eISSN:2708-888X
http://journals.khalijedental.com.ly/index.php/ojs/index

Original article

Parotid and Sublingual Salivary Gland as an Indicator of Antioxidant Efficacy in Experimentally Induced Diabetic Rats

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ABSTRACT

The parotid and sublingual glands were considered the most appropriate tissues to study the effects of diabetes mellitus among the major salivary glands. Globally, diabetes mellitus is a major healthcare concern. It affects bodily tissues and organs in a variety of pathological ways, particularly the major salivary glands. Thirty adult, healthy albino rats were divided into three equal groups of ten animals each. Group I was a control group that did not receive any treatment, group II was experimentally induced to have diabetes mellitus using alloxan, a chemical agent that is more or less safe and permanent, and group III was experimentally induced to have diabetes but treated with ascorbic acid (Vit. C), as an antioxidant drug. All rat groups were sacrificed and tissue samples from their parotid and sublingual glands were prepared. The tissue samples studded histologically using Haematoxylin and Eosin (H&E) stain, histopathologically using Periodic acid Schiff's (PAS) reaction to detect carbohydrates, and immunohistochemically using Beta cell lymphoma-2 (BCL2) as an anti-apoptotic marker. Based on the histology, histopathological, and immunohistochemical significant lesions, the obtained results showed common alterations in the parotid and sublingual glands tissues of diabetic rats. However, compared to untreated animals, rats treated with the antioxidant vitamin C showed fewer tissue changes and lesions. According to the study's findings, vitamin C functions as a helpful antioxidant to assist avoid complications from diabetes.

Keywords: Salivary Glands, Diabetic Rats, Antioxidant, Histopathology, Immunohistochemistry.

Citation: EL-Gusbi G, Hamad L, Ayad M, Shredah M, Soliman A. Strontium Ions Release from Novel Bioactive Glass-Containing Glass Ionomer Cements: A Preliminary Study. 2024;8(2):297–305.
https://doi.org/10.47705/kjdmr.248221

Received: 11/10/24; **accepted**: 04/12/24; **published**: 17/12/24

تم اعتبار الغدد النكفية وتحت اللسان من أكثر الأنسجة ملاءمة لدراسة تأثيرات مرض السكري بين الغدد اللعابية الرئيسية. على مستوى العالم، يعد مرض السكري من أهم المشاكل الصحية. فهو يؤثر على الأنسجة والأعضاء الجسدية بطرق مرضية متنوعة، وخاصة الغدد اللعابية الرئيسية. تم تقسيم ثلاثين فأرًا أبيض بالغًا سليمًا إلى ثلاث مجموعات متساوية تضم كل منها عشرة حيوانات. كانت المجموعة الأولى مجموعة تحكم لم تتلق أي علاج، والمجموعة الثانية تم تحريضها تجريبيًا على الإصابة بمرض السكري باستخدام الألوكسان، وهو عامل كيميائي آمن إلى حد ما ودائم، والمجموعة الثالثة تم تحريضها تجريبيًا على الإصابة بمرض السكري ولكن تم علاجها بحمض الأسكوربيك (فيتامين سي)، كدواء مضاد للأكسدة. تم التضحية بجميع مجموعات الفئران وتم تحضير عينات الأنسجة من الغدد النكفية وتحت اللسان. تم فحص عينات الأنسجة نسيجيًا باستخدام صبغة الهيماتوكسيلين والإيوسين(H&E) ، ونسيجيًا باستخدام تفاعل شيف الدوري الحمضي (PAS) للكشف عن الكربوهيدرات، ومناعيًا كيميائيًا باستخدام ليمفوما الخلايا بيتا 2 (BCL2) كعلامة مضادة للموت الخلوي. بناءً على الآفات المهمة في علم الأنسجة والهيستولوجيا والهيستولوجيا والمناعية الكيميائية، أظهرت النتائج التي تم الحصول عليها تغيرات شائعة في أنسجة الغدة النكفية وتحت اللسان لدى الفئران المصابة بمرض السكري. ومع ذلك، بالمقارنة مع الحيوانات غير المعالجة، أظهرت الفئران المعالجة بمضاد الأكسدة فيتامين سي تغيرات وإصابات أقل في الأنسجة. وفقًا لنتائج الدراسة، يعمل فيتامين سي كمضاد للأكسدة للمساعدة في تجنب المضاعفات الناجمة عن مرض السكري.

INTRODUCTION

The salivary glands are exocrine glands that make, modify and secrete saliva into the oral cavity which hat has digestive, lubricating and protective functions [1]. They are divided into two main types: the major salivary glands, which include the parotid, submandibular and sublingual glands, and the minor salivary glands, which line the mucosa of the upper digestive tract and the overwhelming entirety of the mouth [2]. Rat's salivary glands comprises three primary pairs and numerous smaller glands. The parotid, submaxillary (submandibular or mandibular), and large sublingual glands are the main glands. The minor sublingual, buccal, palatine, and lingual glands are among the minor glands that are not visible on a gross examination [3]. The salivary gland is a tubule-alveolar exocrine complex made of parenchyma and stroma. The gland is divided into lobes and then further subdivided into lobules by the stroma, which is made up of a large connective tissue capsule that is rich in collagen fibers and numerous septa [4].

The strongest connection between oral and systemic health is thought to be saliva and salivary glands. In addition to providing an aqueous solvent required for taste, saliva also lubricates food to aid in deglutition, moistens the buccal mucosa, which is crucial for speaking, and secretes antimicrobial compounds such lactoferrin, lysozymes, and IgA [5]. The two main categories of protein discharges seen in saliva are mucous and serous. The ptyalin enzyme, which is required for starch digestion, is present in the serous discharge. By creating a barrier against desiccation, lubricating, and protecting the oral tissues from mechanical and chemical shocks, the mucous secretion, which contains mucin, is the other secretion [6]. Additionally, histatins, which are antifungal proteins, found in saliva, are strong inhibitors of candida, which is typically maintained at very low levels in the mouth [7].

Diabetes mellitus, which has various adverse effects on the body's tissues and organs, is a major global health concern. Hyperglycemia is the result of a chronic metabolic disorder that impairs body cells' capacity to use blood glucose. The major salivary glands were one of the organs that were thought to be a good place to study the effects of diabetes mellitus [8]. Type 1 and type 2 diabetes (T1 D and T2D) are the two most prevalent types of the disease at the moment. While Type 2 diabetes is a dual illness that arises when the B-cells are unable to correct for insulin resistance by increasing their secretion of insulin, Type 1 diabetic patients experience pancreatic B cell death, which is typically immune-mediated [9]. Diabetes mellitus has been shown to significantly affect the metabolism of fats and carbohydrates in the tissues of both diabetic patients and experimental animals. Moreover, there a decrease in proteins in salivary gland granules, particularly several of the well-known proteins linked to apoptosis, such as the BCl2 family [10]. Antioxidants protect diabetic cells from oxidative stress, scavenge free radicals directly, and increase the synthesis of enzymes known as superoxide-dismutase-like components [11, 12].

The relationship between diabetes mellitus and the primary salivary gland dysfunction is clearly complex and unclear. Therefore, the aim of the current study was to evaluate the histological and histopathological differences in the experimentally induced diabetic rats' parotid and sublingual salivary glands, and the impact of antioxidant (Vitamin C) therapy on these anticipated histological alterations examined.

METHODS

Animals

A total number of thirty rats of body weights ranged between 200-220 g, (4-6 months age), obtained from colony reared in animal house of National Research Center, Egypt. Rats were kept in polycarbonate cages in a controlled laboratory setting. They fed a standard, balanced diet and given water for at least a week prior to the experiment, and they kept at room temperature with a consistent 12-hour light/dark cycle according to the procedure outlined by Hassanain et al. [13].

Study design

Rats in the experiment were divided into three equal groups of ten each: Control group (GI); Rats in this group were given a single injection of sterile saline (0.9% Na Cl) at a dose of 1 milliliter per kilogram, but they were not given any other therapy. Induced diabetic rats (GII); A fresh solution of 5% alloxan monohydrate dissolved in physiological saline was used to experimentally induce diabetes mellitus in this group. According to the procedure outlined by Elfadaly et al., [14], each rat was given an intra-peritoneal injection of 1 ml of the prepared alloxan solution (200 mg/kg body weight) as a single dose under fully aseptic circumstances after fasting for the whole night. Vitamin C treated diabetic rats (GIII); Initially, this group of rats received the same amount of alloxan as group II. One week later, the rats received s/c injections of 40 mg/kg of body weight of ascorbic acid (Vitamin C) for varying durations of one, two, four, eight, and ten weeks. Two weeks following the final ascorbic acid treatment, the animals were sacrificed [15].

Diabetes detection

To measure blood glucose levels, three samples were obtained from each rat: one before diabetes induction, one 24 hours following induction, and one right before scarification. According to He et al. [16] rats with blood glucose levels of 200 mg/dl or more classified as diabetics, while the normal range is 70–110 mg/dl. After being allowed to live for ninety days, the diabetic rats were sacrificed.

Preparation of tissue samples

Ether inhalation anesthesia was used to sacrifice each of the three groups' animals then parotid and sublingual gland were gently removed while the head was stretched. Small tissue samples 0.5 x 0.5 cm cut and immersed in 10% formalin solution. It underwent additional processing and was embedded in paraffin wax. Sections of tissue specimen measuring 5–6 um were prepared for histological and immunohistochemical analysis [17].

Histological study

Haematoxylin and Eosin (H&E) stains are a standard technique for examining the general histological structure of the glands, identifying acidophilic and basophilic structures, and detecting carbohydrates using Periodic Acid Schiff's (PAS) reaction in accordance with methods of Abd El Wahab et al. [18].

Immunohistochemical analysis

Beta cell lymphoma-2 (Bcl-2) was localized using Avidin-biotin peroxidase immunohistochemical responses as an anti-apoptotic marker (Sigma Laboratories). A dark tint in the cytoplasm of the acinar and ductal epithelial cells signified a successful Bcl-2 immunoreaction [19].

RESULTS

Examination of H&E stained parotid and sublingual gland tissue sections of the control rats displayed mixed acini, serous and mucous acini with a flat nucleus, and the overall normal architecture of the main salivary glands (black arrow). Pyramidal cells with flat nuclei and weak, foamy basophilic cytoplasm lined the acini's small lumen (blue arrows). The duct system consisted of several duct types: the kidney-shaped granular convoluted tubules lined by columnar epithelium with eosinophilic cytoplasm, the striated ducts lined by columnar cells with central rounded nuclei and eosinophilic cytoplasm with prominent basal striations, and the intercalated ducts, which were small and rounded and lined by cuboidal epithelium with central rounded nuclei. On occasion, the blood vessels surrounding these ducts were visible (Fig 1A & Fig. 2A).

Examination of the PAS stained parotid and sublingual gland tissue sections of the control rats showed an intense positive reaction (carbohydrate presence) in both ducts and acini, with the presence of carbohydrates being more noticeable at the basement membrane. Particularly, the striated ducts displayed a more concentrated positive response surrounding the

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lumen, +ve results in the acinar cells black arrow but –ve result in the duct (red arrow) (Fig 1 B & Fig. 2B).
Histo-Chemical stain

Examination of the immune-histo-chemical stained parotid and sublingual gland tissue sections of the control rats for detection of BCL2 as anti-apoptotic marker revealed a strong positive Bcl-2 immunoreaction that appeared as a clear brown color in the cytoplasm of acinar and ductal cells
(Fig. 1C & Fig. 2C).

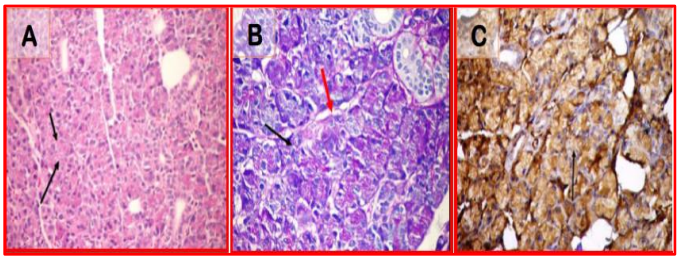

Fig (1): Control rat's parotid gland showed normal structure-stained H&E (**A**), strong positive PAS reaction (**B**) and clear brown positive Bcl-2 immunoperoxidase immunoreaction **(C) (X400)**.

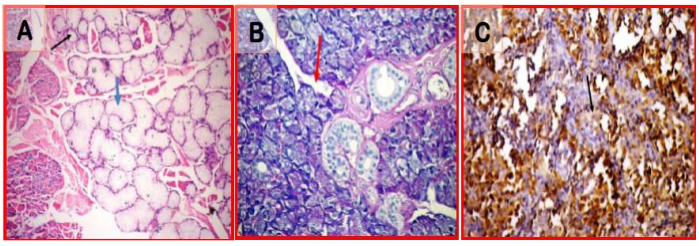

Fig (2): Control rat's Sublingual gland showed normal structure stained H&E, mucous acini (black arrow) & serous acini (Blue arrow).(**A**), strong positive PAS reaction, in the acinar cells (black arrow) but –ve result in the duct (red arrow) (**B**) and clear brown positive Bcl-2 immunoperoxidase immunoreaction **(C) (X400)**.

Examination of H&E stained parotid and sublingual gland tissue sections of the experimentally induced diabetic rats revealed a lack of the typical gland structure. The mixed acini displayed dilated inter-acinar blood vessels (black arrows), serous acini vesiculation, and a reduction in size. In certain portions, there were large gaps between the acini, areas of intra-acinar fatty cell infiltrations and vacuolations, and distortion and loss of some acini and ducts. Some cells had tiny, very basophilic nuclei (Fig 3A & Fig. 4A).

Examination of the PAS stained parotid and sublingual gland tissue sections of the experimentally induced diabetic rats demonstrated a moderate, reduced, or nonexistent (negative) PAS reaction in the acini and ducts when compared to the control group because these diabetic animals produced fewer or less carbohydrates (Fig 3B & Fig. 4B).

Sections of diabetic rats' parotid and sublingual gland tissue stained with the immune-histo-chemical stain for detection of BCL2 as anti-apoptotic marker revealed a strong positive Bcl-2 immunoreaction revealed light brown, weakly positive Bcl-2 immunoreaction around intra-acinar fatty cell infiltrations in certain regions. However, compared to the control group, the majority of others displayed a decrease in the Bcl-2 immunoreaction (Figs. 3C & Fig. 4C).

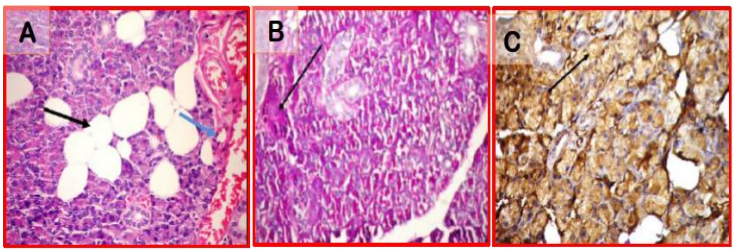

Fig (3): Diabetic albino rat's parotid gland showed loss of normal structure congestion of the periacinar and interlobular blood vessels (Blue arrow) with the presence of large fat globule (Black arrow) stained with H&E (**A**), +ve PAS reaction in the acinar cells (arrow) (**B**) and marked immunoperoxidase reactive materials (brown color) in the acinar cells **(C)** (X400).

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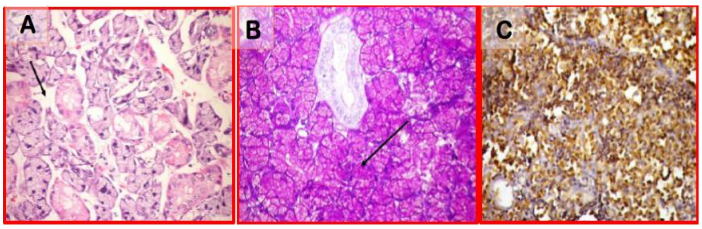

Fig (4): Diabetic albino rat's sublingual gland showed necrosis of the acini and ducts epithelial lining phy and vacuolations of some acini (black arrow) stained H&E (**A**), strong +ve PAS reaction in the acinar cells (Arrow (**B**) and very slight immunoperoxidase reactive in the acinar cells **(C)** (X400).

Examination of H&E stained parotid and sublingual gland tissue sections of the Vitamin C treated diabetic rats displayed somewhat normal general architecture. Predominantly serous acini and duct system, no wide spaces in between the acini were observed and no areas of distortion or loss were seen. The intercalated ducts were noticed in between the acini, the granular convoluted tubules were lined by simple columnar epithelium with eosinophilic cytoplasm, and basal rounded nuclei. In addition, salivary gland showed less change, nearly like control group with normal acini except accumulation of large fat globule, and dilated intra acinar spaces (Fig 5A & Fig. 6A).

Periodic acid Schiff's stain

Examination of the PAS stained parotid and sublingual gland tissue sections from diabetic rats after treatment with vitamin C showed from weak to strong positive reaction (presence of carbohydrates) in the acini and ducts the reaction clear and more concentration at their basement membrane and around the lumen of some striated ducts (Fig 5B & Fig. 6B).

Examination of the immune-histo-chemical stained parotid and sublingual gland tissue sections from diabetic rats after treatment with vitamin C for detection of Bcl-2 showed, from moderate to strong positive Bcl-2 immunoreaction in the cytoplasm of most of acinar and ductal cells (Figs. 5C & Fig. 6C).

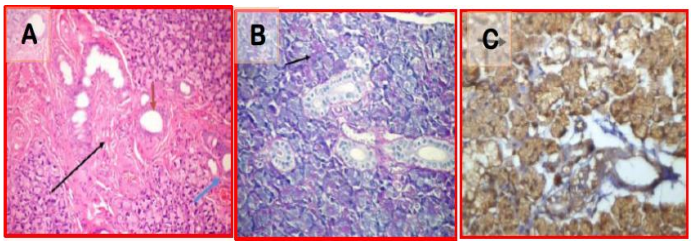

Fig (5): Parotid gland of the diabetic vitamin C treated rats showed almost severe connective tissue proliferation of the interlobular tissue (Black arrows) with few inflammatory cells infiltration stained H&E (**A**), clear slight positive PAS reaction in the acinar cells (Arrow) (**B**) and marked immunoperoxidase reaction (black arrow) in the acinar cells **(C)** (X400).

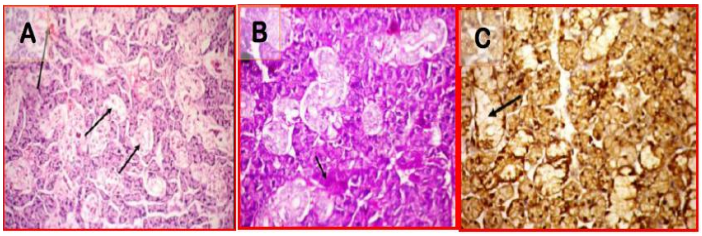

Fig (6): Sublingual gland of the diabetic vitamin C treated rats showed congestion and severe necrosis of the mucinous acini (Arrows) H&E stain (**A**), strong positive PAS reaction in the acinar cells (arrow) (**B**) and slight immunoperoxidase reactive materials **(C)** (X400).

DISCUSSION

The rat was used as the experimental model for this study due to its ease of handling, breeding, and housing. Additionally, it has a long lifespan and is comparatively disease-free, yet when no other diseases are visible, remarkable changes take place [20]. The strongest connection between oral and systemic health thought to be saliva and salivary glands. Saliva plays a crucial part in maintaining the oral cavity's health [21]. Diabetes consequences can be extremely debilitating, but they are usually not life threatening, complications frequently get worse throughout the course of a patient's lifetime and linked to high medical expenses [22]. One of the most prevalent metabolic diseases is diabetes mellitus. It may cause by insufficient insulin or by body cells that are resistant to insulin despite excessive synthesis of it

[23]. It was important to highlight the impact of diabetes mellitus on the main salivary glands, one of the body's essential organs, given the rising prevalence of the disease in the population and its numerous harmful effects [24]. Diabetes mellitus can cause dental caries and dryness of the oral mucosa because it alters the normal structure and function of the salivary glands, resulting in decreased salivary flow and a decrease in the body's natural defenses against bacterial infections [25].

There are several ways to induce diabetes mellitus, including complete or subtotal pancreatectomy, high-dose glucose injections, and chemical medications like alloxan or streptozotocin [26]. In this study, the preferred medication for causing diabetes mellitus was alloxan. Compared to other diabetogenic drugs, it had a lower mortality rate and a higher tolerance by the experimental animals. Additionally, it was easily administered through a variety of methods, and because it destroyed the beta cells in the islets of Langerhans, it had a quick and durable diabetogenic effect [27]. Chemically generated diabetes using alloxan is important to highlight each of these experimental diabetes models' advantages and disadvantages [28].

In the current work, histological examination of the control rats' parotid and sublingual glands using H& E stain revealed showed the typical overall morphology as mixed acini with a narrow lumen lined by pyramidal cells that had basal rounded nuclei and pale basophilic granular cytoplasm. The excretory ducts were lined with pseudo-stratified columnar cells and exhibited a wide lumen. These findings were consistent with other authors' earlier reports of normal histological features of parotid and sublingual glands [29, 30 & 31].

The experimentally induced diabetic rats showed loss of the normal parotid and sublingual glands architecture; in the form of certain acini and ducts becoming distorted or lost, or the formation of large gaps between the acini. Following alloxan diabetic induction, the histological changes were ascribed to the development of large spaces between the acini and the reduction in acinar. The duct size brought on by the reduction in secretions released from the acinar and ductal cells because of cellular degeneration, nuclear and cytoplasmic atrophy, and disorder of the cell membrane [8, 32]. The loss of acini by the presence of macrophages containing acinar cell debris because of the degeneration occurred after diabetes induction. Moreover, extensive cellular degeneration, nuclear and cytoplasmic atrophy and disorganization of the cell membrane resulted from diabetes induction [33].

Concerning Periodic acid Schiff (PAS), the stained sections of the parotid and sublingual glands of the control rats group shown an intense positive response in the ducts and acini, with a greater concentration in the basement membrane. On examining the parotid and sublingual salivary glands of the experimentally induced diabetic rats group with the same stain, Comparing the acini and ducts to the control group, a moderate reactivity was seen. Due to absolute or relative insulin insufficiency, diabetes caused a persistent impairment in carbohydrate metabolism, as seen by the variation in PAS reaction across the various examination groups. Additionally, the diabetic group's glycoprotein concentration was much lower than that of the control groups [34, 35].

Concerning the immune-histo-chemical examination, the parotid and sublingual salivary glands stained sections of control group, revealed a positive Bcl-2 immunoreaction that appeared as a brown color in the cytoplasm of acinar and ductal cells. In contrast to the control group, the majority of tissue sections in the experimentally generated diabetic group displayed a decrease in the Bcl-2 immunoreaction. These findings were consistent with earlier research showing that the anti-apoptotic protein Bcl-2 first inhibited apoptosis by preventing cytochrome-c release from the mitochondria. Hyperglycemia resulted in down-regulated Bcl-2 expression and elevated Bax proteins in diabetic rats [36, 37].

Ascorbic acid (Vitamin C), is a crucial antioxidant ingredient that helps prevent and treat a number of chronic diseases [38]. Vitamin C, has been suggested to protect cells from oxidative stress through the

following mechanisms: scavenging free radicals and promoting their activity [39].

In the current study the Vitamin C treated diabetic rats' parotid and sublingual salivary glands tissue showed a slightly normal histological general architecture by H&, a strong positive PAS reaction, and a diffusely positive Bcl-2 immunoreaction, The suggested methods by which antioxidants shield cells from oxidative stress included absorbing free radicals and increasing their activity [40]. Furthermore, antioxidants are crucial for preserving the salivary glands' natural structure and function. They can also help the salivary glands fully recover from the negative effects of diabetes and return to its original state [41].

CONCLUSION

This study proved that diabetes mellitus is consider as a severe metabolic illness that reduced saliva production and has a number of noticeable impacts on function and tissues of parotid and sub lingual salivary glands inform of tissue alterations and common complications indicated by the histological, histo-pathological and immune-histochemical examinations. However, the antioxidant (Vitamin C) treatment largely reverses and lessens these changes. Therefore, diabetic patients should obtain antioxidants in addition to their prescribed medications in order to prevent problems and enhance their quality of life.

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