes mellitus still considered as a common disease with oral manifestations, so it is mandatory for dentists to be oriented of these manifestations to make an early diagnosis and treat them properly. Therefore, the objective of the present experiment was to estimate effects of EGF on diabetic oral keratinocytes of albino rats using transmission electron microscope.

MATERIALS AND METHODS

- Ethical approval of this study was obtained from the Institutional Review Board at Faculty of Oral and Dental Medicine Al Azhar University, Girls branch in Nasr City, Egypt. The code number is REC-PD-21-04

Materials

- Alloxan (ALX monohydrate, Sigma Aldrich pharmaceutical company)
- Epidermal Growth Factor (EGF) was lyophilized from 1 mg\ml solution after excessive dialysis against 20Mm phosphate buffer, PH 7.4 and 130mM NaCl obtained from (Sigma Aldrich)

Methods

Eighteen adult male albino rats weighing between (200±20 g) (6-7 week olds) were used in this study. In stainless steel cages under standard conditions of a relative humidity and temperature the albino rats were housed (3 rats / cages). They were fasted for 16-18 h with free access to water before to the induction of diabetes.

Diabetes will stimulate in normal rats by intra peritoneal injection of alloxan. Each rat was injected with 1 ml of the prepared alloxan solution (200mg/kg bodyweight) as a single dose in order to induce DM. DM was measured in albino rats by measuring level of glucose with a glucometer after 72 hrs of alloxan monohydrate injection. Experimental rats having level of blood glucose above 300 mg/dl were considered to be diabetic and involved in our study.

The rats were divided into three groups:

- (Group I); Control group: rats received only normal saline. (6 rats)
- (Group II); Diabetic group: rats without any treatment. (6 rats).
- (Group III); diabetic rats treated with EGF at the dose of 1.25 µg of EGF (sigma)\kg. (intra peritoneal injection) (Using an insulin injector, a 1.25 microgram/kg dose of EGF containing the saline solution (6 rats) for 2 months.

After the end of experimental study, the rats euthanized and buccal mucosa specimens were obtained for transmission electron microscope (TEM) examination at The Regional Center for Mycology and Biotechnology, Al Azhar University, Nasr City, Egypt.

All specimens were embedded in the resin block then thin sectioned by a process known as ultramicrotomy, sections of 50 - 70 nm thickness were possessed on metal mesh 'grids' and stained with electron dense stains before monitoring in the TEM. The blocks were cut into semithin sections (1 µm) with a glass knife, using an ultramicrotome. The sections were stained by Toluidine Blue for observation under LM, and for detecting of a certain area for ultrathin sectioning (Figure 1). Ultrathin sections were cut by using a diamond knife at 50-70 nm and placed/collected on a grid of metal.

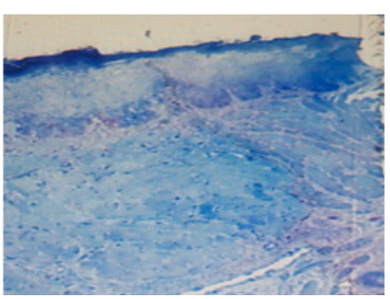

Fig. 1: Semi thin section of buccal mucosa of albino rats

There are no relevant financial interests in this research. We have no actual or potential conflict of interest in relation to this research.

RESULTS

Ultra-structural examination by (TEM) of group I showed normal histological features of the epithelium of buccal mucosa that consist of flattened granular cell layer with flat nuclei revealed electron dense keratohyline granules, which covered by cornified (keratin) layer. Spinous cell layer had definte cell membrane, definite nuclear membrane and normal desmosomal junctions between adjacent cells. The cytoplasm appeared with normal architecture of mitochondria, free ribosomes and abundant tonofilaments. Basal cell layer resting on basement membrane, intact basal lamina connected with basal cells by well developed hemidesmosomes(Figure 2).

Ultrastructural examination (TEM) of buccal mucosa specimens of group II (diabetic G) revealed there was atrophy of epithelial cell layers and loss of its normal arrangment. The overlying layer of keratin appeared in wavy and irregular pattern. Granular cell layer had coarse keratohyline granules of variable size and density also, remaining of superfacial apoptotic granular cell were absorved. Prickle cells also, showed deformed nuclei with peripheralized chromatin and loss of chromatin distribution in some of them. The cytoplasm had vacuolated mitochondria and abnormal nuclear cytoplasmic ratio. Wide intercellular space in some places (loss of cell boundaries and desmosomal junctions) was observed. Basal cells appeared with pyknotic nuclei, irregular basal lamina, and loss of appearance of hemi desmosome in some areas. Lose of normal cell orientation was obviously observed (Figure 3).

Ultrastructural examination (TEM) of buccal mucosa specimens of group III showed slight similarity with histological features of the control rats. Granular cell layer had obviously increase in keratohyline granules and some cells showing apoptosis. Prickle cells showed narrower intercellular spaces in comparison to that of group II. Restored some of cytoplasmic organelles as mitochondria and tonofilament. The cell boundaries and desomsomal junctions between cells were observed. The basal cells showed relative restored hemidesomsomal junction with underlying lamina propria (Figure 4).

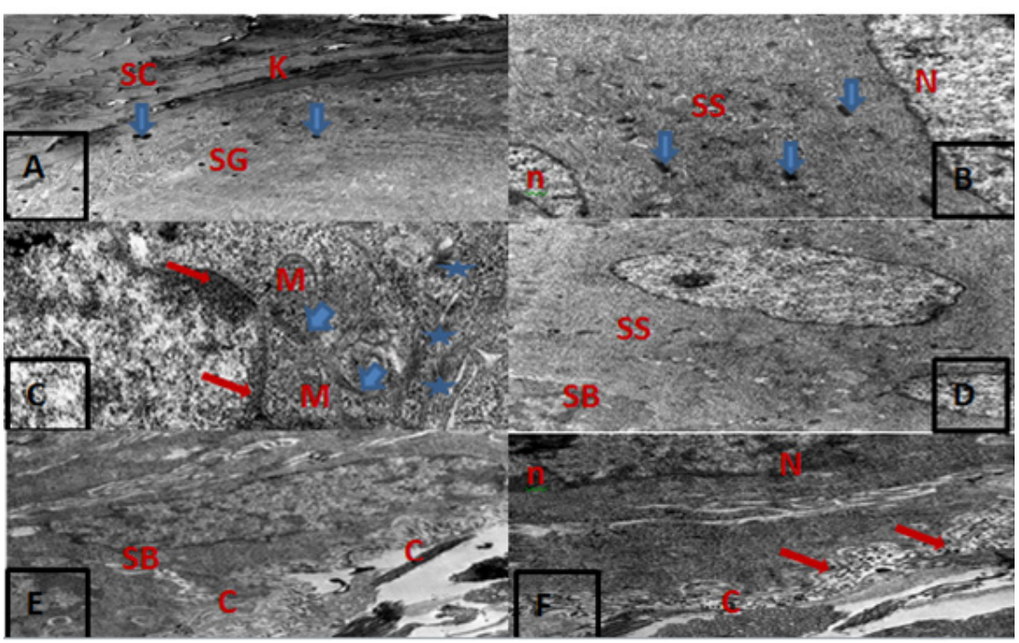

Fig. 2: Transmission electron micrograph of buccal mucosa of control group showing:
(A) Stratum cornium(SC) and normal keratin squamous (K) with keratohyline granules(blue arrow) in underlying granular layer(SG)
(B) Normal appearance of spinous cell layer(SS), normal nuclear cytoplasmic ratio, electron dense cytoplasm with prominent nucleoli(n) , nucleus (N) regular nuclear membrane and normal desmosomal junction(blue arrow)
(C) Normal desmosome between adjacent cells (star), mitochondria of normal appearance (M), heterochromatine of the nucleus(red arrow), tonofilaments (blue arrow)
(D) Basal cell layer (SB), and overlying prickle cell layer (SS)
(E) Basal cell with well-defined cell membrane, normal interdigitation between adjacent cell membranes and anchoring fibrils (C) in underlying lamina propria.
(F) High magnification of basal cell layer showing normal cell boundary and nuclear membrane, well developed hemidesmosomes (arrow) with the underlining collagen fibers (C) of lamina propria.

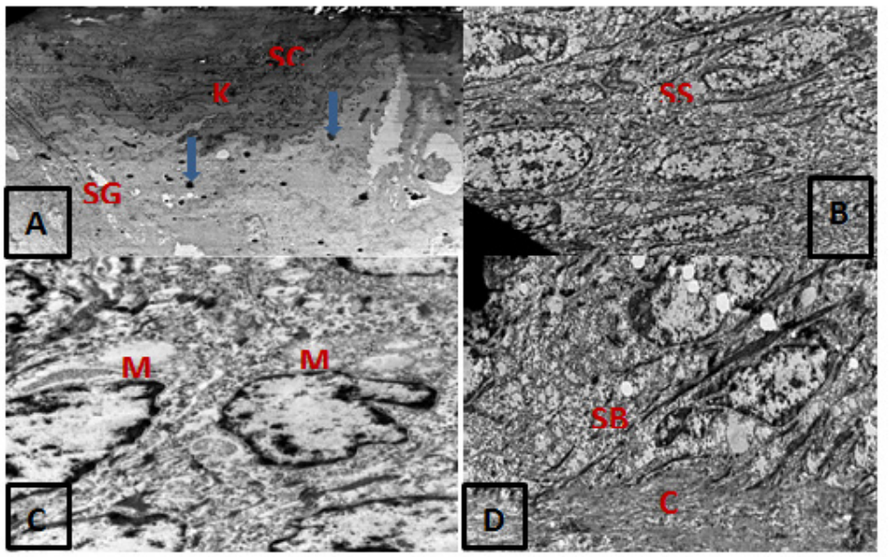

Fig. 3: Transmission electron micrograph of buccal mucosa of diabetic group showing:
(A) Stratum cornium (SC) and keratin layer (K) with coarse keratohyline granules of variable size and density (blue arrow) in underlying granular layer, remaining of superfacial apoptotic granular cell were absorved. The overlying layer of keratin appears wavy and irregular.
(B&C) Spinous cell layer (SS), abnormal nuclear cytoplasmic ratio. The intercellular space was markedly increased, loss of cell boundaris and desmosomal junctions, irregular nuclear membrane. Spinous cells showing Pleomorphism, deformed nuclei, loss of chromatin and vacuolated mitochondria
(D) Basal cell layer(SB) appeared with irregular cell membrane, loss of cell organelles, some apoptotic cells and loss of hemidesmosomes in some area.

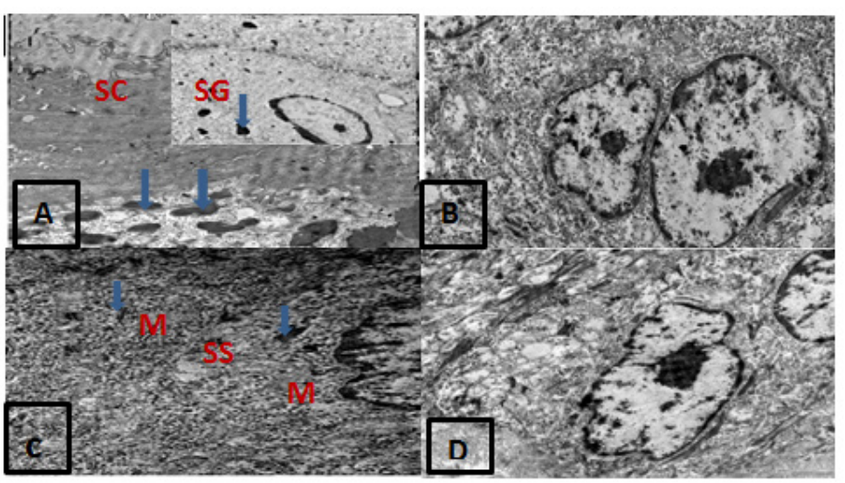

Fig. 4: Transmission electron micrograph of buccal mucosa of EGF treated group showing
(A) Normal keratin layer (SC), granular cell layer(SG) had obviously increase in keratohyline granules (arrow)
(B&C) Spinous cell layer, spinous cells showed narrower intercellular spaces in comparison to that of group II. Restored mitochondria (M), cell boundaries, desomsomal junctions between cells and divided cell.
(D) Basal cell layer (SB) the basal cells showed relative restored hemidesomsomal junction with underlying lamina propria. Some mitochondria (M) had restored, other still vacuolated.

DISCUSSION

There was marked correlation between buccal mucosa alterations and diabetes mellitus, so this affection consider as a part of systemic disorders of DM[5]. In this study group II showed increase thickness of both keratin and granular cell layer (with variable sizes and shapes of keratohylian granules, as it was known that the epithelium of the buccal mucosa of albino rats is keratinized stratified squamous epithelium,[16] In DM there is decrease in salivary flow rate and pH value of saliva which may have an irritant effect.

Furthermore, the hyperkeratosis occur in diabetic group may be a result of repeated infections of buccal cavity due to immunological injury[17,18]. Gül *et al*., 2008 showed that diabetic mice have increase in keratin associated proteins and keratin complexes genes expression, as prolonged exposure to elevation of glucose levels may impair cellular behavior and increase thickness of epithelium[19].

The affection is not only in the keratin quantity but also quality. Only little researches have demonstrated whether filaggrin gene and its mutations may be included in diabetes. There is a relationship between filaggrin gene mutations and type 1 DM. Type 1 DM is related to increased expression of proinflammatory cytokines such as interleukin (IL)-1β, IL-18t, umor necrosis factor (TNF)-α and interferon (IFN)-γ which produce mutations in filaggrin gene[20,21].

Diabetes leads to atrophy in the epithelium of the oral mucosa and loss of orientation of epithelium layering. This was explained according to the fact that diabetes inhibits the mitosis of the epithelial cells by decreasing the number of cells in synthesis phase during DNA replication[5].

Hyperglycemia in diabetes participates to the high level of advanced glycation end products (AGEs), which displays a significant role in consistence of reactive oxygen species (ROS)[22] which in turn results in tissue damage and loss of chromatin due to activation of caspase-3. Then, the caspase-3 stimulates endonuclease that cleaves DNA strands[22,23].

Akimoto *et al*., 2008 showed that hemidesmosomes in the basal cells of epithelium were diminished in number in DM. This may be lead to alteration in components of the basement membrane and integrins due to elevation in their degradation by proteases elevated in diabetic corneas as suggested by Agrawal and Tsai, 2003 and that cause altered both structure and function of basement membrane[24,25].

In our study, notice improvement in the structure of buccal mucosa of diabetic rats treated by EGF restoring hemidesosome between basal cells and underlying lamina may be attributed to effect of EGF to stimulate synthesis of type-IV collagen without impacting collagen disintegration, and increases the production of a type-IV collagen substratum in primary cultures of mammary cells of rat[26].

The obtained results in EGF treated groups may be explained on the bases. The cascades of cellular proceedings that considered as a part of the mitogenic responses including initiation of DNA synthesis of extracellular macromolecules are produced by EGF. Studies on animals and humans showed that EGF displays have various biological effects including stimulation of cell proliferation, differentiation and maturation. EGF treatment also may be novelty for pancreatic regeneration in DM as it increases the synthesis of secretory granules in the beta cells and have healing effect in the mitochondrion and in granular endoplasmic reticulum in pancreas in rats treated by alloxan[27].

This in concert with (Farzaneh agha-hosseini, *et al*. 2015)[28] where they found that EGF receptor is implicated in activating pathways increasing proliferation of cells, survival cells, migration and differentiation in most epithelial tissues, so the biological activities of EGF depends upon its binding to its receptors. Furthermore, EGF is a pivotal factor in the healing cascade, working on epithelium and also enhancing the preservation of damaged epithelium[29].

CONCLUSION

The structural alterations detected in this study confirmed the harmful effects of diabetes showed in oral keratinocytes of buccal mucosa. EGF cause relatively improvement of the damaged epithelium.

CONFLICT OF INTERESTS

There are no conflicts of interest.

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الملخص العربى

تقييم الفحص المجهري الالكترونى للخلايا الكيراتينية الفموية للفئران البيضاء المصابة بداء السكري والتي تم علاجها بعامل النمو الطلائى

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الهدف:مرض السكري له آثار ضارة على أنسجة الجسم المختلفة ، أحد هذه الأنسجة هو الغشاء المخاطي للفم.هدفت الدراسة إلى تقدير تأثير عامل النمو الطلائى على الخلايا الكيراتينية الفموية للفئران البيضاء المصابة بداء السكري (تقييم الفحص المجهري الإلكتروني).

المواد والطرق: اختيار ١٨ ذكورالفئران البيضاء البالغة مقسمة إلى ثلاث مجموعات. لم تتلق المجموعة الأولى (مجموعة الفئران الضابطة) أي أدوية ، بينما المجموعة الثانية (مجموعة الفئران التي أصيبت بداء السكري) بعد حقنها جرعة واحدة من حقن الألوكسان داخل الغشاء البروتونى. علاوة على ذلك ، فإن المجموعة الثالثة مصابة بداء السكري وتم حقنها بعامل النمو الطلائى. تم تشريح الغشاءالفموى (الخدي)من جميع الفئران ومعالجتها لتقييم الفحص المجهري الإلكتروني.

النتائج: أظهر الفحص المجهري الالكترونى فى المجموعة الضابطة أن الخلايا الكيراتينية الفمويةظهرت بصورة طبيعية والتى تتكون من طبقة الخلايا الحبيبية المسطحة ذات نواة مسطحة والتى اظهرت حبيبات كثيفة من الكيراتوهيلاين والتى تغطى بالطبقة الكيراتينيه.بينما أظهرت المجموعة الثانية المصابة بداء السكري ضمور في جميع طبقات الخلايا وفقدان ترتيبها الطبيعى كما ظهرت الطبقة العلوية من الكيراتين بنمط متموج و غير منتظم بينما أظهرت المجموعة الثالثةالمعالجة بعامل النمو الطلائى تشابها ملحوظا مع الصفات النسيجية للمجموعة الضابطة.

الاستنتاج: أظهرت النتائج التأثير الضارلمرض السكري على الخلايا الكيراتينية الفمويةكما أظهرت أن عامل النمو الطلائى يسبب تحسنًا نسبيًا في الخلايا التالفة (الخلايا الكيراتينية الفموية).