



A Rat Experimental Model for Investigation of the Effect of Diabetes on Submandibular Salivary Glands Treated with Epidermal Growth Factor

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Abstract

BACKGROUND: Despite the plethora of research around the negative effects of diabetes on different body organs, this topic still attracts a lot of attention to find potential remedies that could counteract or reverse the damaging effect of diabetes.

AIM: In this study, we developed a reliable experimental rat model that can be used for the investigation of the ability of epidermal growth factor (EGF) in restoring the normal architecture of oral tissues after being damaged by diabetes.

METHODS: Eighty adult male albino rats (average weight ± 220 gm) were used in the current study. Twenty rats served as control and received no treatment. Diabetes was induced in 40 rats using a single injection of 65 mg/kg of streptozotocin. Out of the 40 diabetic rats, 20 rats received a single daily intraperitoneal injection of epidermal growth factor (EGF) (10 μ g/Kg) for 8 weeks. Furthermore, 20 healthy rats received the same dose of EGF and served as positive controls. The submandibular salivary glands of all rats were examined for immunohistochemical detection of myosin in the glandular structure.

RESULTS: The EGF-treated group showed comparable myosin expression to the control group. The diabetic group revealed deterioration of all components of the submandibular salivary glands. Finally, the diabetic + EGF group has demonstrated restoration of the myosin expression levels in the submandibular salivary glands to a level that is not significantly different from healthy (nondiabetic) rats in the control group ($p > 0.05$) and significantly higher than the diabetic group ($p < 0.0001$).

CONCLUSION: The findings of the present study confirm previous studies and validate the use of our animal model as predictable experimental tool to investigate the effects of diabetes and EGF on different oral tissues. It also highlights the importance of further research investigating EGF as a promising treatment modality for the restoration of the condition and functions of tissues damaged by diabetes not only in the oral cavity but also around the whole body.

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Introduction

Animal models provide a unique opportunity to research to study the development of diseases and their complication under controlled conditions that limits the influence of genetic and environmental factors on the progression of diseases, including diabetes [1], [2], [3], [4]. In diabetes research, different animal models have been developed and used to study type I diabetes by spontaneously developing autoimmune diabetes or ablation of beta cells of the pancreas, while type II diabetes animal models have different degrees of obesity, insulin resistance, and pancreatic beta cells failure [5], [6], [7]. Therefore, it has been recommended to use multiple animal models in the study of diabetes due to the great variability in individuals suffering from diabetes within the human population [7].

Diabetes mellitus (DM), also known simply as diabetes, is a group of metabolic diseases in

which there are high blood sugar levels over a prolonged period [8]. It characterized by persistent hyperglycemia, glucosuria, and polyuria [9]. This results in elevated blood glucose levels (hyperglycemia), which leads to a number of complications, including nephropathy, neuropathy, cardiomyopathy, retinopathy, atherosclerosis, microangiopathy, and diabetic foot ulcers [10]. Moreover, diabetes is associated with an increased susceptibility to bacterial infections, inflammation, caries, xerostomia, and periodontal diseases and is also related with malfunctioning of salivary glands resulting in deficient salivary flow [11].

Investigations demonstrated that insulin and diabetes affect the function and structure of rat parotid gland found that after 1 month, of diabetes induction in rats, there was an inhibition glands growth in diabetic rats [12], [13]. By histological examination of the gland, there were an excessive accumulation of lipid droplets within the acinar cells,

cytoplasm together with increase of lysosomes. Moreover, it was found that dense vacuoles and crystalloids were found in the apical cytoplasm of the parotid gland striated duct cells [12], [13]. The effect of diabetes on salivary flow and protein secretion by salivary glands results in an increased susceptibility to mucosal infections, tooth demineralization, and caries [14]. It was concluded that reduced statherin secretion by salivary glands in diabetic mice might be partly responsible for a less effective protection of the oral tissues, resulting in a higher incidence of caries and oral infections associated with diabetes [14].

Epidermal growth factor (EGF) is a powerful protein that has a significant role in wound healing through facilitating the proliferation and migration of fibroblasts [15]. EGF was identified while studying nerve growth factor in submaxillary glands of male Swiss-Webster mice. EGF is known to be produced in human salivary glands [16]. EGF binds specifically to its receptor, EFG receptor, which is found on the surface of the cells. The high affinity between the EGF and its receptor stimulates and activates the intrinsic protein-tyrosine kinase activity of the receptor [17]. This activation then starts a signal transduction cascade which ends in various biochemical alterations within the cell. These changes include increase in protein synthesis and in glycolysis as well as an increase in gene expression which eventually leads to DNA synthesis and cell proliferation [18].

Myosins include a family of ATP-dependent motor proteins and are best known for their role in muscle contraction and their involvement in a wide range of other eukaryotic motility processes. They are responsible for actin-based motility. The term was originally used to describe a group of similar ATPases found in striated and smooth muscle cells [19]. Myosins participate in a variety of cellular processes, including cytokinesis, organellar transport, cell polarization, transcriptional regulation, intracellular transport, and signal transduction. They bind to filamentous actin and produce physical forces by hydrolyzing ATP and converting chemical energy into mechanical force [20]. In a previous study that was conducted by our research team, we showed that cytokeratin was highly expressed in streptozotocin (STZ)-induced diabetic rats treated with EGF. Myosin being an integral component within the cytoskeleton of the parenchymal elements of salivary glands helps maintain its integrity.

The aim of the present study was to validate the diabetes-EGF experimental rat model and to detect if EGF could reduce the well-known side effects of DM induced by STZ on the submandibular salivary gland of adult male Albino rats or not. This was verified through immunohistochemical detection of myosin in the parenchyma of the gland.

Methods

Animals and housing

Eighty male Albino rats of adult age, 3 months old (average weight 220 g), were used in this study. Albino rats were sourced from Kasr ElAini experimental animals' unit, Faculty of Medicine, Cairo University, were fed natural diet and supplied drinking water ad libitum throughout the whole experimental period.

Experimental groups

The albino rats were divided equally ($n = 20$) into four groups as follows:

Control group

Control group was injected with the same number of saline injections that is equivalent to EGF injections.

Diabetic group

DM was induced by subjecting the rats a single intraperitoneal injection of 65 mg/kg of freshly prepared STZ (Sigma-Aldrich Co) dissolved in 0.1 M sodium citrate buffer after subjecting the rats to 16-hr fasting period prior to the STZ injection [21], [22].

Epidermal growth factor group

Rats were subjected to a single injection of EFG intraperitoneally in daily dose (EFG, human - animal component free - recombinant, expressed in *Escherichia coli*) provided by Sigma-Aldrich, Inc., in a dose of 10 µg/Kg for 8 weeks [23].

Diabetic + epidermal growth factor group

Rats were treated with STZ as the diabetic group rats. After confirming their diabetic condition, they were subjected to a single injection of EFG as per the EGF group.

At the end of the experiment, animals of all groups were euthanized, and their submandibular salivary glands were dissected.

Image and statistical analysis

Immunohistochemical detection of myosin was performed in the glandular tissue using staining reaction incubated by antimyosin antibody and color developed by 3-Amino-9-ethylcarbazole (AEC) (Figure 1a). The specimens were examined and photographed using ZEISS Primo Starlight microscopy mounted by Tucsen IS 1000 10.0MP Camera in the Centre of Innovative dental sciences, Faculty of Dentistry, The British University in Egypt.

The myosin immunointensity in the glandular specimens from different groups was analyzed using Image J (1.46 a, NIH, USA) software. For each selected section, 6 microscopic fields were captured at a magnification $\times 200$.

Statistical analysis of the myosin immunoexpression was performed and described in terms of mean and standard deviation. Analysis of variance (ANOVA) test was used to compare groups followed by independent samples test as *post hoc* for two-group comparisons. All statistical analysis was done using Statistical Package for the Social Sciences version 28 (SPSS Inc., Chicago, IL, USA).

Results

Negative control

Submandibular salivary glands sections taken from animals from the control group and incubated with non-specific serum and color developed by AEC showing no staining reaction for all glandular components (Figure 1b).

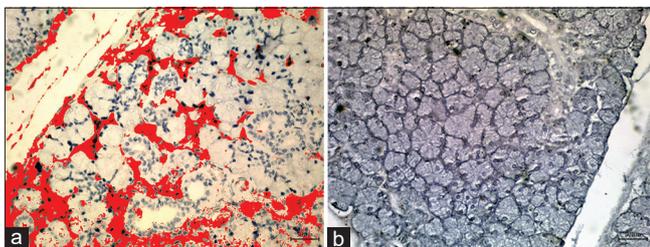


Figure 1: (a) Showing an example of masking myosin expression by red colour and the grey background with no immune reaction to Myosin. (b) A photomicrograph of (control group) incubated with non-specific serum and colour developed by AEC showing no staining reaction of submandibular salivary gland elements (Original magnification, $\times 200$).

Control group

Negative staining of the cytoplasm of mucous secretory cells was evident in the EGF group. While negative to weakly positive staining of the cytoplasm opposite to the lumen and intercellular canaliculi of serous secretory cells was observed in the same group. Strongly positive staining reaction was found around nearly all the secretory cells and intercalated ducts representing staining of the myoepithelial cells (MECs). The intercalated ducts, striated ducts, granular convoluted tubules, and excretory ducts demonstrated weakly positive staining reaction. Moderately to strongly positive staining reaction was evident within the vascular walls surrounding the excretory ducts and connective tissue cells showed weakly positive staining reaction (Figure 2a).

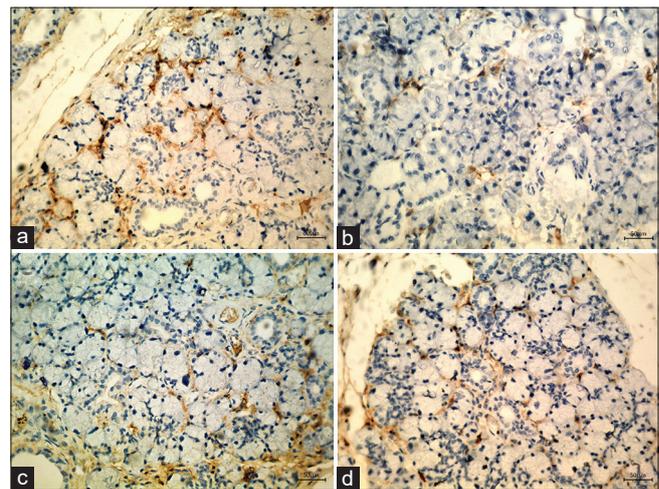


Figure 2: A photomicrograph of a Submandibular salivary gland from the incubated with antimyosin antibody and color developed by AEC. (a) Control group showing weakly +ve reaction of serous acinar cells, and strongly +ve reaction in myoepithelial cells. (b) Diabetic group showing -ve to weakly +ve reaction of serous acinar cells, and weakly +ve reaction in the myoepithelial cells. (c) Epidermal growth factor group showing -ve to weakly +ve reaction of serous acinar cells, and strongly +ve reaction in the myoepithelial cells. (d) Diabetic + epidermal growth factor showing weakly +ve reaction of serous acinar cells, and strongly +ve reaction in the myoepithelial cells. (Original magnification $\times 200$)

Diabetic group

Negative staining of the cytoplasm of mucous secretory cells. While negative to weakly positive staining of the serous secretory cells. The MECs surrounding the periphery of the acini and the intercalated ducts showed weakly positive staining reaction in their cell bodies and weaker staining reaction of their cell processes. The intercalated ducts, striated ducts, granular convoluted tubules, and excretory ducts revealed negative to weakly positive staining reaction. The walls of blood vessels presented weakly positive reaction as well as connective tissue cells surrounding the excretory ducts (Figure 2b).

Epidermal growth factor group

Negative staining of the cytoplasm of mucous secretory cells was evident in the EGF group. While negative to weakly positive staining of the cytoplasm opposite to the lumen and intercellular canaliculi of serous secretory cells was observed in the same group. Strongly positive staining reaction was found around the secretory cells and intercalated ducts representing MECs' staining. The intercalated ducts, striated ducts, granular convoluted tubules, and excretory ducts showed weakly positive staining reaction. Moderately to strongly positive staining reaction was detected in the walls of blood vessels surrounding the excretory ducts while connective tissue cells showed weakly positive staining reaction (Figure 2c).

Diabetic + epidermal growth factor group

Examination of sections taken from the Diabetic rats treated with EFG and incubated with

anti-myosin antibody revealed obvious improvements in comparison to those of the diabetic group. Negative staining of the cytoplasm of mucous secretory cells, while negative to weakly positive staining reaction of the cytoplasm was evident opposite to the lumen and intercellular canaliculi of serous secretory cells.

The MECs with their elongated cell bodies and the spider-like extensions of their processes showed strongly positive staining reaction around nearly all the secretory cells and intercalated ducts. The intercalated ducts, striated ducts, granular convoluted tubules, and excretory ducts revealed weakly positive staining reaction. The walls of blood vessels represented moderately to strongly positive reaction while connective tissue cells showed weakly positive staining reaction.

Statistical results

Histomorphometric analysis of the submandibular salivary gland of the three groups revealed that the highest mean area percent occupied by myosin immunostaining was recorded in the control group, whereas the lowest value was recorded in the diabetic (Table 1). ANOVA test revealed a statistically significant difference in the mean area percentage of expression of myosin immunostaining ($p < 0.0001$).

Table 1: Mean values and standard deviation of the area percentage of expression of myosin immunostaining in the submandibular salivary glands of the different groups

Group	Control	Diabetic	EGF	Diabetic + EGF
Number of cases	20	20	20	20
Maximum	33.24	12.21	35.87	29.75
Minimum	31.41	9.48	32.14	23.13
Mean \pm SD	30.72 \pm 3.05	11.01 \pm 1.59	31.86 \pm 3.55	24.27 \pm 3.95

SD: Standard deviation, EGF: Epidermal growth factor.

A pairwise comparison was held between myosin mean area expression percentage in the control group and the other experimental groups using an independent *t*-test. A statistically significant decrease in the myosin expression area percentage in the diabetic group ($p < 0.0001$) and the diabetic + EGF ($p < 0.05$) when compared to the control group. There was no statistically significant difference between the myosin expression in the control and EGF groups. Furthermore, there was a statically significant decrease in the myosin expression area percentage in the Diabetic + EGF group

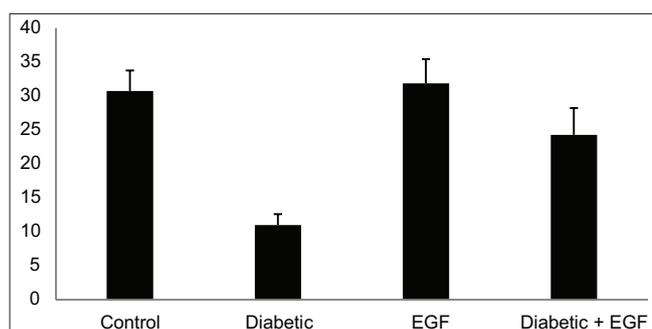


Figure 3: Bar chart showing the mean myosin expression area percentage in submandibular gland between all the groups

when compared to the control group ($p < 0.05$). Finally, there was a significant increase in myosin expression area percentage in the EGF and the Diabetic + EGF group when compared to the diabetic group ($p < 0.0001$) (Figure 3).

Discussion

A successful choice of an animal model requires careful consideration of a number of intrinsic and extrinsic factors associated with disease being studied as well as the similarities and differences between the humans and the animal model in responding to the disease [24]. Procedural simplicity and reproducibility have been identified as important criteria in an excellent animal model [25], [26]. The animal model used in this study fulfills the above requirements as it only involves two steps, induction of diabetes and EGF injections. Furthermore, the animal model should mimic the anatomy, histology physiology, and genetics of humans [27]. The animal model we used is ideal as rats have identical oral features to humans [28], [29]. Immunohistochemical staining of rodent salivary glands requires a careful choice of secondary antibodies and negative control staining [29]. Therefore, a common immunohistochemical marker (Myosin) that is readily available in rats and human salivary glands parenchyma/stroma as well as negative control staining was performed.

STZ was the drug of choice in this study to induce diabetes as it had low mortality rate and high tolerance by the experimental animals than alloxan. Furthermore, it can be given easily by different routes, and its diabetogenic action was rapid and permanent as it is known to destroy the beta cells of islets of Langerhans [30]. Furthermore, for a functional restoration of the damaged salivary glands, the diabetic rats were treated with injection of EGF intraperitoneally in daily doses for two months in accordance with that used by Ohlsson *et al.*, (1997) [22].

In the present study, different glandular elements of the control animals stained positively for myosin, these are the MECs, the serous acinar cells and intercalated duct cells, assuming that these contractile proteins might be involved in the process of transport of cellular products and the exocytosis of secretory granules. This finding is in agreement with what has been reported by Ogawa (2003) [31] who explained that contraction of the filaments helps to expel the secretory granules towards the apical part of the cells along the luminal border and opposite the inter-canalicular spaces.

In the present investigation, the myosin was demonstrated heavily in the MECs, pericytes as well as smooth muscle cells of the blood vessels walls. Similar findings were reported by (Dewar *et al.*, 2011) [32]

who found the same results on the MECs associated with normal breast ductules and vascular smooth muscle. In addition, these findings were concomitant with Grandi *et al.*, (2000) [33] who investigated the immunocytochemical characteristics of normal MECs of human major and minor salivary glands. Immunoreactivity of MECs was observed exclusively with fully differentiated smooth muscle antibodies.

The submandibular salivary glands of the diabetic rats showed fainter staining patterns with varying degrees (compared with the controls), denoting a decrease in the intracellular content of myosin. Furthermore, the defect in contraction of the MECs due to the decrease in its myosin content may result in impairment of the process of cytoplasmic exocytosis of acinar cells and defective excretory function of the duct system due to improper squeezing forces of the MECs, making it not enough to clear the duct lumen. These results were approved by histological and ultrastructural findings in this investigation where the accumulation of secretory granules in the acinar cells as well as most of duct lumens which were dilated and filled with stagnant secretion.

The production of EGF in the submandibular gland and its circulating level were studied in diabetic mice. EGF concentrations in the submandibular gland and plasma were reduced to 13% and 30% of the control levels, respectively. In STZ-treated diabetic mice, they were reduced to 18% and 20% of controls, respectively, 5 weeks after the drug injection. In addition, histological examination of the submandibular glands indicated that the size of the granular convoluted tubules, which produce EGF, was substantially reduced in the diabetic mice. Insulin administration to STZ-treated mice almost completely reversed the decrease in EGF content in the submandibular gland and increased the size of the granular convoluted tubules in the gland. It was concluded that EGF deficiency occurs in DM and that insulin may be important in maintaining the normal level of EGF in the submandibular gland and plasma [34].

Salivary-derived growth factors, including EFG, are known to play a major role in maintaining oral health, promotion wound healing [35], and protecting the mucosal integrity [36]. Salivary levels of EGF in diabetic patients versus healthy controls were studied. The salivary EGF concentration was significantly lower for the diabetic patients compared to control patients suggesting that reduced levels of salivary EGF in diabetic patients may contribute to the development of oral and systemic complications of diabetes, opening the pathway for future clinical applications [9]. Furthermore, the submandibular salivary gland under the effect of induced DM was studied using routine hematoxylin and eosin (H and E) stain, histopathologically for the detection of carbohydrate using Periodic acid Schiff's (PAS) reaction and immunohistochemically for the detection of anti-apoptotic marker, Beta cell lymphoma-2 (BCL) to investigate the DM effects and Vitamin C

(Ascorbic acid) on both the structure and function of the submandibular salivary gland. The obtained results revealed tissue alterations and common complications in the submandibular glands of diabetic rats. On the other hand, the antioxidant (Vitamin C)-treated rats had less tissue alterations and lesions than untreated animals [37].

The remarkable feature of the results of the present study is that it demonstrates the total or subtotal prevention of diabetic changes in the submandibular salivary gland by treatment with EFG. This is in agreement with previous studies in the literature that investigated the effects of EGF on different tissues in diabetic patients [38], [39], [40], [41], [42], [43]. In addition to the above, previous studies using the same experimental rat animal model revealed results that are consistent with the present study [44], [45]. EGF restored normal cytokeratin expression [44] and the ultrastructural picture of submandibular salivary glands in diabetic rats [45]. The consistency in results validates the use of our diabetes-EGF experimental rat model in future studies involving other oral tissues as well as different body organs. The simplicity, ease of development, and predictability of reliable outcomes encourages further studies that could involve restoration of taste functions, delaying the progression of periodontal disease, and counteracting diabetes-induced xerostomia (dry mouth) using EGF. Furthermore, combined therapeutic methods, including the use of EGF and silver nanoparticles (AgNPs) [46] in the management of diabetic wound healing and healing of dental extraction sockets will also be studied using our experimental rat model. In general, treatment with EGF is very promising. Before moving to the next level of clinical trials, further studies are necessary to provide more data related to the cost-effectiveness, best route of administration, and optimal dosage before EGF can be approved in regular clinical practice.

Conclusion

The experimental rat model described in the current study is a simple, reliable, and predictable animal model that can be used in diabetes and EGF research. EFG administration in a daily dose of 10 µg/Kg body weight for 2 months intraperitoneally led to the enhancement of all of the submandibular salivary glands components. This was proven by immunohistochemical localization of myosin. The findings from the present study confirm previous results from studies that used scanning electron microscopy and cytokeratin expression and the same animal model.

Author Contributions

M.M.B.: Conceptualization, funding acquisition, methodology, study design, data curation, investigation, formulating the discussion section, project administration, writing—original draft, writing—review and editing. M.S.: Formal analysis, validation, investigation, formulating the discussion section, writing—review and editing. S.A.W.: Validation, investigation, data curation, formulating the discussion section, writing—review and editing. M.M.A.-A.: Conceptualization, methodology, study design, data curation, investigation, formulating the discussion section, project administration, writing—original draft, writing—review and editing. All authors have read and agreed to the published version of the manuscript.

Institutional Review Board Statement

This project was reviewed and granted ethical approval by the Suez Canal University Research Ethics Committee (SUEZ-REC 23/2014).

Data Availability Statement

The data presented in this study are available on request from the corresponding author.

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