The Influence Of Ozonized olive oil gel on the dorsal surface of the tongue ofstreptozotocin induced diabetic albino rats.

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Abstract: Background: It has been a growing interest recently in use of non-medication methods in management of medical diseases as diabetes. Nevertheless, most studies have focused only on their ability to maintain blood glucose levels, and have not been investigated for their beneficial effects on secondary complications of diabetes such as oral lesions. Therefore, the current study aimed. **The aim of the current study is** to evaluate the possible effect of ozone in ameliorating the histological changes in the lingual papillae of diabetic rats experimentally induced by streptozotocin. **Methods**: Thirty adult male albino rats were divided into 3 equal groups: group I; (control group), group II; (diabetic group):diabetes was induced by a single intraperitoneal injection of streptozotocin in a dose of 65 mg/kg and group III;(Ozone group) rats were treated as in diabetic group for 4 weeks then ozone gel was applied on the tongue mucosa daily for 2 weeks. By the end of the experimental periods all animals were sacrificed and the tongue of all rats were dissected and processed for light and scanning electron microscopic examinations. **Results**: Examination of dorsal surface of diabetic rats' tongues revealed numerous filiform papillae with evidently disturbed orientation and inclination. Some of them depicted notched or bifurcated ends; others were severely destructed with desquamation of its epithelial covering. There were hyperkeratosis and markedly reduced CT papillae. Disfigured fungi form papillae with vacuolated taste buds depicting peripheral arrangement of the cells and empty center were also seen. Dorsal surface of rats' tongues of the ozone group revealed almost normal direction, distribution and structure of the papillae and taste buds. **Conclusion**: From the present study we concluded that diabetes has a deleterious effect on tongue papillae and taste buds. Fortunately ozone gel provided considerable treatment of these effects.

[Heba Mohamed Eltokhey. T**he Influence of Ozonized olive oil gel on the dorsal surface of the tongue ofstreptozoptocininduced diabetic albino rats.** *J Am Sci* 2013;9(10):193-201]. (ISSN: 1545-1003). http://www.jofamericanscience.org. 25

Keywords: Ozone, Diabetes, Dorsal surface of the tongue, Tongue papillae, and Taste buds.

1. Introduction

Diabetes mellitus is a syndrome characterized by disordered metabolism and inappropriately high blood sugar (hyperglycaemia) resulting from either low levels of the hormoneinsulin or from abnormal resistance to insulin's effects coupled with inadequate levels of insulin secretion to compensate.(1)

In 2006, according to the World Health Organization, at least 171 million people worldwide suffer from diabetes. Its incidence is increasing rapidly, and it is estimated that by the year 2030, this number will be doubled. Diabetes mellitus occurs throughout the world, but is more common (especially type 2) in the more developed countries. The greatest increase in prevalence is, however, expected to occur in Asia and Africa, where most patients will likely be found by 2030. (2,3)

Diabetes can cause many complications including hypoglycemia, ketoacidosis or nonketotic hyperosmolar coma, cardiovascular diseases, chronic renal failure, retinal damage, nerve damage, and microvascular damage, which may cause impotence and poor healing.(4)

Thickened blood vessels in diabetic patients can impair the efficiency of the flow of nutrients and removal of wastes from body tissues. This impaired blood flow can weaken the gums and bones, making them more susceptible to infection. (2)

The most common oral complications are the periodontal diseases, dental caries, asymptomatic enlargement of salivary glands and dry mouth. In addition, impairment of taste, atrophic lesions of the tongue, leukoplakia, lichen orisplanus, and tumors might be the consequence of chronic inflammation and changes in innervation. Burning mouth syndrome has been attributed secondarily to diabetes, poor glycemic control, and diabetic neuropathy. (5)

Free radical mediated oxidative stress is mainly involved in the pathogenesis of diabetic complications. Proteins and lipids are among the prime targets for oxidative stress. (6)

It is currently well documented that free radical mediated oxidative stress is involved in the pathogenesis of diabetic complications.The mechanisms involved in the increased oxidative stress in diabetes include not only oxygen free radical generation due to non-enzymatic glycosylation, auto-

oxidation of glycation products, but also changes in the tissue content and activity of antioxidant defense systems. Increased levels of the products of oxidative damage to lipids have been detected in serum of diabetic patients, and their presence correlates with the development of complications.[7,8]

Moreover, many studies [9,10] attributed oral complications in patients with uncontrolled diabetes to increased glucose concentrationsin the saliva (salivary hyperglycemia). When the normal environment of the oral cavity is alteredbecause of a decrease in salivary flow or alteration in salivarycomposition, a healthy mouth becomes susceptible to atrophic changes and cracking oralmucosa is an eventual complication from insufficient salivaryproduction. They reported that mucositis, ulcers and desquamation,as well as an inflammed, depapillated tongue, are common manifestations.

Ozone therapy is one of the modern non-medication methods of treatment. It is being used for more than 100 years. Medical reports on successful application of ozone in therapy of different diseases and studies of its effects caused a rapid growing interest in it. Some other factors were responsible for its wide spreading, such as simplicity of performance, good tolerance by patients, absence of side-effects or adverse reactions and high medical-social and economic efficiency. Even though ozone therapy is still being ignored by most of medical establishment because of facts that gaseous ozone is quite toxic and has strong oxidative properties. [11]

Ozone causes the synthesis of biologically active substances such as interleukins and prostaglandins. It influences cellular and humoral immune system, activates function of macrophages and increases sensitivity of micro-organisms to phagocytosis.[12,13]

At he cellular level, ozone brings about the rise of pO2 in tissues and improves transportation of oxygen in blood, which results in change of cellular metabolism, activation of aerobic process and use of energetic resources. It prevents formation of erythrocytes aggregates and increases their contact surface for oxygen transportation. Ozone causes secretion of vasodilator such as NO, which are responsible for dilatation of arterioles and venules. It also activates mechanisms of protein synthesis, increase amount of ribosomes and mitochondria in cells. These changes explain elevation of functional activity and regeneration potential of tissue and organ in response to ozone. [14]

2. Material and Methods

Thirty adult male albino rats with body weight ranging between 180-200 gm were housed under the same conditions as regards light cycles (12h) and temperature (21-23°C). They received standard diet with free access to water and divided into three equal groups:

Group I: Control group that received a single intraperitoneal injection of saline.

Group II: Diabetic group; diabetes was induced by a single intraperitoneal injection of streptozotocin (Sigma Chemical Co., Egypt) in a dose of 65 mg/kg body weight. [11]After 4 weeks, blood samples were collected using capillary tube for the assessment of blood glucose levels.

Group III: Ozone group; rats were treated as in diabetic group. After 4 weeks, blood samples were collected using capillary tube for the assessment of blood glucose levels. Then, Ozonized olive oil gel was daily applied as a very thin film on the dorsal surface of the tongue for 2 weeks. The Experimental protocol with designed in accordance with the guidelines for the responsible use in animals in research as a part of the scientific research ethics recommendation.[15]

By the end of experimental periods, animals of each group were exposed to light ether anesthesia. Tongues were carefully dissected and processed for light and scanning electron microscopic examination.

I] Preparation of the specimen for examination by light microscopy:

Specimen from all rats' tongue were fixed in 10% formol saline. Paraffin blocks were prepared and 5µ sections were stained using:

Haematoxylin and Eosin (H&E) stain. [16]

II] Preparation of the specimen for examination by scanning electron microscopy

Specimen from all rats' dorasal surface of tongue mucosa were immediately fixed in 2.5% gluteraldhyde in 0.1M phosphate buffer (pH 7.4). The sample were treated with 8N hydrochloric acid at 60° for 30 minutes to remove mucus from the surface of the tongue and prepared for scanning electron microscope study. [16] The dorsal surface of the tongue was examined and photographed with JEOL, JSM-53009 scanning electron microscope in EM unit, Faculty of Science, Alexandria University.

2. Results

The blood glucose level in rats of diabeteic group was high (above 300 mg/dl)that confirm establishment of diabetes.[17]

H&E stain

Dorsal surface of the tongue of control group showed normal arrangement and shape of papillae. Filiform papillae appeared sharp conical covered with stratified squamous epithelium with thin regular keratin layer. Well formed connective tissue and

muscle fibers running in different direction were also noticed (Figure 1a). Fungiform papillae were seen in-between filiform ones with broader surface and wide vascular connective tissue core. The covering keratin appeared relatively thin. A single well defined taste bud was seen at the summit of each papilla (Figure 1b).

Examination of the tongues of diabetic rats revealed loss of the normal appearance of filiform papillae. Most of them showed flattening, loss of their characteristic conical shape, with evident hyperkeratosis. Their epithelial lining showed marked thickening with many mitotic figures and ill defined CT papillae. Most of the examined fungiform papillae appeared dome shaped covered with thickened keratin layer. Taste buds appeared vacuolated with peripheral arrangement of the cells and empty center. Some fungiform papillae appeared elongated with narrow tips with vacuolated taste buds and separation of its covering keratin (Figures 2, 3).

Dorsal surface of the tongue of the ozone group showed almost normal structure of filliform papillae including their covering epithelium and keratin (Figure 4a&b). However there were some regions that showed short ill definedfiliform papillae surround elongated fungiform papilla. The taste bud showed almost regular arrangement of taste cells (Figure 5a&b).

Scanning electron microscopic results:

Examination of the dorsal surface of the control rats' tongues showed numerous sharp conical projections of filiform papillae with uniform keratinized tips arranged in parallel regular rows depicting constant antero-posterior direction towards the tongue root (figure 6). The covering epithelial cells appeared with smooth linear edges. Few rough keratinized cells were seen at their bases. The interpapillary epithelial surface exhibited fine regular lines (figure 7)

Scattered fungiform papillae with broad apices were seen among numerous filiform ones. Thin epithelial smooth cells were depicted around centrally located well defined regular gustatory pore surrounded by shallow indentation (figure 8).

Examination of dorsal surface diabetic rats' tongues revealed numerous filiform papillae with evidently disturbed orientation and inclination (Figure 9). Some of them depicted bifid or bifurcated tapering ends. Others were covered by constricted keratin. Blunt eroded tips of some filiform papillae were also seen. Severely destructed filiform papillae with desquamation of its epithelial covering were depicted (figure 10).

The interpapillary ridges are irregular, sharp and heavily keratinized.Fungiform papillae with wrinkled heavily keratinized epithelial covering depicted. The gustatory pore appears depressed irregular and ill-defined (figure 11a&b).

Examination of dorsal surface rats' tongues of the ozone group revealed almost normal direction and distribution of filiform papillae (figure 12). However, few filiform papillae depicted blunt or serrated tips (figure 13). The fungiform papillae showed almost control image with regular smooth covering and well defined taste buds (figure 14).

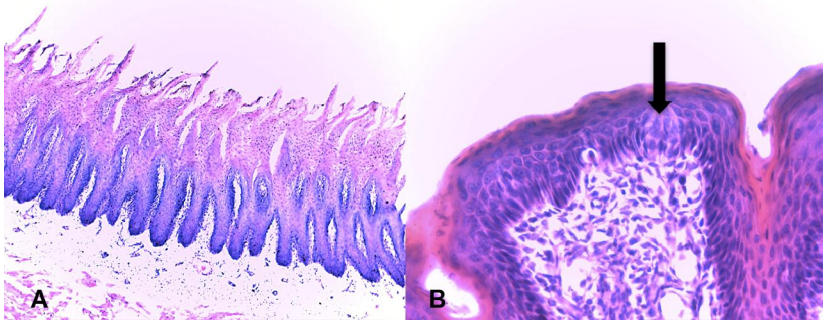

Figure 1: Photomicrographs of the dorsal surface of the control tongue showing:
a- sharp conical projections of filiform papillae with thin smooth keratinized epithelial covering. Skeletal muscle fibers running in different directions are seen underneath the papillae.
b: A fungiform papilla showing normal barrel like intraepithelial taste bud (arrow)
H&E stain. Mic Mag a- X 100; b- X 400

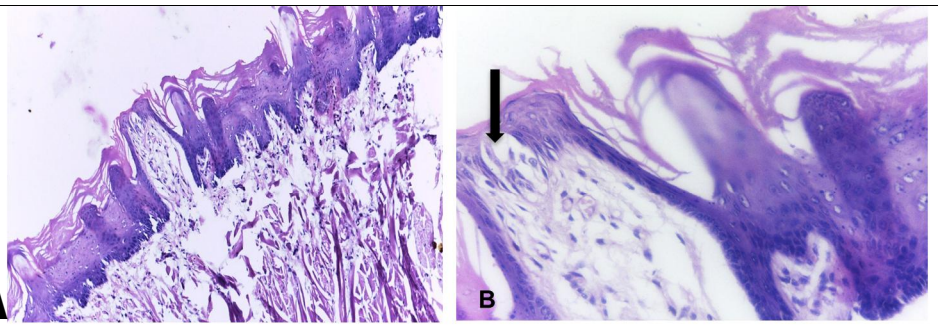

Figure 2:
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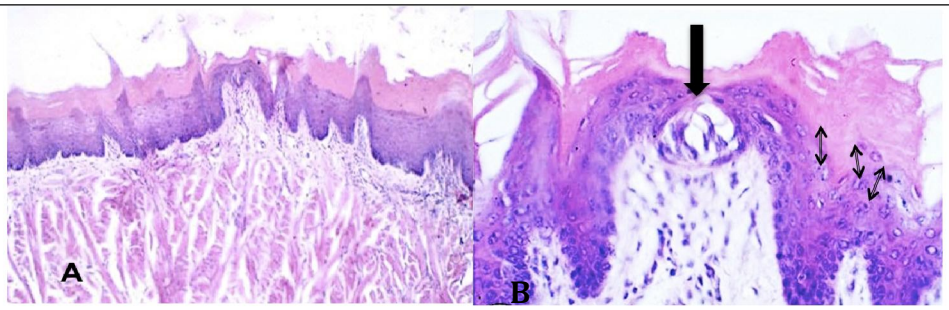

Figure 3:
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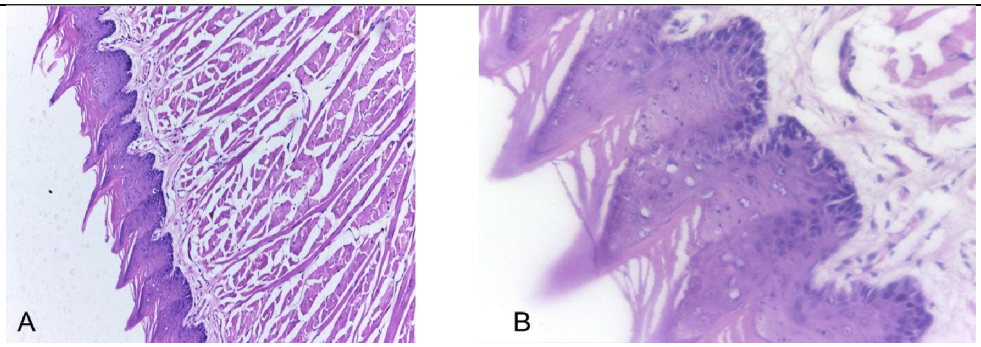

Figure 4a&b: Photomicrographs of the dorsal surface of rat tongue of the Ozone group showing almost normal finger like appearance of filiform papillae covered with thin keratin and fairly normal connective tissue papillae
H&E stain. Mic Mag a- X 100; b- X 400

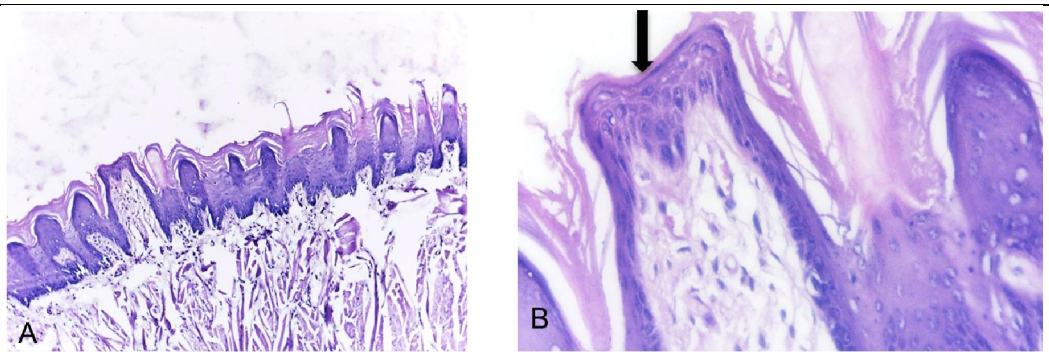

Figure 5 a &b: Photomicrographs of the dorsal surface of rat tongue of the Ozone treated group showing: short ill definedfilliform papillae surround elongated fungiform papilla. The taste bud showed almost regular arrangement of taste cells
H&E stain. Mic Mag a- X 100; b- X 400

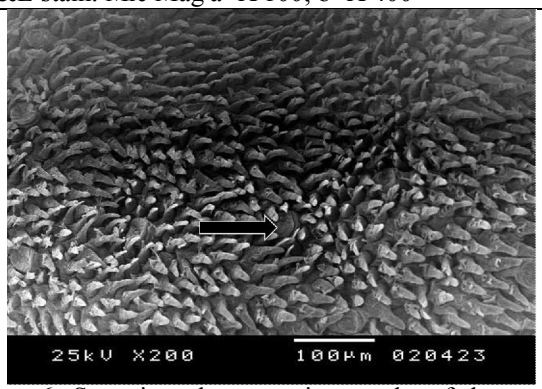

Figure 6: Scanning electron micrographs of the control rat's tongue showing the regular parallel rows of long conical filiform papillae with tapering ends and uniform antero-posterior inclination. Mushroom-like fungiform papillae are interposed in between the numerous filiform ones (arrow).MicMag:X 200

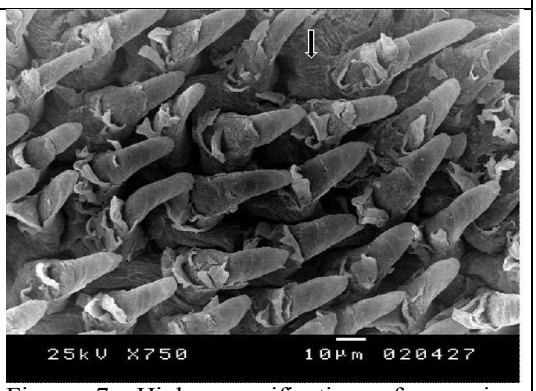

Figure 7: High magnification of scanning electron micrographs of the control rat's tongue showing sharp and regularly conical filiform papillae with keratinized tips. Note: the interpapillary surface depicts fine regular lines(arrow). MicMag:X 750

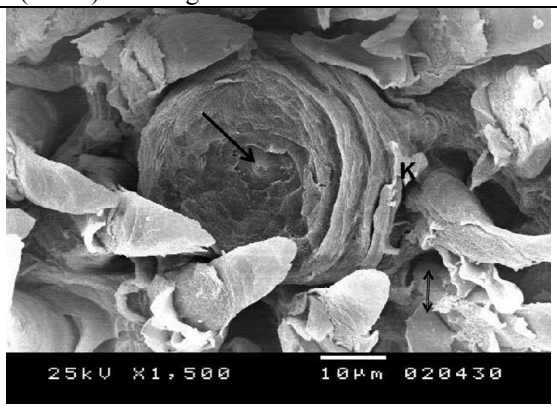

Figure 8: Scanning electron micrographs of the control rat's tongue showing top surface of a fungiform papilla covered by several regular epithelial cells. Some of these cells show keratinization (k). A well defined regular gustatory pore surrounded by shallow indentation in its center is also seen (arrow). Note few rough keratinized cells seen at the bases of the surrounding filiform papillae (double headed arrow). Mic Mag: X1,500

Figure 9: Scanning electron micrographs of diabetic rat's tongue showing numerous filiform papillae with evidently disturbed orientation and inclination.
Mic Mag X 100.

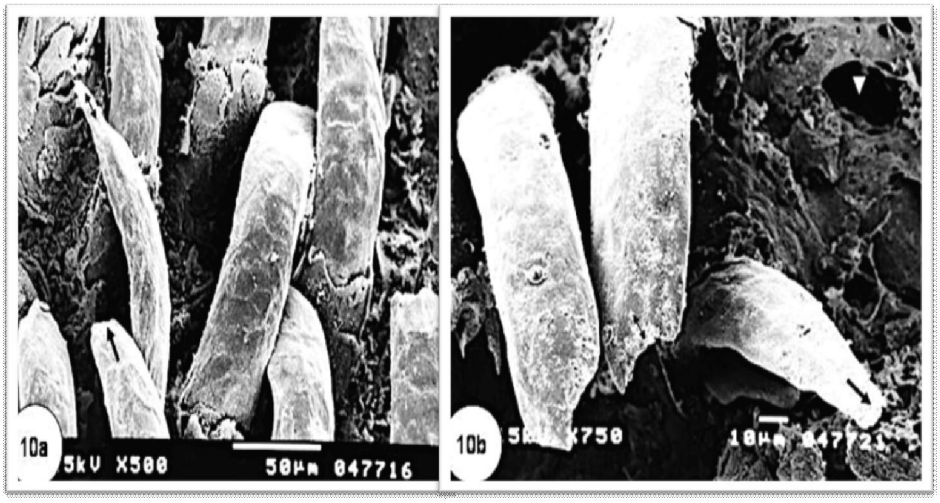

Figure 10: Scanning electron micrographs of other diabetic rat's tongue showing:
a- High magnification of filiform papillae. Most of them show keratinized blunt ends (arrow) while few exhibit pointed ends with constricted keratin covering (double headed arrow).
Note the rough prominent interpapillary ridges
b- Higher magnification of filiform papillae with irregular blunt tips or concave eroded ones (double headed arrow).
Note an adjacent area devoid of lingual papillae (arrow head)
Mic Mag: a- X 500; b- X 750

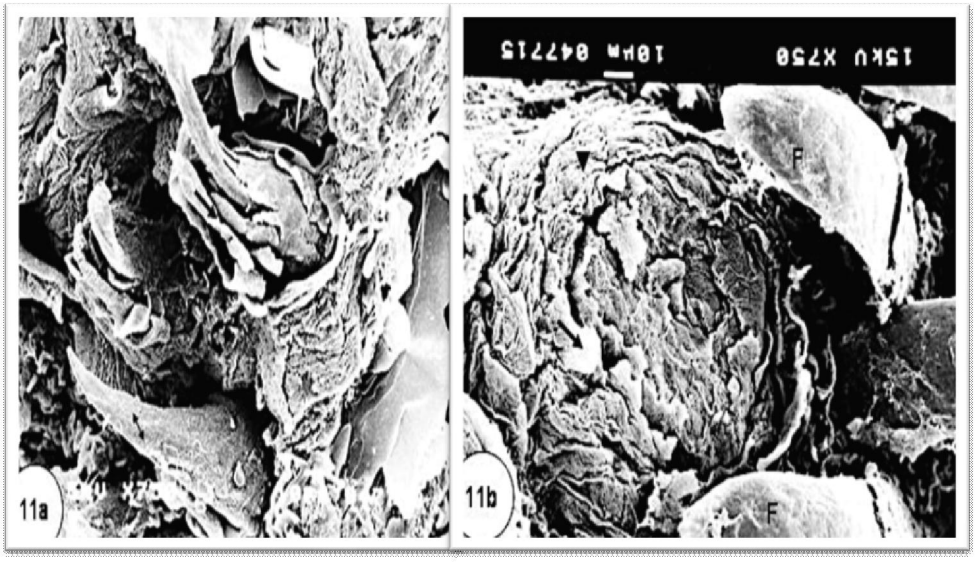

Figure 11: Scanning electron micrographs of diabetic rats' tongues showing:
a-Completely disorganized distribution of filiform papillae which appear severely destructed with desquamat its epithelial covering (arrow). The interpapillary ridges are irregular, sharp and heavily keratinized (curved a A single nearly normal filiform papilla is also seen (double headed arrow).
b-Fungiform papilla with wrinkled keratinized epithelial covering (arrow). The gustatory pore appears dep irregular and ill-defined (double headed arrow). F; filiform papillae
Mic Mag: a&b- X 750

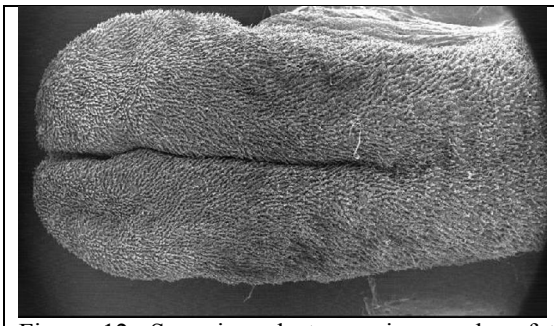

Figure 12: Scanning electron micrographs of the ozone rat's tongue showing almost normaluniformantero-posterior inclination of regular parallel rows of long conical filiform papillae with tapering ends and.
Mic Mag X 50

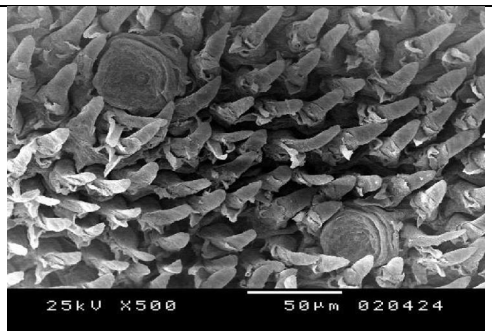

Figure 13: Scanning electron micrographs of the ozone rat's tongue showing nearly normal filiform papillae with scattered fungiform papillae.
Mic Mag X 500

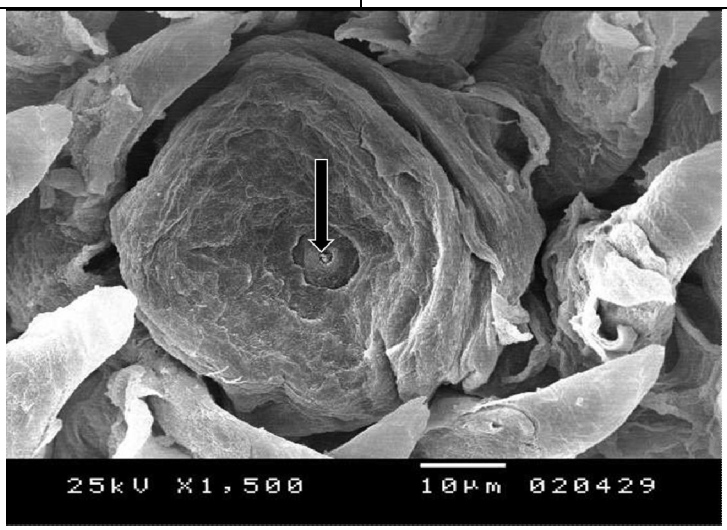

Figure 14: Scanning electron micrographs of the ozone rat's tongue showing almost normal fungiform papilla with regular epithelial covering and a well defined regular gustatory pore (arrow). Some filiform papillae still depict blunt or serrated tips (arrow head).
MicMag:X 1500

4. Discussion:

It has beena growing interest recently in use of non-medication methods in management of medical diseases as diabetes. Nevertheless, most studies have focusedonly on their ability to maintain blood glucose levels, andhave not been investigated for their beneficial effects on secondarycomplications of diabetes such as oral lesions. Therefore, the current study aimed to evaluate the possible curative effect of ozone in ameliorating the histological changes in the lingual papillae of diabetic rats experimentally induced by streptozotocin. Both light and scanning electron microscopes were utilized to study the morphological changes in the lingual papillae and taste buds.

The detected increased blood glucose level in diabetic rat after one week of streptozoptocin injection in coincided with that found by Clara et al. [(17)]They stated thatblood glucose averaged 6.4 ± 0.2 mmol/l in basal conditions and rose to 23.3 ± 1.9 mmol/l 15 h after STZ administration.

We used in this study ozonized olive oil gel instead of ozonized water as the half life of ozone is extremely short for achieving sufficient disinfection or effect exerting a bactericidal effect on cells. Thus we focused on the newly developed ozone containing gel capable for maintaining the ozone effect for a long time. [(18)]

The present results reflected atrophic changes in the lingual papillae of diabetic rats in the form of distorted filiform papillae with alteration in the normal inclination. They exhibited -flat, splitted, bifid or branched tips. This unique pattern was depicted in light microscopic examination covered with excessive keratin. Severe degenerated papillae with eroded apical parts or completely desquamated

covering cells were also depicted by scanning electron microscopic examination. In consistence with our results, Batbayar *et al.* [5] attributed these complications to chronic inflammation, changes in innervations and microvasculature secondary to diabetes.

The observed flattening of the filiform papillae in the present study were also recorded by Naganuma *et al.* [19]They observed flattening of the papillae with exfoliation as an atrophic change in a scanning electron microscopic study on the effect of aging on taste buds. The altered blood and nerve supply associated with diabetes could give effect like aging.

Examination of the diabetic group revealed hyperkeratosis in the form increased thickness of keratin layer as depicted by light and scanning electron microscopes. In accordance with our results, Rodgers *et al.* [20] ,in a study on expression of intracellular filaments, reported that keratin-associated proteins and keratin complexes gene expression were increased in diabetic mice.

The observed increase in mitotic figure in diabetic group may be explained by the accelerated rate of cell death and cell replacement in diabetic. This result was also concluded by Dennis *et al*. They found that diabetes increases mitosis as well as apoptosis of the epithelial cells and lamina propria of olfactory bulb. [21]

Alteration in the histological structure of the fungiform papillae in the diabetic group was also recorded in the current work. Some papillae lost their characteristic mushroom shape and appeared thin and elongated. In agreement with our results, Nagato *et al.*[22] in a study on morphogenesis of the rat fungiform papillae after denervation observed that the atrophic fungiform papillae can be transformed to a form resembling filiform papillae. They also reported that, the filiform-like papillae derived from the fungiform ones showed various shapes, sizes, and orientations.

In the current work, taste buds of the fungiform papillae of the diabetic group exhibited alteration as many of them showed shrinkage and vacuolation with peripheral arrangement of the cells leaving an empty center. Scanning electron microscopic examination of the fungiform papillae revealed arrays of rough irregular microplicae, while the gustatory pore appeared depressed irregular and ill-defined.

In consistence with our observations, Pai *et al.* [23] studied the innervations of the vallate papillae and taste buds in diabetic and control rats by a morphometric and quantitative immunohistochemical analysis. They concluded that the innervation of taste cells was significantly reduced in diabetic animals. They explained these changes by neuropathy defects and/or morphological changes in the taste buds. Moreover, another study on the effect of denervation of the fungiform papillae recorded changes that ranged from disappearance of the pores to their shrinkage and atrophy of the pore-associated epithelial structures. [24]

The documented involvement of oxidative stress in the development of diabetic complications elucidated clearly the amelioration in the histological architecture of the lingual papillae of the ozone group in the present study. Ozone is a well-documented naturally-occurring anti-oxidant with numerous pharmacological activities such as anti-inflammatory, anti-carcinogenic and anti-bacterial effects. [25]

The obvious protective effect of ozone on the lingual papillae in the present results may be attributed to its antibacterial, immunostimulation and strong antioxidant profile. The main use of Ozone dentistry is relays on its antimicrobial properties. It is proved to be effective against both gram positive, gram negative bacteria, viruses and fungi. [26]

The marked improvements observed in tongues of ozone group were also concluded by Filippi A. [27] He studied the influence of ozonized water on the epithelial wound healing process in the oral cavity. It was found that ozonized water applied on the daily basis can accelerate the healing rate in oral mucosa. This effect can be seen in the first two postoperative days. The comparison with wounds without treatment shows that daily treatment with ozonized water accelerates the physiological healing rate.Other authors claimed that application of Ozone for a period of 10S was capable of reducing the number of streptococcus mutans and streptococcus sobrinus *in vitro*. [28]

Therefor, ozonized olive oil gel can compensate the diabetic atrophy of oral mucosa, improve immue system and guard against bacterial invasion. All these support effect resulted in almost normal tongue mucosa and taste buds.

Agents that exert multiple actions, includindg ozone,such as antimicrobial, antihypoxic, analgesic, immunostimulating etc could be considered more effective in prevention of diabetic complications on the lingual papillae via its wide range of biological activities.

The histological results of the current work supplemented by the biochemical ones strongly recommend ozone as an efficient protector against the detrimental effects of diabetes induced by streptozotocin on the lingual papillae most probably via its antioxidant and anti-inflammatory effects.

Conclusion:

In comparison with classical medicine modalities such as antibiotics and disinfectants,

ozone therapy is quite inexpensive, and according to the results of the present work and to many case reports and scientific studies it is very promising especially in diabetic patients. Further research is needed to standardize indications and treatment procedure in ozone therapy.

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