

Submandibular Glands as an Evident of the Effects of Antioxidant on Alloxan-Induced Diabetic Rats

¹Ghada A.M. EL-Gusbi, ²Mohamed T.H. Shredah and ³Abd EL-Hafez E. Soliman

¹Department of Oral Biology, Faculty of Dentistry, Alfath University, Libya

²Oral Biology Department, Faculty of Dentistry, Damanhour University, Egypt

³Department of Oral Biology, Faculty of Dentistry, Alazhar University, Egypt

Abstract: The submandibular salivary gland is one of the major salivary glands that were considered as a scope to be studied under the effect of diabetes mellitus. Diabetes mellitus is a significant healthcare problem concern worldwide. It has different pathological effects on body tissues and organs especially on the major salivary glands. A total number of 30 adult healthy albino rats were classified into 3 equal groups (10 animals each); group I as a control group which injected with sterile saline as a single dose of 1ml/kg, group II experimentally induced diabetic mellitus using alloxan and group III that experimentally induced diabetic and treated with ascorbic acid (Vitamin C). All groups of rats were sacrificed and tissue samples from their submandibular gland were examined histologically using routine Haematoxylin and Eosin (H&E) stain, histo-pathologically for detection of carbohydrate by using Periodic acid Schiff's (PAS) reaction and immune histo-chemically for detection of anti-apoptotic marker, Beta cell lymphoma-2 (BCL₂) to investigate the diabetes mellitus effects on both the structure and function of the submandibular salivary gland. The obtained results revealed tissue alterations and common complications in submandibular glands of diabetic rat. On the other hand the antioxidant (Vitamin C) treated rats had less tissue alterations and lesions than untreated animals.

Key words: Submandibular Salivary Glands • Diabetic Rats • Antioxidant • Histopathology • Immunohistochemistry

INTRODUCTION

The rat submandibular salivary gland is tightly attached to the sublingual salivary gland. So, they appear to be one structure (submandibular and sublingual organ complex) lies in the ventral cervical region with the head extended [1].

Saliva and salivary glands are considered the strongest link between oral and systemic health. Saliva is a fluid lubricates food to assist deglutition, moistens the buccal mucosa that is important for speech, provides an aqueous solvent necessary for taste and also secretes antimicrobial agents as IgA, lysozymes and lactoferrin [2]. Saliva contains two major types of protein secretions; serous and mucous ones. The serous secretion is containing ptyalin enzyme that is necessary for digesting starch. The other secretion is the mucous secretion containing mucin that lubricates and protects the oral tissues by forming chemical and mechanical

insults and barrier against desiccation [3]. Moreover, saliva also contains histatins (antifungal proteins) that are potent inhibitors of candida which is normally kept at extremely low level in the mouth [4].

Diabetes mellitus is a significant healthcare problem concern worldwide that has different pathological effects on the body tissues and organs. It is a chronic metabolic disease that affects the ability of body cells to utilize blood glucose resulting in hyperglycemia. Salivary glands are considered as a scope to be studied under the effect of diabetes mellitus [5]. Moreover salivary proteins play an important part in oral health maintenance, thus specific changes in salivary protein composition in diabetic individuals might alter the ecological balance in favor of cariogenic bacteria and toward the initiation and progression of the disease process [6].

Currently, there are two common forms of the diabetes: type 1 and type 2 diabetes (T1 D and T2D).

Type 1 diabetic patients have pancreatic B cell destruction, which is usually immune-mediated while Type 2 diabetes is a dual disease that develops when the B-cells can no longer compensate for insulin resistance by increasing their insulin secretion [7]. The diabetic patients are unable to produce adequate saliva. They suffer from long term problems including xerostomia (dry mouth), mucositis and rampant dental caries, infections of the mouth and pharynx as well as difficulty in swallowing, speech and taste. These conditions dramatically reduce quality of life and can also be the source of systemic infections [8]. The induction of diabetes mellitus experimentally using some chemical compounds that selectively damage pancreatic B-cells, is very convenient and simple. The most usual substances to induce diabetes in the rat are alloxan and streptozotocin. However, many investigators found that alloxan constituted a class of diabetogenic agents can be used which exhibited the most potent, quite satisfactory safe diabetogenicity and have been widely used for induction of experimental diabetes [9].

Diabetes mellitus was found to have a considerable effect on carbohydrates and lipid metabolism in both experimental animals' tissues and in the diabetic patients. Also proteins were decreased in salivary gland granules especially some of the well-recognized apoptosis associated proteins include the Bcl₂ family [10]. Apoptosis is a morphologically distinct form of programmed cell death that plays an important and major role in cellular development and homeostasis, the process involved in regulation of salivary gland structure when affected by some stimuli. DNA fragmentations (apoptosis) were detected in the salivary glands' epithelial cells with induction of diabetes [11].

About 40% of the populations take vitamins as a supplement and recently much public and scientific interest has been antioxidant nutrient, such as vitamin C, vitamin E, carotenoids and selenium are believed to play a role in the prevention and treatment of variety of chronic diseases [12]. The antioxidants mechanisms in diabetic cells protection by saving these cells from oxidative stress and directly remove the free radicals in scavenge manner and it enhance the production of eliminating enzymes called superoxided- dismutase like component [13,14].

However, the relationship between diabetes mellitus and the major salivary gland dysfunction is obviously complex and unclear. Therefore, the present work was conducted to assess the histological, histopathological variations in submandibular salivary glands of the experimentally induced diabetic rats. The immunohistochemical examination also performed to

elucidate the cells that undergo apoptosis and furthermore, the effects of anti-oxidant (Vitamin C) therapy on these expected histological changes were investigated.

MATERIALS AND METHODS

Experimental Animals: A total number of thirty adult (4-6 months age) healthy albino rats, their body weights ranged between 180-220 g were obtained from laboratory animal's colony, Faculty of Veterinary Medicine, Cairo University, Egypt. Rats were housed in polycarbonate cages (5 rats / cages) in controlled laboratory environment according to method described by Renno *et al.* [15]; they kept at room temperature with a constant 12 hour light / 12 hour dark cycle; fed a standard balanced diet and had water for at least one week before being used in the experiment.

Experimental Design: Experiment the animals were classified into three equal groups (10 animals each)

Group I: (Control group): Rats in this group received no treatment but only were injected with sterile saline (0.9% NaCl) as a single dose of 1ml/kg.

Group II: (Experimentally induced diabetic rats): This group was exposed to experimental induction of diabetes mellitus by using a freshly prepared solution of 5% alloxan monohydrate dissolved in physiological saline. Each rat after overnight fasting was injected intra peritoneal with a single dose of 1 ml from the prepared alloxan solution (200mg/kg body weight) as a single dose under complete aseptic conditions in order to induce diabetes mellitus according to method described by Lenzen *et al.* [16].

Group III: (Ascorbic acid treated diabetic rats): This group of rats was firstly injected with the same dose of alloxan as group II and after 1 week, animals were treated with 40 mg/kg of body weight of ascorbic acid (Vitamin C) which injected s/c for different periods of 1, 2, 4, 8 and 10 weeks. The animals were sacrificed two weeks after the last injection of the ascorbic acid administration [17].

Induction of Diabetes: From each rat, 3 samples were taken for measuring blood glucose level; the first was before diabetic induction, the second was 24h after induction while the third was immediately before

scarification. The normal blood glucose level is 70-110 mg/dl, whereas the rats which their blood glucose level reached 200 mg/dl or more were considered to be diabetic according to Rizk *et al.* [18]. The diabetic rats were allowed to survive for 90 days then they were being sacrificed.

Tissue Samples: All rats from the 3 groups were sacrificed at the appropriate time by ether inhalation anesthesia. With the head extended, the submandibular gland of one side of the neck was dissected out from each animal carefully. Small block of tissue from each gland (10×10×3mm) was fixed in 10% formalin solution for about 18 hours. Further processing and embedding in paraffin wax was carried out. Tissue specimen sections of 5-6 µm were prepared and submitted for the histological and immunohistochemical examinations.

Histological Examination: for studying the general histological structure of the glands and identify acidophilic and basophilic structures by using as a the routine method of Haematoxylin and Eosin (H&E) stains and also for detection of carbohydrate by using Periodic acid Schiff's (PAS) reaction according to procedures of Bancroft and Marylin [19].

Immunohistochemical Examination: Avidin-biotin peroxidase immuno-histochemical reactions were carried out for localization of Beta cell lymphoma-2 (Bcl-2) as anti-apoptotic marker (Sigma Laboratories). The positive results of Bcl-2 immunoreaction were indicated by a brown coloration in the cytoplasm of the acinar and ductal epithelial cells [20].

Microscopical Examination: Appropriate slides which prepared from stained sections with H&E, PAS and immunohistochemical reaction were microscopically examined using light Olympus microscope with digital camera for photo capturing (Olympus LTD Tokyo, Japan).

RESULTS

Group I (Control Group)

Haematoxylin and Eosin Stain: Examination of H&E stained tissue sections of the submandibular salivary gland from the control albino rats showed the general normal architecture of the major salivary glands, it revealed mixed acini, serous and mucous acini with flat nucleus (black arrow). The acini had narrow lumen and lined by pyramidal cells with faint foamy basophilic cytoplasm with flat nuclei (white arrows). The duct system was composed of different types of ducts; the intercalated

ducts which were small and rounded lined by cuboidal epithelium with central rounded nuclei, the granular convoluted tubules appeared kidney shaped lined by columnar epithelium with eosinophilic cytoplasm and the striated ducts lined by columnar cells with central rounded nuclei and eosinophilic cytoplasm containing prominent basal striations. The blood vessels were occasionally seen around these ducts (Fig 1, A).

Periodic Acid Schiff's (PAS): The PAS stained sections revealed strong positive reaction (presence of carbohydrates) in both acini and ducts which was observed more at their basement membrane. The striated ducts specially showed more concentrated positive reaction around the lumen (Fig 1, B).

Histo-Chemical Stain: the immune-histo-chemical stained sections of the submandibular salivary gland from control group rats for detection of BCL₂ as anti-apoptotic marker showed a strong positive Bcl-2 immunoreaction that appeared as a clear brown color in the cytoplasm of acinar and ductal cells (Figs. 1, C).

Group II (Experimentally Induced Diabetic Rats)

Haematoxylin and Eosin Stain: Examination of haematoxylin and eosin stained sections in the experimentally induced diabetic albino rats' submandibular salivary glands showed loss of the normal gland architecture. The mixed acini showed reduction in size and vesiculation of serous acini and dilated inter acinar blood vessel (black arrows). Wide spaces in between the acini, distortion and loss of some acini and ducts areas of intra-acinar fatty cell infiltrations and vacuolations were noticed in some sections. Small and deeply basophilic nuclei were seen in some cells (Fig 2, A).

Periodic Acid Schiff's (PAS) Stain: PAS stained sections of submandibular salivary glands from diabetic rats showed moderate, less or no (negative) PAS reaction in the acini and ducts in comparison with that observed in the control group due to less carbohydrates formation of these diabetic animals (Fig 2, B).

Histo-Chemical Stain: immunohistochemical stained sections of submandibular salivary glands from diabetic rats showed faint brown weak positive Bcl-2 immunoreaction around areas of intra-acinar fatty cell infiltrations observed in some sections. Whereas most others showed a decrease of the Bcl-2 immunoreaction in comparison with that observed in the control group (Fig 2, C).

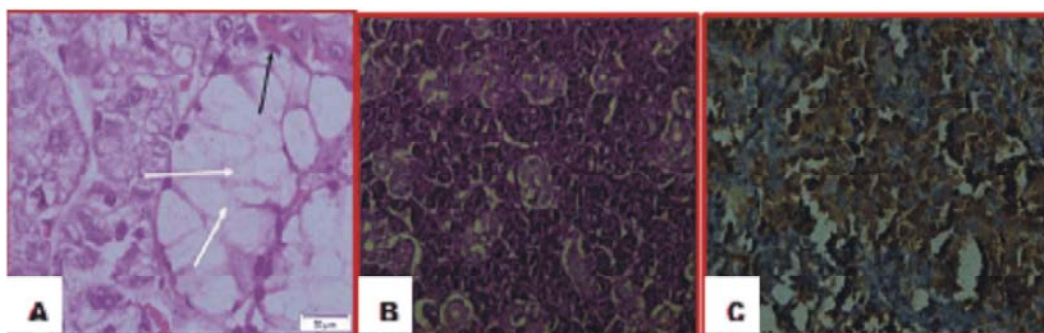


Fig. 1: A photomicrograph of a sections in the control albino rat's submandibular gland showed normal structure stained H&E(A), strong positive PAS reaction (B) and clear brown positive Bcl-2 immunoreaction (C).

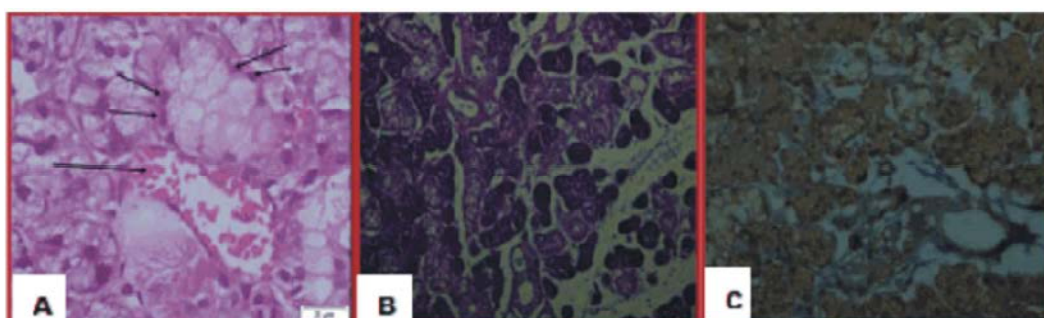


Fig. 2: A photomicrograph of a sections in the diabetic albino rat's submandibular gland showed loss of normal structure stained H&E(A), negative PAS reaction (B) and faint brown weak positive Bcl-2 immunoreaction (C).

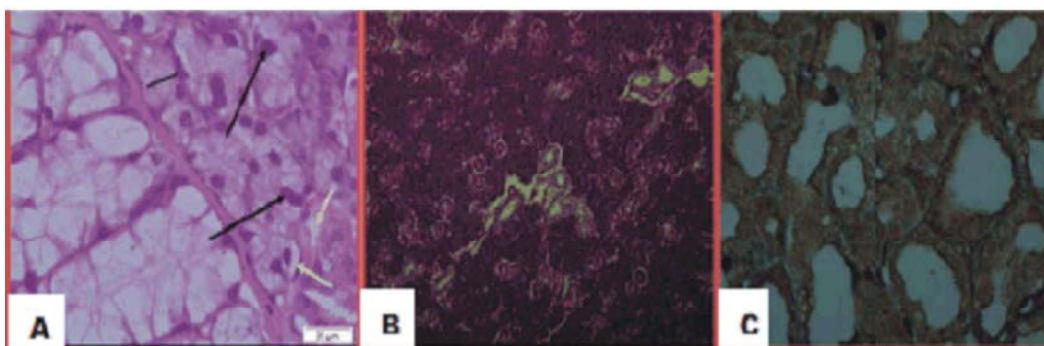


Fig. 3: A photomicrograph of a sections in the diabetic vitamin C treated albino rat's submandibular gland showed almost normal structure stained H&E(A), clear positive PAS reaction (B) and diffuse brown moderate positive Bcl-2 immunoreaction (C).

Group III: Ascorbic acid (Vitamin C)

Treated Diabetic Rats:

Haematoxylin and Eosin Stain: Vitamin C treated diabetic albino rats' submandibular salivary gland showed that the gland exhibited its somewhat normal general architecture consisted of predominantly serous acini and duct system. No wide spaces in between the acini were observed. No areas of distortion or loss were seen. The serous acini had narrow lumen and lined by pyramidal cells (black arrow)

with pale basophilic granular cytoplasm and basal rounded nuclei while some acini had intra-acinar vacuolations (white arrow). Blood vessels were seen around these ducts and the excretory ducts showed minimal disruption in their pseudo stratified epithelial lining with some flattened dark nuclei. The gland tissues showed less change and nearly like control group with normal acini except accumulation of large fat globule and dilated intra acinar spaces (Fig 3, A).

Periodic Acid Schiff's (PAS) Stain: PAS stained sections of submandibular glands from diabetic rats after treatment with vitamin C showed from weak to strong positive reaction (presence of carbohydrates). The reaction clear in acini and ducts and more concentration at their basement membrane and around the lumen of ducts (Fig 3, B).

Histo-Chemical Stain: Immunohistochemical stained sections of submandibular glands from diabetic rats after treatment with vitamin C showed from moderate to strong positive Bcl-2 immunoreaction in the cytoplasm of most of acinar and ductal cells (Fig 3, C).

DISCUSSION

Saliva and salivary glands are considered the strongest link between the oral and systemic health. Although the submandibular salivary gland is considered the 2nd largest salivary gland, it produces about 60% of saliva. Saliva has an important role to keep healthy conditions of the oral cavity. Diabetes mellitus disrupted the normal salivary gland structure and function as decreased salivary flow and consequently the natural defense mechanisms against bacterial infection decreased in diabetic patients, thus, it was possible that diabetic patients could be suffering from dental caries and dryness of oral mucosa [2, 21].

The rat was chosen as an experimental model in this research because it can be housed, bred and handled without difficulties. Also, it has a long life span and is relatively disease-free, yet remarkable changes occur when no other diseases are apparent [22]. The induction of diabetes mellitus could be achieved by different methods as total or subtotal pancreatectomy, injection of high doses of glucose and drugs including alloxan or streptozotocin, while Alloxan was the drug of choice to induce diabetes mellitus in this study due its low mortality rate and high tolerance by the experimental animals than other diabetogenic agents. Also, it could be given easily by different routes and its diabetogenic action was rapid and permanent as it destructed the beta cells of islets of Langerhans [23].

In the current work, histological examination of the control albino rats' submandibular glands using H&E stain revealed the normal general architecture in form of mixed acini had narrow lumen and lined by pyramidal cells with pale basophilic granular cytoplasm and basal rounded nuclei. The excretory ducts appeared with wide lumen and lined by pseudo stratified columnar cells. These results were in accordance with many previously reported by many authors also in normal submandibular

gland tissues [24, 25&26]. While the experimentally induced diabetic albino rats showed loss of the normal gland architecture; in form of appearance of wide spaces in between the acini, distortion and loss of some acini and ducts. These alterations were attributed to the appearance of wide spaces in between the acini to the decrease in acinar and duct size occurred after alloxan diabetic induction due to the decrease in released secretions from the acinar and ductal cells as a part of generalized failure of growth, cellular degeneration, nuclear and cytoplasmic atrophy and disorganization of the cell membrane following induction of diabetes [27, 28].

Concerning Periodic acid Schiff (PAS), the stained sections of the submandibular salivary gland of the control group revealed strong positive reaction in both acini and ducts that was observed more at their basement membrane. On examining the submandibular salivary gland of the experimentally induced diabetic group with the same stain, there was a moderate reaction in acini and ducts as compared with that observed in the control group. These variation in PAS reaction in the different examine groups referred to that diabetes resulted in a chronic defect in carbohydrate metabolism due to absolute or relative insulin deficiency and significant reduction in the glycoprotein concentration in diabetic group than control groups [29, 30].

Concerning the immune-histo-chemical examination, the submandibular salivary glands stained sections of control group, showed a positive Bcl-2 immunoreaction that appeared as a brown color in the cytoplasm of acinar and ductal cells. Whereas most tissue sections in the experimentally induced diabetic group showed decrease of the Bcl-2 immunoreaction in comparison with that observed in the control group. These results were agreed with that previously proved, whereas the Bcl-2 (an anti-apoptotic protein) was initially inhibit apoptosis by prohibiting cytochrome-c release from the mitochondria and hyperglycaemia caused down regulated expression of Bcl-2 and increased Bax proteins were detected in diabetic rats [31, 32].

Ascorbic acid (Vitamin C) was one of important antioxidant nutrient, play a role in the prevention and treatment of variety of chronic diseases [12]. In the current study the Vitamin C treated diabetic albino rats' submandibular salivary gland tissue somewhat exhibited its normal histological general architecture by H&E, strong positive PAS reaction and diffuse positive Bcl-2 immunoreaction. The proposed mechanisms by which the antioxidants protect the cells from oxidative stress were including, remove the free radicals in scavenge manner

and were promote the activity of the free radicals[33]. Moreover, that the antioxidant is an important factor in maintaining normal structure and function of salivary glands and may cause complete recovery from adverse effects occurred through diabetes and the gland was returned to its normal picture completely [17].

CONCLUSION

From this study, we concluded that diabetes mellitus caused causes marked effects on the structure and function of the submandibular salivary gland inform of tissue alterations and common complications indicated by the histological, histo-pathological and immune-histochemical examinations. On the other hand the antioxidant (Vitamin C) treatment more or less overcome and decrease these alterations So, diabetic patients should receive antioxidant beside their treatment appropriately to protect them from such complications and to improve quality of their life.

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