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IMPACT OF COD LIVER OIL SUPPLEMENTATION ON THE HISTOLOGICAL CHANGES OF THE TONGUE DORSAL SURFACE IN INSULIN TREATED STREPTOZOTOCIN INDUCED DIABETIC RATS

Heba Fathy El-Imam* *and* Nesreen Nabil Abdel-Salam**

ABSTRACT

Background: Diabetes mellitus (DM) is one of the most common chronic metabolic disease that affecting all age. However, it has many complications such as stomatitis, delayed wound healing, mucosal neuro-sensory disorders. Cod liver oil (CLO) is an important source of ω-3 fatty acids that have anti-inflammatory effect.

Objective: The present study designed to investigate the protective effect of cod liver oil (CLO) supplementation in ameliorating the histological changes of the tongue dorsal surface in streptozotocin (STZ) induced diabetic rats. **Design:** Forty adult male Swiss albino rats were randomly divided into four groups (n=10). Group (I) rats were received single intraperitoneal injection of 1 ml/kg citrate buffer. Group (II) rats were received single intraperitoneal injection of 60mg/kg STZ freshly dissolved in 1 ml/kg citrate buffer, blood samples were obtained and fasting glycaemia was measured to confirm the development of diabetes. Group (III) after confirmation of diabetes rats were received subcutaneous injection of human insulin with a dose (5 IU/kg/d). Group (IV) rats were treated as in group (III) and received pure cod liver oil with a dose (60mg/Kg /d) by intra-gastric intubation. After four weeks, animals were scarified and tongues were dissected and the prepared sections were examined histologically by H&E, histochemically by Masson Trichrome stain (MTC) and morphomtrical analysis. Data obtained from morphomtrical analysis were statistically described in terms of mean ± standard deviation (± SD).

Results: Histological examination of Group I revealed the normal features of the dorsal surface of tongue and shape of filiform papillae. In Group II has evident hyperkeratosis and loss of conical shape of filiform papillae. Group (III) has better epithelium and keratin layer and shape of filiform papillae. Group IV has histological features resembling nearly those of group I. The morphometric analysis confirmed the previous results as group I showed the highest mean epithelium thickness, followed by Group IV, then Group III and the least value was for Group II.

Conclusions: diabetes has a deleterious effect on dorsal surface of the tongue. Insulin can't completely inhibit the complications of diabetes. However, ω-3 fatty acids present in CLO has protective effect on these abnormalities caused by diabetes.

KEY WORDS: Diabetes, streptozotocin, insulin, cod liver oil, tongue.

* Lecturer of Oral Biology, Faculty of Dentistry, Egyptian Russian University, Cairo, Egypt.
** Lecturer of Oral Biology, Faculty of Dentistry, Mansoura University, Mansoura, Egypt.

INTRODUCTION

Diabetes mellitus (DM) is one of the most prevalent chronic metabolic disease that is common throughout the world affecting all age groups and more common in adults [1]. DM is manifested by abnormally elevation in blood glucose levels (hyperglycemia) resulting from either a deficiency in insulin secretion from the pancreas or totally absent and called insulin dependent (type 1) or an impaired cellular resistance to the action of insulin and called insulin non- dependent (type 2) [2].

DM is characterized by a lack of insulin causing elevated blood glucose, often with associated insulin resistance. Over time, especially in genetically susceptible individuals, such chronic hyperglycemia can cause tissue injury, one pathological response to tissue injury is the development of fibrosis, which involves predominant extracellular matrix (ECM) accumulation [3]. DM cause many complications including hypoglycemia, ketoacidosis, cardiovascular diseases, chronic renal failure, retinal damage, nerve damage, and microvascular damage, which may cause impotence and poor healing [4].

Many inflammatory diseases and soft tissue lesions in oral cavities are associated with DM, these complications include periodontal diseases (periodontitis and gingivitis), salivary dysfunction leading to a reduction in salivary flow and changes in saliva composition with taste dysfunction, oral fungal and bacterial infections, oral mucosal lesions in the form of stomatitis, geographic tongue, benign migratory glossitis, fissured tongue, lichen planus, and angular chelitis,[5] delayed mucosal wound healing, mucosal neuro-sensory disorders, dental carries and tooth loss[6]. Also, diabetes caused taste disorders by morphological changes in taste buds[7]. Free radical mediated oxidative stress including proteins and lipids is mainly involved in the pathogenesis of diabetic complications[8].

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

Diabetes also disturbs natural antioxidant defence systems by changing the antioxidant enzyme activities in tissues including heart, liver, lung and kidney [10]. Antioxidants, including vitamins C, E, A, have a role in preventing or reversing disturbances in tissue antioxidant defence in diabetic animals [11]. Concerning in the management of diabetes without any side effect lead to an increasing demand for natural products with effective results and fewer side effects [12].

Dietary intake of omega-3 fatty acids, found in fish, flax seeds, walnuts, soy, canola, and greens is protective against the development of type 1 diabetes in children as omega-3 fatty acids can reduce the incidence of inflammation [13]. Fish oil (FO) is a compound rich in omega-3 (n-3) fatty acids which are polyunsaturated fatty acids PUFAs represented by eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) that are considered essential fatty acids because they are essential to human health but cannot be manufactured by the body so, it must be obtained from food [14] . It regulates many functions in the body including blood pressure, blood clotting, and modulation of inflammatory response. Moreover, FO has been used for the treatment of several pathologies such as rheumatoid arthritis, autoimmune diseases, allergic asthma, hypertension, cardiovascular diseases and in cancer therapy such as mammary and colon tumors [15].

Cod liver oil (CLO) is an important source of long-chain omega-3 fatty acids (eicosapentaenoic and docosahexaenoic acids) and vitamins A, E, and D [16]. Long-chain omega-3 fatty acids become

incorporated into the cell membranes and have anti-inflammatory properties that has a role in the prevention of type 1 diabetes, such as decreased expression of human leukocyte antigen (HLA) [17]. Thus, oral administration of CLO decreased the deleterious effects of pancreatic damage by lowering glucose, suppressing the release of inflammatory cytokines TNF-·, IL-6 that are involved in cell damage and development of diabetes and also by slightly elevating plasma insulin [18].

The present study was designed to investigate the protective effect of cod liver oil (CLO) supplementation in ameliorating the histological changes of the tongue dorsal surface in streptozotocin induced diabetic rats.

MATERIAL AND METHODS

Animals

Forty adult male Swiss albino rats (200-250gm) were selected for this study. The animals were purchased from the Egyptian Organization for Biological and Vaccine Production and kept under normal laboratory conditions. The rats were housed in separate metal cages in Ain-Shams animal house under controlled temperature, humidity and dark-light cycle. Rats were kept under good ventilation and adequate diet consisting of fresh vegetables, dried bread and tap water.

Material

1. Streptozotocin or (STZ) (Sigma Chemical Co., St. Louis, MO, USA). Each vial of sterilized Streptozotocin powder contains 1gr. of Streptozotocin active ingredient.
2. Human insulin (Mixtard® 30, Novo Nordisk, Denmark)
3. Cod liver oil (CLO) supplied by (Arctic Cod Liver Oil®, Nordic Naturals, Inc., USA). Each 1ml contained 27mg EPA, 21mg DHA, 2.1mg vitamin A, 20mg vitamin D, and 15.5mg vitamin E

Experimental procedure:

The experimental animals were randomly divided into four groups (ten rats each) as following:

Group I (Negative control group):

The rats of this group received a single intraperitoneal injection of 1ml/kg body weight citrate buffer (0.01 M; pH 4.5) under ether anesthesia[19].

Group II (Positive control group): (Diabetic untreated group):

The rats were fasted for 16 h before the induction of diabetes by a single intraperitoneal injection of 60mg/kg body weight STZ freshly dissolved in 1ml/kg body weight citrate buffer (0.01 M; pH 4.5) under ether anesthesia [20].

After the injection, the animals were given normal feeding. Blood samples were obtained via vein puncture of tail vein. Fasting glycaemia was measured by the glucose oxidase method using a clinical glucometer to confirm the development of diabetes mellitus. Plasma glucose level greater than 300 mg/dl confirmed the occurrence of diabetes.

Group III (Insulin treated group):

After confirmation of diabetes rats received subcutaneous injection of human insulin (rDNA), (Mixtard® 30, Novo Nordisk, Denmark) with a dose (5 IU/kg body weight/day) four weeks [21]. The rats received the last insulin dose 24 hours before being sacrificed.

Group IV (Cod liver oil treated group):

Rats were treated as in group (III) and received pure cod liver oil with a dose (60 mg/Kg body weight/day) by intra-gastric intubation for four weeks [22]. In addition to insulin injection of the same dose of the last group.

At the end of the experimental period, after four weeks, the animals were sacrificed by ketamine over dose [23].

Tongues were carefully dissected and processed for light microscopic examination.

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

1. Haematoxylin and Eosin (H&E) as routine stain.
2. Masson Trichrome histochemical stain (MTC).
3. Morphomtrical analysis (Haematoxylin and Eosin):

   Epithelial thickness was measured from the basal membrane to the granular layer and used to indicate proliferative activity in 3 microscopic fields per slides. Keratin thickness layer was measured at the tip of the filiform papillae and used as indicator of epithelial maturation / keratinization [24]. The liner measurement tool available in the image tool 3.0 and was done at (University of Ain-Shams, Cairo, Egypt) and the adjustment procedures were repeated before the end of data collection.
4. Statistical analysis data were expressed as mean ± SD. Analysis were performed by an examiner unaware of which group each image belonged to. An analysis of variance (ANOVA) and least significance difference (LSD) for multiple test were used to compare the groups.

RESULTS

The blood glucose level in rats of diabetic group was high (above 300mg/dl) that confirm establishment of diabetes

1- Haematoxylin and Eosin stain (H&E) results:

Group I (Control group)

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 directions were also noticed (fig 1: a, e)

Group II: (Diabetic untreated group):

Filiform papillae showed flattening with loss of their characteristic conical shape and evident hyperkeratosis were noticed. Their epithelial lining showed marked thickening with many proliferating cells and ill-defined connective tissue papillae. Mild inflammatory cellular infiltration were also seen (fig 1: b, f)

Group III: (Insulin treated group)

Filiform papillae of showed more regular arrangement, better epithelium and keratin configuration. Nearly proper connective tissue appearance (fig 1: c, g)

Group IV: (Cod liver oil treated group)

Filiform papillae showed normal structure including their covering epithelium, keratin layer and conical shape appearance. Proper well-formed connective tissue papillae. (fig 1: d, h)

2- Masson's trichrome stain:

Group I (control):

Dorsal surface of rat tongues showed normal filiform papillae, thin keratin and proper connective tissue support (fig 2: a)

Group II (Diabetic untreated group):

Dorsal surface of rat tongues showed hyperkeratosis and mild fibrosis of connective tissue support (fig 2: b).

Group III (Insulin treated group):

Dorsal surface of rat tongues showed almost normal keratin thickness and mild fibrosis of connective tissue support (fig 2: c).

Group IV (Cod liver oil treated group)

Dorsal surface of rat tongues showed normal keratin thickness and proper connective tissue support.

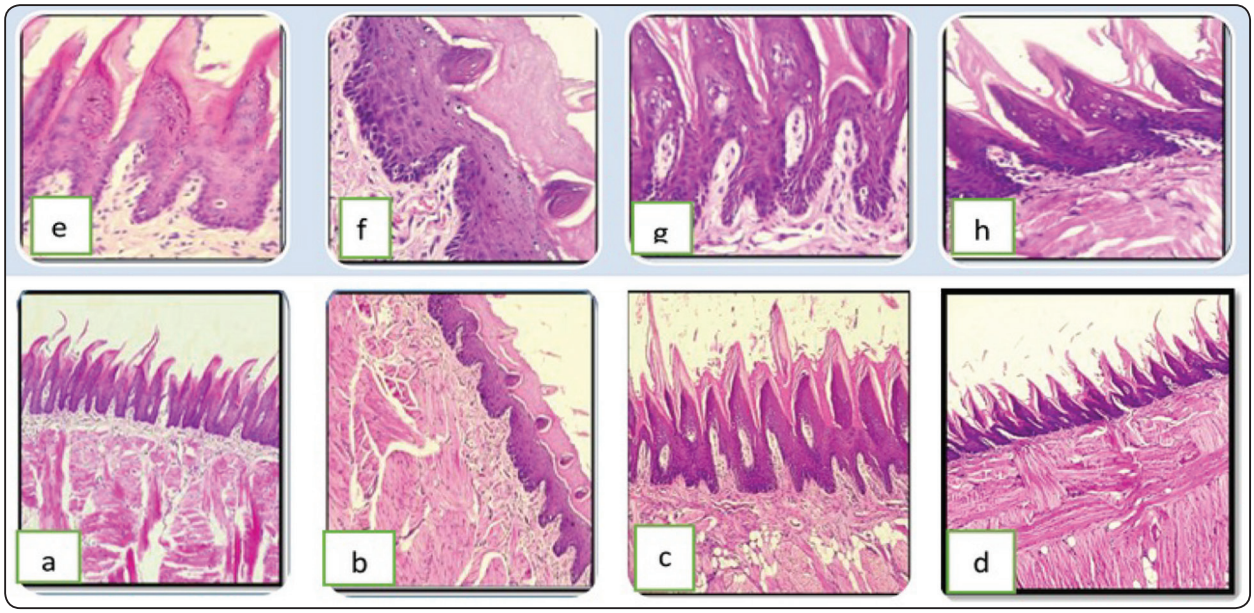

Fig. (1) Photomicrographs of the dorsal surface of the tongue from all examined groups showing:

a. Group I: normal arrangement and conical shape of filiform papillae and well-formed connective tissue and muscle fibers running in different direction.
b. Group II: loss of their normal conical shape with hyperkeratosis and distorted connective tissue papillae.
c. Group III: more regular arrangement, better epithelium and keratin configuration. Nearly Proper connective tissue appearance.
d. Group IV: normal structure of filiform papillae including normal thickness covering epithelium, keratin layer and conical shape appearance, well-formed connective tissue papillae.

Mic. Mag. a, b, c, d X 100 - e, f, g, h X 400

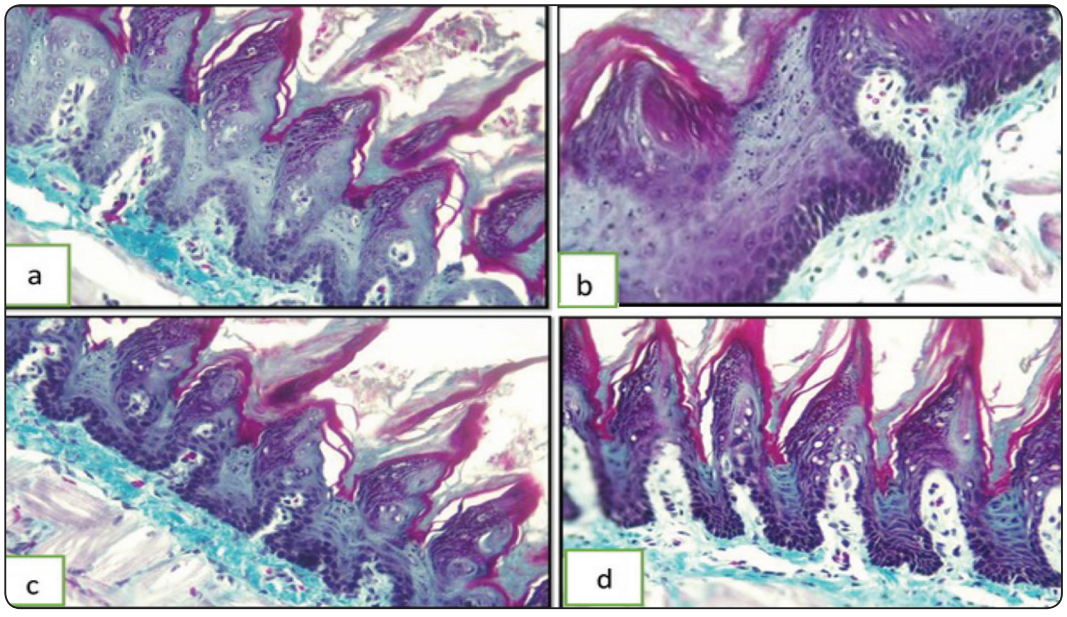

Fig. (2) Photomicrographs of the dorsal surface of the tongue from all examined groups showing:

a. Group I: normal filiform papillae, thin keratin and proper connective tissue support.
b. Group II: hyperkeratosis and mild fibrosis of connective tissue support.
c. Group III: almost normal keratin thickness and mild fibrosis of connective tissue support.
d. Group IV: normal keratin thickness and proper connective tissue support.

Statistical results:

Data was analyzed using Statistical Package for Social Science software computer program version 23 (SPSS, Inc., Chicago, IL, USA). Data were presented in mean and standard deviation. One way Analysis of variance (ANOVA) and tukey were used for comparing data. *P* value less than 0.05 was considered statistically significant.

	Group I	Group II	Group III	Group IV	P
Epithelial Thickness Evaluation	1399±78.70	705.2±141.0	976.9±80.17	1170±55.35	<0.001*
P1		<0.001*	<0.001*	<0.001*	
P2			<0.001*	<0.001*	
P3				<0.001*	

Data expressed as mean±SD SD: standard deviation P: Probability *:significance <0.05

Test used: One way ANOVA followed by post-hoc tukey

P1: significance relative to group I-P2: significance relative to group II- P3: significance relative to group III

	Group I	Group II	Group III	Group IV	P
Area Fraction Analysis	9.490±1.58	14.52±2.42	11.56±1.93	10.57±1.76	<0.001*
P1		<0.001*	0.1	0.6	
P2			0.009*	<0.001*	
P3				0.66	

Data expressed as mean± SD SD: standard deviation P: Probability *:significance <0.05

Test used: One way ANOVA followed by post-hoc tukey

P1: significance relative to group I-P2: significance relative to group II- P3: significance relative to group III

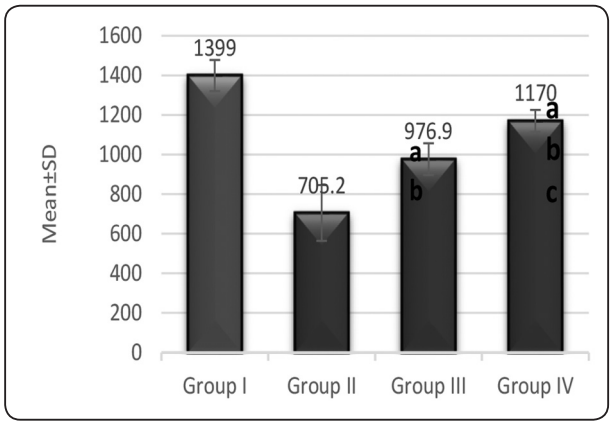

Fig. (3) a: significance relative to group I b: significance relative to group II c: significance relative to group III

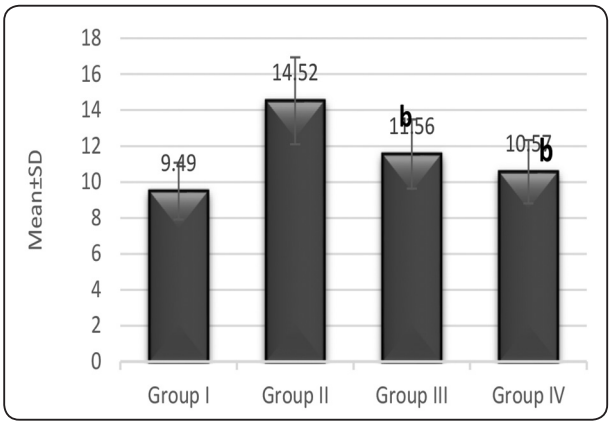

Fig. (4) a: significance relative to group I b: significance relative to group II c: significance relative to group III

DISCUSSION

Our study aimed to evaluate the impact of cod liver oil supplementation on the histological changes of the tongue dorsal surface in insulin treated streptozocin induced diabetic rats. Light microscope was utilized to study the morphological changes in filiform papillae of the tongue dorsal surface histologically and histochemically.

In the present study diabetes mellitus was selected because this disease decreases the patient's general metabolism as stated by **Graves et al., 2007**[25] and changes the cellular and humoral immunity, increasing the risk of infections especially when the patient is submitted to surgical procedures. [26] Moreover, the delay in cell proliferation, the decrease of collagen metabolism and all other granulation tissue components, such as glycoproteins and mucopolysaccharides, are direct consequences of the disease that severely affects the tissue repair process. [27]

One of the objectives of our study was to develop a rat model for type 1 DM or insulin-dependent diabetes that may represented the clinical pathogeneses seen in humans who have autoimmune destruction of the pancreatic β-cells. This model was achieved by injection of 60 mg/kg body weight STZ which is the effective dose for development of animal model with type 1 diabetes this was in accordance with **Islam and Choi, 2007**. [28]

In the present study, Induction of diabetes mellitus was confirmed after one week of streptozocin injection and was in coincided with that found by Clara et al. who stated that blood glucose averaged 6.4±0.2 mmol/l in basal conditions and elevated to 23.3±1.9 mmol/l 15h after STZ injection. It resulted in marked affection of the dorsal surface of the tongue due to diabetes systemic effects as reported by **Fahmy and Soliman, 2007**. [29]

Histologically filiform papillae are widely distributed on the dorsal surface of the tongue and they undergo flattening, loss and atrophic changes more than other papillae. It was stated by **Taiwo et al., 2009** [30] that the filiform papillae has high metabolic activity, so any changes in vascular insufficiency, nutritional deficiency, or drug toxicity may result in atrophy of these papillae. So that it is considered the mirror that reflects the general health of the body.

In our results, examination of the tongues of group II (diabetic rats) showed flattening of filiform papillae with loss of their characteristic conical shape and evident hyperkeratosis. Their epithelial lining showed marked thickening with many proliferating cells and ill-defined connective tissue papillae. These results were in accordance with **Batbayar et al, 2004** [31] who explained these complications to chronic inflammation, changes in innervations and microvasculature secondary to diabetes.

In contrary to our results, **Hamilton and Blackwood, 1977** [32] mentioned that rats with chemically induced diabetes show a reduction in cell proliferation 3-4 weeks after the reported onset of the diabetic state, but with no epithelial atrophy**. Reuterving et al., 1986** [33] reported that periodontal disease characterized by an inflammatory process in the oral tissue of rats with chemically induced diabetes. A large number of studies done by **Görfi, et al., 1996** [34] in rats with two-week of diabetes observed the presence of inflammatory cells adjacent to the oral epithelium, with an inflammatory infiltrate in connective tissue characterized by a reduced occurrence of fibroblasts and collagen and an increase in plasma cells compared to healthy animals. These observations are in agreement **Seppala et al., 1997.** [35]

In contrast to these findings, rats with five weeks of streptozotocin-induced diabetes showed no effective inflammatory process, although there was reduced cell proliferation and reduced thickening of the epithelial lining as mentioned by **Shirai et al., 1998** [36]. Likewise, a study done by **Mori et al., 1999** [37] who observed the effects of chemically induced diabetes in rats subjected to mechanical

pressure of the oral mucosa and revealed that no inflammatory cells or epithelial atrophy, although there was a reduced rate of cell proliferation. A clinical study of cellular polymorphism suggested that the microscopic alterations produced by diabetes in the oral epithelium could be used to diagnose this disease as stated by **Alberti et al., 2003** [38].

Histological (H&E) stain and morphomtrical analysis of group II (diabetic group) in our results revealed hyperkeratosis in the form increased thickness of keratin layer. This finding was in accordance with **Rodgers *et al.*, 2006** [39] who studied the expression of intracellular filaments, collagen, and collagenase genes and explained that keratin associated proteins and keratin complexes gene expression were increased in diabetic rats.

Our results also revealed atrophy in the epithelium of the oral cavity by decreasing the number of prickle cells. This could be explained by **Nagy et al., 2000** [40] who stated that diabetes inhibits the mitosis of the epithelial cells by decreasing the number of cells in synthesis phase (S-phase) in which deoxyribonucleic acid (DNA) is replicated. Furthermore, in diabetic models there is decrease in the concentration of epidermal growth factor in saliva which affect more the rate of cell division.

In most histological studies, collagen, when viewed in sections stained with hematoxylin and eosin, could not be identified. So, with the application of Masson's trichrome stain (MTC), the concentration and the blue stain of collagen fibers became more identifiable. Our trichrome histochemical stain results of diabetic group (group II) showed hyperkeratosis and mild fibrosis of connective tissue support in agreement with **Hoda et al., 2007** [41] who demonstrated the histological changes in the lingual papillae of diabetic rats experimentally induced by streptozotocin and the protective effect of Curcumine.

Also our results were in accordance with **Grotendorst et al., 1985** [42] who proved that wound healing of diabetic group 14thday using MTC revealed decreased connective tissue thickness, and separation of muscles from underlying connective tissue. Disorganized granulation tissue mass with decreased number of fibroblasts, inflammatory cells and short irregular collagen fibers with mild staining reaction.

Our Histological and histochemical results of group III showed that insulin did not completely inhibit the complications of diabetes. This finding was in accordance with **Lebovitz and Face, 2011** [43] who stated that there are no better clinical outcomes in treatment with insulin compared to those with other anti-hyperglycemic regimens. They explained this finding to the fact that insulin treatment is not durable in maintaining glycemic control. Moreover, insulin did not completely inhibit the abnormalities in the oxidative metabolism in diabetic rats as insulin treatment partially inhibits lipid peroxidation which can increase tissue oxidative stress. This finding was agreed well with **Jain et al., 2006**. [44]

Our histological and histochemical results of group IV showed that filiform papillae showed normal structure including their covering epithelium thickness, keratin layer and conical shape appearance with well-formed proper connective tissue papillae, results were in consistence with **Amany, Marwa, 2017** [21] who revealed that CLO inhibited to a great extent the abnormalities in the oxidative metabolism recorded in Group II compared to the effect of insulin as observed in insulin treated group, this could be explained according to previous studies which proved that patients with recently diagnosed type 1 diabetes, commonly retain limited capacity to secrete insulin for several months or years [44].

CONCLUSION

From the present study we concluded that diabetes has a deleterious effect on dorsal surface of the tongue. Insulin can't completely inhibit the complications of diabetes. However, ω-3 fatty acids present in CLO can provided considerable effect on the abnormalities caused by diabetes.

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