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THE POSSIBLE EFFECT OF OMEGA-3 FATTY ACIDS VERSUS INSULIN ON EXPRESSION OF ACTIVATED CASPASE-3 AND RUSSELL BODIES DEVELOPMENT IN BUCCAL MUCOSA OF STREPTOZOTOCIN-INDUCED DIABETIC ALBINO RATS

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ABSTRACT

Background: Damage of tissue repair and dysfunction of the oral mucosa are usually associated with the diabetic condition. Apoptosis plays a functional role in limiting diabetic repair. Russell bodies, an uncommon finding that may be associated with chronic inflammatory conditions. Although insulin is the mainstay of diabetes treatment, it has prominent side effects. Episodes of severe hypoglycemia and increased mortality rate are associated with insulin therapy. Cod liver oil (CLO) is an important source of long-chain omega-3 (ω-3) fatty acids that have antioxidant and anti-inflammatory properties.

Objective: The present study was designed to determine the adaptive apoptotic alterations accompanying diabetes in the buccal mucosa with the possibility of occurrence of the so-called Russell bodies and the possible role of long-chain ω-3 fatty acids versus insulin supplementation in enhancement of the buccal mucosa in streptozotocin (STZ)-induced diabetic rats.

Design: Sixty adult male Swiss albino rats (200-250 gm) were selected for this study. The animals were randomly divided into four groups (fifteen rats each): **Group I** (Control group), **Group II** (Diabetic untreated group), **Group III** (Insulin treated group) and **Group IV** (Cod liver oil treated group). At the end of the experimental period (four weeks), the rats were sacrificed and the specimens were obtained from the mucosa of the cheek of both sides. The sections were examined histologically, immunohistochemically and histomorphometrically. **Statistical analysis:** Data obtained from histomorphometric analysis were statistically described in terms of mean ± standard deviation (± SD).

Results: Histopathologic examination of Group I revealed the normal histological features of the buccal mucosa. In Group II several histological changes in the epithelial layer of the buccal mucosa were noticed. These changes include; atrophy in the epithelium, evidences of cells undergo degeneration, nuclear changes, ill-defined cell membrane, cytoplasmic vacuolations,

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thickening of stratum granulosum (hypergranulosis) which was accompanied by hyperkeratosis. Moreover, the lamina propria showed differential orientation and arrangement of the collagen fiber bundles, areas of degeneration and hyalinization in addition to fibroblasts that showed signs of degeneration. Furthermore, the lamina propria was strongly infiltrated by inflammatory cells and invaded by multiple Russell bodies scattered throughout the connective tissue (C.T) and dilated blood vessels (BVs); lined by swollen endothelial cells and engorged with red blood cells (RBCs) were observed. Some of these changes were still observed in Group III that received insulin treatment. Treatment with CLO in Group IV resulted in histological features resembling nearly those of the control group (Group I). The least immuno-expressions for caspase-3 and CD-138 were detected in Group I, followed by Group IV, then Group III and subsequently Group II. The histomorphometric analysis supported 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. On the other hand, Group I showed the least mean keratin thickness and the least mean area percentage of both caspase-3 and CD-138 immunoreactivities, followed by Group IV, then Group III and the highest values were for Group II. There was statistically highly significant difference between the studied groups.

Conclusions: Diabetes has deleterious effect on the structure of the buccal mucosa. The raised levels of capase-3 in diabetic buccal mucosa are related to increased Russell bodies (Mott cells) formation. Insulin can't completely inhibit the complications of diabetes. However, ω-3 fatty acids present in CLO can inhibit to a great extent the abnormalities caused by diabetes.

KEY WORDS: Diabetes, buccal mucosa, apoptosis, Russell bodies, Mott cells, insulin, cod liver oil, omega-3 fatty acids.

INTRODUCTION

Diabetes mellitus (DM) is one of the most common endocrine metabolic disorders and its prevalence has been increasing worldwide ***(Vernillo, 2001)***. It is the fifth most common chronic disease and the sixth most frequent cause of death among the elderly persons ***(Chávez et al., 2001)***. In the Middle East and North Africa region, 1 in 10 adults has diabetes; Egypt has the highest prevalence of diabetes, at 10.9%. There are 34.6 million people with diabetes in the Middle East and North Africa, a number that will almost double to 67.9 million by 2035 if concerted action is not taken ***(International Diabetes Federation, 2013)***. DM is a metabolic disorder manifested by abnormally high levels of glucose. The hyperglycemic state developed from either a deficiency in insulin secretion (type 1) or an impaired cellular resistance to the action of insulin (type 2). This is usually associated with a number of complications including; retinopathy, nephropathy, peripheral neuropathy, angiopathy and impaired wound healing. Moreover, tissue repair is damaged and dysfunction of the oral mucosa mostly occurs due to alterations in salivary flow and constituents, reduced immune defenses and a greater tendency to infections ***(Little et al., 2002; Greenburg and Glick, 2003; American Diabetes Association, 2010)***.

Apoptosis is method of programmed cell death required for the function of multicellular organisms. It involves a series of biochemical events leading to a characteristic cell morphology and death. However, abnormal apoptosis has been implicated in diabetes and plays an important role in several diabetic complications. These include apoptosis of neuronal cells in diabetic neuropathy, myocardial apoptosis, which plays a role in cardiac pathogenesis and apoptosis of mesangial cells that occurs in diabetic nephropathy ***(Cai et al., 2002; Li et al., 2002; Yamagishi et al., 2002; Alikhani et al., 2005 a; Graves et al., 2006)***. Furthermore, abnormal apoptosis has been implicated in periodontal diseases ***(Jarnbring et al., 2002; Bantel et al., 2005)***. The molecular events regulat-

ing apoptosis are complex and involve genes that are both pro-apoptotic and anti-apoptotic. Caspases (cysteine aspartate-specific proteases) are a family of cytosolic interleukin-1β-converting enzyme family proteases, involved in the initiation and execution of apoptosis. Fourteen caspases have been identified so far, all of which share some common properties: they are all aspartate-specific cysteine proteases; they all have a conservative pentapeptide active site 'QACXG' (X can be R, Q or D); their precursors are all zymogens known as procaspases ***(Yuan and Ding, 2002)***. Caspase-3 is essential for normal brain development and is also required for some typical hallmarks of apoptosis. Caspase-3 is indispensable for apoptotic chromatin condensation and DNA fragmentation in all cell types examined. Thus, caspase-3 is essential for certain processes associated with the dismantling of the cell and the formation of apoptotic bodies, but it may also function before or at the stage when commitment to loss of cell viability is made ***(Alan and Reiner, 1999)***. Caspase-3 activation was determined in the retina of alloxan-diabetic rats (2-14 months duration) and in the isolated retinal capillary cells (endothelial cells and pericytes), ***(Kowluru and Koppolu, 2002)***. In addition, a study carried out by ***Cai et al.*** in ***2002*** on the effect of hyperglycemia-induced apoptosis in mouse myocardium. Their results revealed that, reactive oxygen species (ROS) were generated in the cells exposed to high levels of glucose and induced apoptotic cell death in the myocardium.

Russell bodies, are eosinophilic, large and homogenous immunoglobulin-containing cytoplasmic inclusions, usually found in plasma cells undergoing excessive synthesis of immunoglobulins ***(Pizzolitto et al., 2007; De Petris and Leung, 2010; Del Gobbo et al., 2011)***. The reason that these immunoglobulins are not eliminated and crystallize in the cytoplasm remains to be established. Plasma cells filled with Russell bodies, also known as Mott cells, have been associated to immunoproliferative disorders such as plasmocytoma ***(Paik et al., 2006)***. The Mott cells can be demonstrated by periodic acid-Schiff (PAS) method and are immunopositive for the antibodies CD-79a and CD-138 as well as kappa and lambda immunoglobulin light chains ***(Erbersdobler et al., 2004; Ensari et al., 2005; Habib et al., 2010)***.

Although insulin is the mainstay of diabetes treatment, it has prominent side effects. Weight gain accompanies insulin treatment. The magnitude of the weight gain is influenced by the level of the initial glycemic control, the glycemic control achieved, the duration of insulin therapy and the insulin regimen used ***(Purnell et al., 1998)***. Furthermore, complications in macrovascular and retinal functions in addition to neuropathic problems are still associated in the patients receiving insulin ***(Jain et al., 2006)***. Moreover, episodes of severe hypoglycemia and increased mortality rate are associated with insulin therapy ***(Finfer et al., 2009)***. In addition, there has been a growing concern about the association of an increase in the incidence of pancreatic cancer with insulin treatment ***(Li et al., 2009)***.

Management of diabetes without any side effect is still a challenge to the medical system, this has led to an increasing demand for natural products with antidiabetic activity and fewer side effects ***(Galletto et al., 2004)***. Food derived antioxidants have a strong potential for long term use as chemo-preventive agents in disease conditions involving oxidative stress ***(Mckim et al., 2002)***. Traditionally, many researches on diet are focused on only few nutrients with well established roles in the formation and maintenance of structural components of oral tissue such as collagen (vitamin C), bone (calcium) and integrity of epithelial tissue (vitamin A). However, few researches have been performed on the putative role of the well-documented healthy fatty acids such as polyunsaturated fatty acids (PUFAs), ***(Kaye, 2010)***. Fish oil (FO) is a compound rich in omega-3 (ω-3) or (n-3) fatty acids which are PUFAs mainly represented by eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA). Previous studies have shown that FO regulates a wide range of functions

in the body including blood pressure, blood clotting, modulation of inflammatory response as well as correct development and functioning of brain and nervous systems. Moreover, FO has been used for the treatment of several pathologies such as glomerulonephritis, rheumatoid arthritis, autoimmune diseases, allergic asthma, hypertension, cardiovascular diseases and as adjuvant in cancer therapy such as mammary and colon tumors ***(Ramesh and Das, 1996; Lordan et al., 2011)***. Additionally, it has been tested an increased intake of ω-3 fatty acids might reduce the risk of developing diabetes in mice received streptozotocin (STZ), ***(Soltan, 2012)***. Among the different types of FO, cod liver oil (CLO) is an important source of long-chain ω-3 fatty acids (EPA & DHA) as well as vitamins A, E and D ***(Hamdy et al., 2007)***. It was reported that CLO has antioxidant effect. Moreover, long-chain ω-3 fatty acids present in CLO become incorporated into the cell membranes and have anti-inflammatory properties that may be relevant for the prevention of type 1 diabetes, such as decreased expression of human leukocyte antigen (HLA) class II molecules on activated human monocytes ***(Hughes and Pinder, 2000; Leite et al., 2014)***.

From the fore mentioned, the present study was designed to determine the adaptive apoptotic alterations accompanying diabetes in the buccal mucosa with the possibility of occurrence of the so-called Russell bodies and the possible role of long-chain ω-3 fatty acids versus insulin supplementation in enhancement of the buccal mucosa in STZ-induced diabetic rats.

MATERIALS AND METHODS

Animals

Sixty adult male Swiss albino rats (200-250 gm) were selected for this study. The steps of this experiment were done according to the rules approved by the Bio-ethical Committee of Faculty of Dentistry, Ain-Shams University. The rats were housed in separate metal cages, five rats per cage (20 cm width and 40 cm length) in Ain-Shams animal house under controlled temperature, humidity and dark-light cycle. This was done under supervision of specialized veterinarian since their housing till getting rid of sacrificed bodies which was done by the incinerator of Ain-Shams. Rats were kept under good ventilation and adequate stable diet consisting of fresh vegetables, dried bread and tap water throughout the experimental period.

Experimental procedure

After one week acclimatization period, the animals were randomly divided into four groups (fifteen rats each):

Group I (Control group): The rats of this group received a single intraperitoneal injection of 1 ml/kg body weight citrate buffer (0.01 M; pH 4.5) under ether anesthesia ***(Soon and Tan, 2002)***.

Group II (Diabetic untreated group): The rats were fasted for 16 h before the induction of diabetes by a single intraperitoneal injection of 60 mg/kg body weight STZ (Sigma Chemical Co., St. Louis, MO, USA) freshly dissolved in 1 ml/kg body weight citrate buffer (0.01 M; pH 4.5) under ether anesthesia. After the injection, the animals were given free access to water and food. Blood samples were obtained via vein puncture of tail vein. Fasting glycemia was measured by the glucose oxidase method using a clinical glucometer. Plasma glucose level greater than 300 mg/dl confirmed the occurrence of diabetes that was determined three days after the drug injection ***(Soon and Tan, 2002)***.

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) for four weeks. The rats received the last insulin dose 24 hours before being sacrificed ***(Pinheiro et al., 2011)***.

Group IV (Cod liver oil treated group): After confirmation of diabetes, rats received pure cod

liver oil (Arctic Cod Liver Oil®, Nordic Naturals, Inc., USA) with a dose (60 mg/Kg body weight/day) by intra-gastric intubation for four weeks ***(Marjan et al., 2012)***.

Animal sacrifice

At the end of the experimental period which was four weeks for all groups, the animals were sacrificed by ketamine over dose ***(Shredah and El-Sakhawy, 2014)***.

Specimen preparation

Hematoxylin and Eosin (H&E) staining

The specimens were obtained from the mucosa of the cheek of both sides and they were fixed immediately in 10% neutral buffered formalin solution. Then the specimens were washed by tap water, dehydrated in ascending grades of ethyl alcohol, cleared in xylene and embedded in paraffin wax. Sections of 4-5 μm in thickness were obtained and mounted on clean glass slides. Tissue sections were then deparaffinized in xylene and rehydrated by ethanol series ending with pure H2O (Millipore Corporation, Temecula, CA, USA) before histological staining with H&E solutions (Sigma, St. Louis, MO, USA), to verify histological details ***(Shredah and El-Sakhawy, 2014)***.

Immunohistochemistry

Immunolabeling for detection of caspase-3 and CD-138 was performed on paraffin sections of 4-5 μm in thickness mounted on positively charged microscope slides. The set of sections were incubated in hot oven for 2 hours at 56°C, deparaffinized in xylene and rehydrated by ethanol series ending with pure H2O (Millipore Corporation, Temecula, CA, USA). After 5 minutes washing in phosphate buffered saline (PBS), sections were incubated in 0.05 mg/ml proteinase K in 0.05 M Tris-HCl, 0.01 M Ethylene diamine tetra acetic acid (EDTA) and 0.01 M NaCl, pH 7.8 for 10 minutes at 37°C. After two washes with PBS, unmasking of the antigens was carried out using antigen retrieval citrate buffer solution for 10 minutes in boiling water. Then the sections were placed in a humid chamber and the endogenous tissue peroxidase was blocked with 3% hydrogen peroxide for 5 minutes. Incubation with bovine serum albumin for 20 minutes was performed to reduce unwanted nonspecific reactions. Without washing, the sections were incubated with the primary antibodies overnight at 4°C. The primary antibodies used were anti-caspase-3 active form (Millipore Corporation, Temecula, CA, USA) and anti-CD-138 (R&D Systems, Minneapolis, MN, USA) with dilutions 1:100. In the next day, after washing in PBS, the sections were incubated with secondary universal antibody (Vectastain Universal Elite ABC-peroxidase kit, Vector Laboratories) and then with the Avidin-Biotin complex (ABC) (Vectastain Universal Elite ABC kit, Vector Laboratories, Burlingame, CA, USA) according to the manufacturer's protocol. The substrate 3,3'-diaminobenzidine (DAB) was applied for the same amount of time on all labeled sections until development of desired brown color. Finally, the sections were counter-stained with Mayer's hematoxylin (Sigma, St. Louis, MO, USA) for 30 seconds to visualize tissue topography. The negative control was obtained by omitting the primary antibody from the protocol outlined above ***(Shredah and El-Sakhawy, 2014)***.

Histological and immunohistochemical examinations were performed using light microscope (Olympus® BX 60, Tokyo, Japan) at (400 x) magnification.

Histomorphometric analysis

The data were obtained using Leica Qwin 500 image analyzer computer system (England). The image analyzer consisted of a coloured video camera, coloured monitor, hard disc of IBM personal computer connected to the microscope

and controlled by Leica Qwin 500 software. The image analyzer was first calibrated automatically to convert the measurement units (pixels) produced by the image analyzer program into actual micrometer (μm) units.

In H&E stained sections, the image analysis system was used to assess the keratin thickness and epithelium thickness. In the immunohistochemistry treated sections, the image analysis system was used to assess the area percentage of caspase-3 immunoreactivity in the epithelial and C.T cells as well as in the Russell bodies of the studied specimens. In addition, the image analysis system was used to assess the area percentage of CD-138 immunoreactivity in the inflammatory cells and Russell bodies of the studied specimens. After grey calibration, the image was transformed into a grey delineated image to choose areas exhibiting positive reactivity with accumulation of all grades of reactivity (minimum, maximum and median grey). Areas of positive reaction were then masked by a blue binary color. Ten fields were measured for each specimen. The studied fields were measured under magnification 40x objective lens. Mean values were obtained for each specimen. All calculations of the area percentage were performed in relation to a standard measuring frame of an area 118476.6 μm^2.

Statistical analysis

Data obtained from histomorphometric analysis were statistically described in terms of mean ± standard deviation (± SD). One-way analysis of variance (ANOVA) test was used to compare between the four groups followed by Tukey's post hoc test. A probability value (P-value) <0.001 was considered as highly significant and P-value ≤ 0.05 was considered significant. Statistical analysis was performed by Microsoft® Excel 2013 (Microsoft® Corporation, NY, USA) and Statistical Package for the Social Science (SPSS® Inc., Chicago, IL, USA) version 20.

RESULTS

Histological results

Group I: Control group

Histopathologic examination of Group I showed normal histological features of the buccal mucosa. The epithelium consists of stratum basale resting on the basement membrane followed by, stratum spinosum, stratum granulosum and stratum corneum. Columnar cells with a centrally located nucleus were observed in stratum basale. Stratum spinosum showed well defined polyhedral cells with a centrally located nucleus. Stratum granulosum was apparently thin and the cells were flat with a centrally located nucleus. Keratohyaline granules could be detected in this layer. Stratum corneum showed apparently thin keratin layer. The epithelium showed well defined rete pegs projecting into the underlying lamina propria. In the lamina propria; densely packed collagen fiber bundles, fibroblast cells and few detected inflammatory cells scattered throughout the connective tissue (C.T) could be noticed. Well defined C.T papillae projecting into the overlying epithelium could be observed **(Fig. 1a)**.

Group II: Diabetic untreated group

Histopathologic examination of Group II revealed atrophy in the epithelium. In stratum basale, the cells showed apparent reduction in the cytoplasmic and nuclear volumes. Some basal cells undergo degeneration leaving spaces. Cells with cytoplasmic vacuolations and displaced pyknotic nucleus could be detected. Moreover, basal cells with nuclei showing pleomorphism, karyorrhexis and others with nuclei showing karyolysis were noticed. Stratum spinosum showed apparent reduction in cells number and the cells lost their prickly appearance. Degenerated cells, cells with pleomorphic nuclei and others with nuclei had marginated chromatin could be detected. Furthermore, cells with karyolytic nuclei could be observed. Apparent thickening in stratum

granulosum (hypergranulosis) with deeply stained and well defined keratohyaline granules could be detected. Stratum corneum showed apparent hyperkeratosis. The epithelium showed poorly defined rete pegs. The lamina propria showed differential orientation and arrangement of the collagen fiber bundles; some were arranged in densely packed bundles, while other bundles were diffusely arranged. Furthermore, apparently large areas of degeneration among some of the collagen bundles could be detected. In addition, areas of hyalinization and fibroblasts showing signs of degeneration were observed. The lamina propria was strongly infiltrated by inflammatory cells and invaded by multiple Russell bodies scattered throughout the C.T. The blood vessels (BVs) were dilated, lined by swollen endothelial cells and engorged with red blood cells (RBCs). The C.T papillae could be hardly detected **(Fig. 1b)**.

Group III: Insulin treated group

Histopathologic examination of Group III revealed apparent increase in epithelium thickness. In stratum basale, some columnar cells with a centrally located nucleus, while others with apparent reduction in the cytoplasmic and nuclear volumes were observed. Cells with cytoplasmic vacuolations and displaced pyknotic nucleus could be detected. Stratum spinosum showed apparent increase in cells number. Stratum spinosum showed many polyhedral cells with a centrally located nucleus. However, some cells with pleomorphic nuclei and others with cytoplasmic vacuolations and displaced nucleus could be detected. Moreover, some cells with nuclei showing karyolysis were observed. Stratum granulosum showed apparent hypergranulosis with somewhat deeply stained keratohyaline granules. Stratum corneum showed apparent reduction in keratin layer thickness. The epithelium showed apparently short and broad rete pegs. The lamina propria showed some diffusely arranged collagen fiber bundles. Furthermore, apparently small areas of degeneration among some of the collagen bundles could be detected. In addition, areas of hyalinization and fibroblasts showing signs of degeneration were observed. The lamina propria was infiltrated by some inflammatory cells and invaded by some Russell bodies scattered throughout the C.T. Somewhat dilated BVs lined by somewhat swollen endothelial cells and engorged with RBCs were observed. Apparently short and narrow C.T papillae could be detected **(Fig. 1c)**.

Group IV: Cod liver oil treated group

Histopathologic examination of Group IV showed more or less normal histological features of the buccal mucosa. Columnar cells with a centrally located nucleus were observed in stratum basale however, few cells with cytoplasmic vacuolations and displaced pyknotic nucleus could be detected. In addition, few basal cells showed apparent reduction in the cytoplasmic and nuclear volumes. Stratum spinosum showed apparent increase in cells number. Stratum spinosum showed well defined polyhedral cells with a centrally located nucleus. However, few cells with cytoplasmic vacuolations and displaced nucleus and others with pyknotic nuclei could be detected. Furthermore, few cells with nuclei showing karyolysis were observed. Stratum granulosum was apparently thin with normal stainability of keratohyaline granules. Stratum corneum showed apparently thin keratin layer. The epithelium showed well defined rete pegs projecting into the underlying lamina propria. The lamina propria showed densely packed collagen fiber bundles with apparently very small areas of degeneration among some of the collagen bundles. Few fibroblasts showing signs of degeneration were observed. Few inflammatory cells and Russell bodies could be detected in the lamina propria. More or less normal BVs were observed. The BVs had apparently small diameter and they were lined by thin endothelial cells. Well defined C.T papillae projecting into the overlying epithelium could be observed **(Fig. 1d)**.

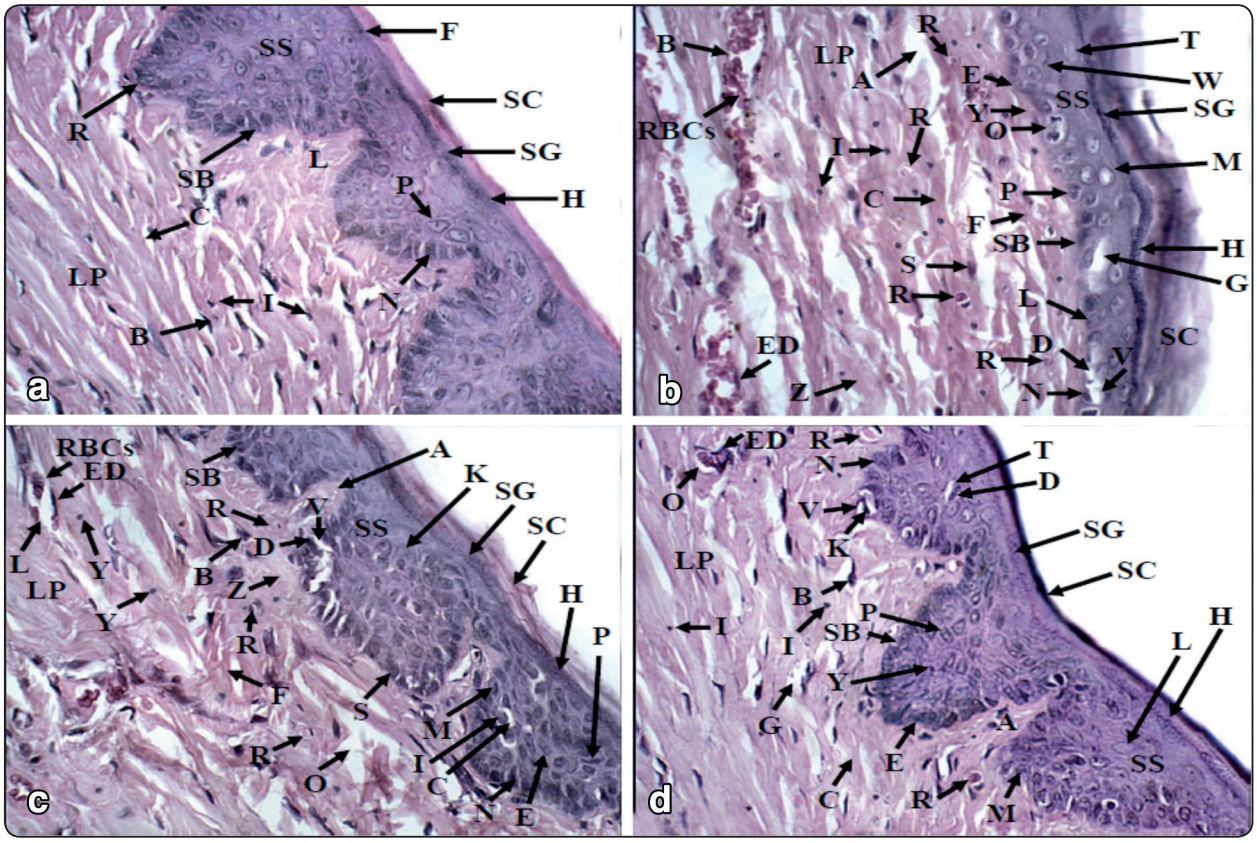

Fig. (1): Photomicrographs of H&E sections:

(a)- Control group (I) showing: normal histological features of the buccal mucosa. Columnar cells with a centrally located nucleus (N) are observed in stratum basale (SB). Stratum spinosum (SS) shows well defined polyhedral cells with a centrally located nucleus (P). Stratum granulosum (SG) is apparently thin and the cells are flat with a centrally located nucleus (F). Keratohyaline granules (H). Stratum corneum shows apparently thin keratin layer (SC). Well defined epithelial rete pegs (R). In the lamina propria (LP); densely packed collagen fiber bundles (C), fibroblast cells (B) and few detected inflammatory cells (I) can be noticed. Well defined C.T papillae (L), (the original magnification x400).

(b)- Diabetic untreated group (II) showing: atrophy in the epithelium. In stratum basale, the cells show apparent reduction in the cytoplasmic and nuclear volumes (SB). Some basal cells undergo degeneration leaving spaces (D). Basal cells with cytoplasmic vacuolations (V) and displaced pyknotic nucleus (N). Nuclei showing pleomorphism (P), karyorrhexis (O) and others showing karyolysis (L). Stratum spinosum shows apparent reduction in cells number and the cells lose their prickly appearance (SS). Degenerated cells (G), cells with pleomorphic nuclei (W), nuclei with marginated chromatin (M) and Karyolytic nuclei (T). Apparent thickening in stratum granulosum (hypergranulosis) (SG) with deeply stained and well defined keratohyaline granules (H). Stratum corneum shows apparent hyperkeratosis (SC). Poorly defined epithelial rete pegs (E). The lamina propria (LP) shows some densely packed collagen fiber bundles (C), while other bundles are diffusely arranged (F). Apparently large areas of degeneration (A). Areas of hyalinization (Z) and fibroblasts showing signs of degeneration (S). The lamina propria is strongly infiltrated by inflammatory cells (I) and invaded by multiple Russell bodies (R). Dilated BVs (B) are lined by swollen endothelial cells (ED) and engorged with RBCs. Hardly detected C.T papillae (Y), (the original magnification x400).

(c)- Insulin treated group (III) showing: apparent increase in epithelium thickness. In stratum basale (SB), some columnar cells with a centrally located nucleus (N), while others with apparent reduction in the cytoplasmic and nuclear volumes (M) are observed. Cells with cytoplasmic vacuolations (V) and displaced pyknotic nucleus (D). Stratum spinosum shows apparent increase in cells number (SS). Many polyhedral cells with a centrally located nucleus (E). Some cells with pleomorphic nuclei (P) and others with cytoplasmic vacuolations (C) and displaced nucleus (I). Some cells with nuclei showing

karyolysis (K). Stratum granulosum shows apparent hypergranulosis (SG) with somewhat deeply stained keratohyaline granules (H). Stratum corneum shows apparent reduction in keratin layer thickness (SC). Apparently short and broad epithelial rete pegs (S). The lamina propria (LP) shows some diffusely arranged collagen fiber bundles (F). Apparently small areas of degeneration (O). Areas of hyalinization (Z) and fibroblasts showing signs of degeneration (B). The lamina propria is infiltrated by some inflammatory cells (Y) and invaded by some Russell bodies (R). Somewhat dilated BVs (L) lined by somewhat swollen endothelial cells (ED) and engorged with RBCs are observed. Apparently short and narrow C.T papillae (A), (the original magnification x400).

(d)- Cod liver oil treated group (IV) showing: more or less normal histological features of the buccal mucosa. Columnar cells with a centrally located nucleus (N) are observed in stratum basale (SB). Few basal cells with cytoplasmic vacuolations (V) and displaced pyknotic nucleus (K). Few basal cells show apparent reduction in the cytoplasmic and nuclear volumes (M). Stratum spinosum shows apparent increase in cells number (SS). Well defined polyhedral cells with a centrally located nucleus (P). Few cells with cytoplasmic vacuolations (T) and displaced nucleus (D) and others with pyknotic nuclei (Y). Few cells with nuclei showing karyolysis (L). Stratum granulosum (SG) is apparently thin with normal stainability of keratohyaline granules (H). Stratum corneum shows apparently thin keratin layer (SC). Well defined epithelial rete pegs (E). The lamina propria (LP) shows densely packed collagen fiber bundles (C). Apparently very small areas of degeneration (G). Few fibroblasts showing signs of degeneration (B). Few inflammatory cells (I) and Russell bodies (R). More or less normal BV is observed (O). The BV has apparently small diameter and it is lined by thin endothelial cells (ED). Well defined C.T papillae (A), (the original magnification x400).

Immunohistochemical results

Caspase-3

Nuclear and/or cytoplasmic caspase-3 expressions were detected in the epithelial and C.T cells of Groups I, II, III and IV with different patterns of reactivity.

In Group I, the keratin layer showed very mild immunohistochemical expression for caspase-3. Few epithelial and C.T cells showed mild nuclear and cytoplasmic immunohistochemical expressions for caspase-3 **(Fig. 2a)**. On contrary, in Group II a strong immuno-expression for caspase-3 was observed in the keratin layer. Moreover, strong immuno-expression for caspase-3 was noticed in the nuclei and cytoplasm of the epithelial and C.T cells. All detected Russell bodies showed strong immuno-expression for caspase-3 **(Fig. 2b)**. In Group III, moderate immuno-expression was noticed in the keratin layer. In addition, moderate immuno-expression was observed in most of the nuclei and in the cytoplasm of the epithelial and C.T cells. All detected Russell bodies showed moderate immuno-expression **(Fig. 2c)**. On the other hand, in Group IV mild immuno-expression was observed in the keratin layer. Furthermore, mild nuclear and cytoplasmic immuno-expressions for caspase-3 were noticed in few epithelial and C.T cells. All detected Russell bodies showed mild immuno-expression **(Fig. 2d)**.

CD-138

In Group I, mild immuno-expression for CD-138 was detected in few inflammatory cells **(Fig. 3a)**. On contrary, in Group II strong immuno-expression for CD-138 was detected in the inflammatory cells and Russell bodies **(Fig. 3b)**. In Group III, moderate immuno-expression for CD-138 was observed in some inflammatory cells and in the detected Russell bodies **(Fig. 3c)**. On the other hand, in Group IV mild immuno-expression for CD-138 was observed in few inflammatory cells and in the detected Russell bodies **(Fig. 3d)**.

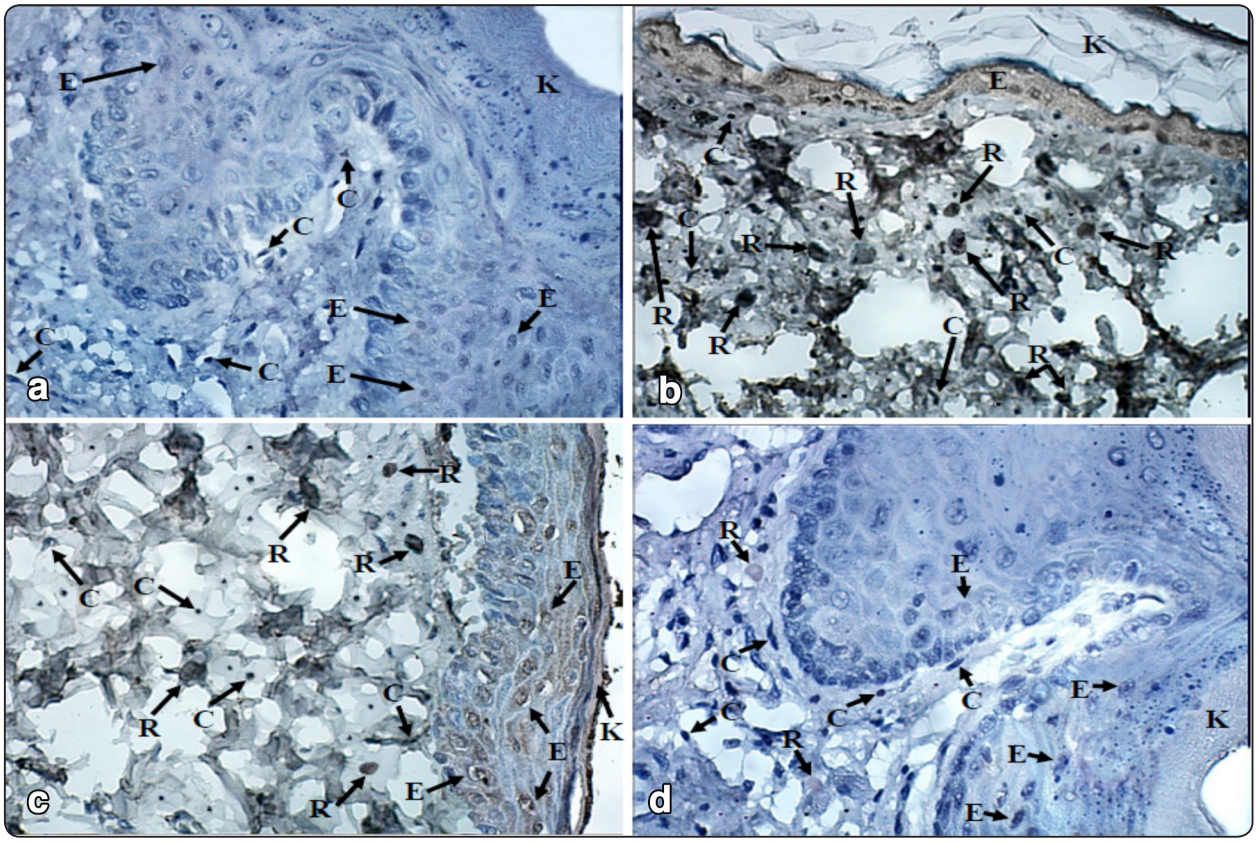

Fig. (2): Photomicrographs of caspase-3 immunolocalization:

(a)- Control group (I) showing: very mild immunohistochemical expression for caspase-3 in the keratin layer (K). Mild nuclear and cytoplasmic immunohistochemical expressions for caspase-3 in few epithelial (E) and C.T cells (C), (the original magnification x400).

(b)- Diabetic untreated group (II) showing: a strong immuno-expression for caspase-3 in the keratin layer (K). Strong immuno-expression for caspase-3 in the nuclei and cytoplasm of the epithelial (E) and C.T cells (C). All detected Russell bodies show strong immuno-expression for caspase-3 (R)**,** (the original magnification x400).

(c)- Insulin treated group (III) showing: moderate immuno-expression in the keratin layer (K). Moderate immuno-expression is observed in most of the nuclei and in the cytoplasm of the epithelial (E) and C.T cells (C). All detected Russell bodies show moderate immuno-expression (R)**,** (the original magnification x400).

(d)- Cod liver oil treated group (IV) showing: mild immuno-expression in the keratin layer (K). Mild nuclear and cytoplasmic immuno-expressions for caspase-3 are noticed in few epithelial (E) and C.T cells (C). All detected Russell bodies show mild immuno-expression (R)**,** (the original magnification x400).

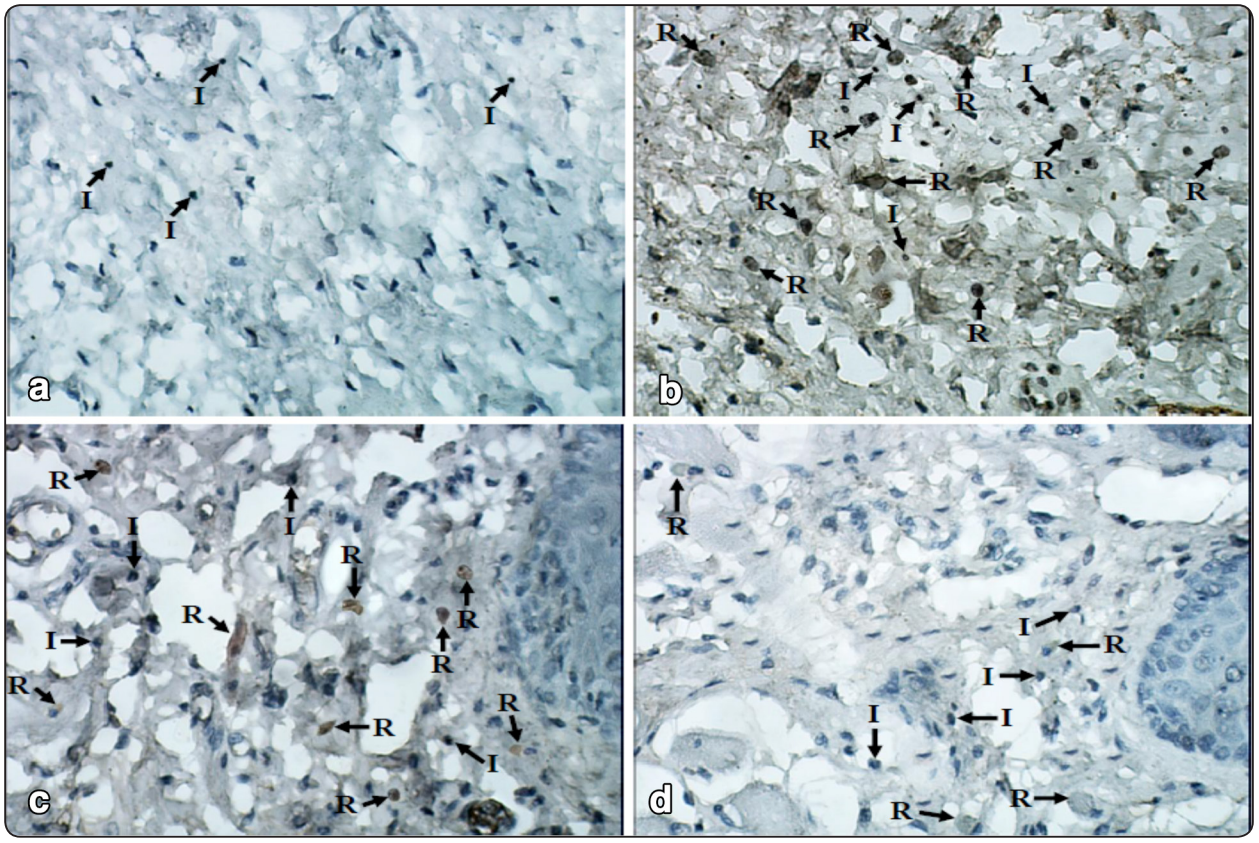

Fig. (3): Photomicrographs of CD-138 immunolocalization:

(a)- Control group (I) showing: mild immuno-expression for CD-138 in few inflammatory cells (I), (the original magnification x400).

(b)- Diabetic untreated group (II) showing: strong immuno-expression for CD-138 in the inflammatory cells (I) and Russell bodies (R), (the original magnification x400).

(c)- Insulin treated group (III) showing: moderate immuno-expression for CD-138 in some inflammatory cells (I) and in the detected Russell bodies (R), (the original magnification x400).

(d)- Cod liver oil treated group (IV) showing: mild immuno-expression for CD-138 in few inflammatory cells (I) and in the detected Russell bodies (R), (the original magnification x400).

Statistical results

Keratin thickness

The control group showed the least mean keratin thickness (μm) followed by CLO treated group, then insulin treated group and the highest value was for the diabetic untreated group. One-way ANOVA showed highly significant difference between the studied groups. Tukey's post hoc test showed highly significant difference between each group when compared with the others (table 1 & fig. 4).

Table (1) Showing the mean ± SD values and results of ANOVA as well as Tukey's post hoc tests for the comparison between the studied groups regarding keratin thickness (μm).

Keratin thickness (μm)	Control	Diabetes	Insulin	Cod liver oil	ANOVA	p-value
Mean	10.307[a]	25.677[b]	14.880[c]	10.866[d]	4344.103	**<0.001****
±SD	0.238	0.592	0.373	0.624		
Min.	9.835	24.582	14.159	10.047		
Max.	10.514	26.390	15.584	11.843		
P1		**<0.001****	**<0.001****	**<0.001****		
P2			**<0.001****	**<0.001****		
P3				**<0.001****		

Different superscript letters in the same row indicate significant difference between each group when compared with the others according to Tukey's post hoc test.

P1: Comparison between control group versus other groups.

P2: Comparison between diabetes group versus insulin and cod liver oil groups.

P3: Comparison between insulin group versus cod liver oil group.

: Highly significant at p-value* ***<0.001.

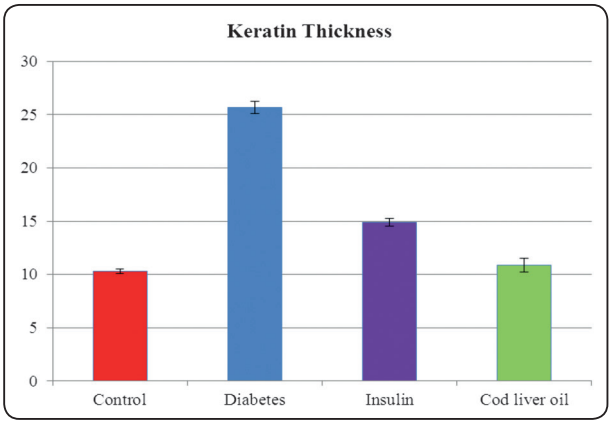

Fig. (4): Bar chart representing mean and SD values of keratin thickness (μm) in the studied groups.

Epithelium thickness

The control group showed the highest mean epithelium thickness (μm) followed by CLO treated group, then insulin treated group and the least value was for the diabetic untreated group. One-way ANOVA showed highly significant difference between the studied groups. Tukey's post hoc test showed highly significant difference between each group when compared with the others (table 2 & fig. 5).

Table (2) Showing the mean ± SD values and results of ANOVA as well as Tukey's post hoc tests for the comparison between the studied groups regarding epithelium thickness (μm).

Epithelium thickness (μm)	Control	Diabetes	Insulin	Cod liver oil	ANOVA	p-value
Mean	78.662[a]	23.090[b]	53.507[c]	77.649[d]		
±SD	0.280	0.322	0.308	0.339		
Min.	78.295	22.633	52.918	76.994		
Max.	79.094	23.462	53.826	77.879	139407.032	**<0.001****
P1		**<0.001****	**<0.001****	**<0.001****		
P2			**<0.001****	**<0.001****		
P3				**<0.001****		

Different superscript letters in the same row indicate significant difference between each group when compared with the others according to Tukey's post hoc test.

P1: Comparison between control group versus other groups.

P2: Comparison between diabetes group versus insulin and cod liver oil groups.

P3: Comparison between insulin group versus cod liver oil group.

******: Highly significant at p-value **<0.001**.***

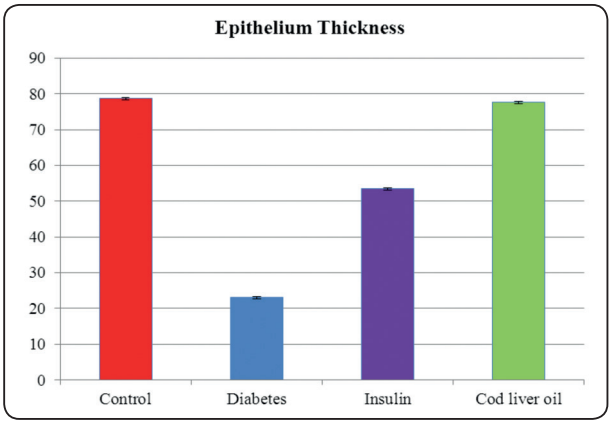

Fig. (5): Bar chart representing mean and SD values of epithelium thickness (μm) in the studied groups.

Caspase-3 immunoreactivity

The control group showed the least mean area percentage of caspase-3 immunoreactivity followed by CLO treated group, then insulin treated group and the highest value was for the diabetic untreated group. One-way ANOVA showed highly significant difference between the studied groups. Tukey's post hoc test showed highly significant difference between each group when compared with the others (table 3 & fig. 6).

TABLE (3) Showing the mean ± SD values and results of ANOVA as well as Tukey's post hoc tests for the comparison between the studied groups regarding caspase-3 immunoreactivity area %.

Caspase-3 immunoreactivity area %	Control	Diabetes	Insulin	Cod liver oil	ANOVA	p-value
Mean	1.640[a]	19.310[b]	9.500[c]	1.870[d]	62261.677	**<0.001****
±SD	0.110	0.255	0.108	0.021		
Min.	1.500	19.000	9.300	1.850		
Max.	1.800	19.900	9.700	1.900		
P1		**<0.001****	**<0.001****	**<0.001****		
P2			**<0.001****	**<0.001****		
P3				**<0.001****		

Different superscript letters in the same row indicate significant difference between each group when compared with the others according to Tukey's post hoc test.

P1: Comparison between control group versus other groups.

P2: Comparison between diabetes group versus insulin and cod liver oil groups.

P3: Comparison between insulin group versus cod liver oil group.

******: Highly significant at p-value **<0.001.***

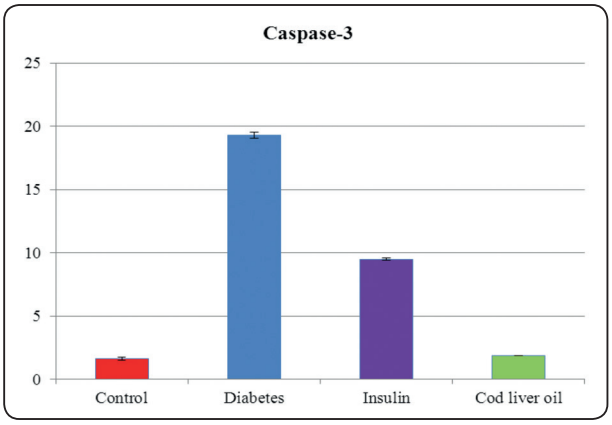

Fig. (6): Bar chart representing mean and SD values of caspase-3 immunoreactivity area % in the studied groups.

CD-138 immunoreactivity

The control group showed the least mean area percentage of CD-138 immunoreactivity followed by CLO treated group, then insulin treated group and the highest value was for the diabetic untreated group. One-way ANOVA showed highly significant difference between the studied groups. Tukey's post hoc test showed highly significant difference between each group when compared with the others (table 4 & fig. 7).

TABLE (4) Showing the mean ± SD values and results of ANOVA as well as Tukey's post hoc tests for the comparison between the studied groups regarding CD-138 immunoreactivity area %.

CD-138 immunoreactivity area %	Control	Diabetes	Insulin	Cod liver oil	ANOVA	p-value
Mean	1.890[a]	12.320[b]	7.600[c]	2.255[d]	9490.672	**<0.001****
±SD	0.107	0.378	0.222	0.051		
Min.	1.700	11.700	7.400	2.200		
Max.	2.000	12.700	8.100	2.300		
P1		**<0.001****	**<0.001****	**<0.001****		
P2			**<0.001****	**<0.001****		
P3				**<0.001****		

Different superscript letters in the same row indicate significant difference between each group when compared with the others according to Tukey's post hoc test.

P1: Comparison between control group versus other groups.

P2: Comparison between diabetes group versus insulin and cod liver oil groups.

P3: Comparison between insulin group versus cod liver oil group.

: Highly significant at p-value* ***<0.001.

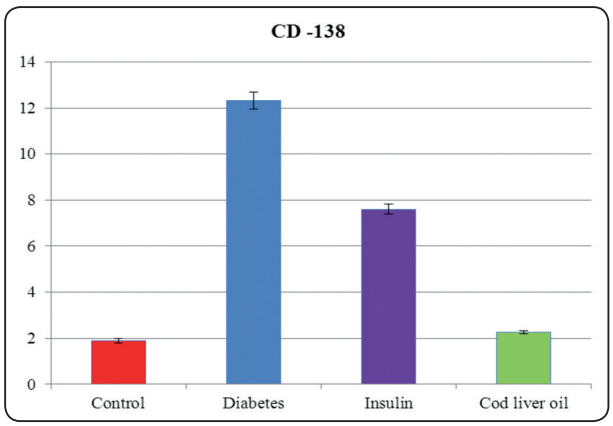

Fig. (7): Bar chart representing mean and SD values of CD-138 immunoreactivity area % in the studied groups.

DISCUSSION

Induction of diabetes mellitus in the present study was confirmed from the third day of STZ injection. It resulted in marked affection of the buccal mucosa. It was reported that affection of the oral cavity due to diabetes is a part of its systemic effect ***(Fahmy and Soliman, 2007)***.

One of the objectives of this study was to develop a rat model for type 1 DM or insulin-dependent diabetes that may mimic 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 ideal dose for development of animal model with type 1 diabetes ***(Homo-Delarche, 2001; Islam and Choi, 2007)***.

The current study revealed several histological changes in the epithelial layer of the buccal mucosa in the diabetic untreated group (Group II). The histological changes in Group II revealed atrophy in the epithelium, evidences of cells undergo degeneration and nuclear changes in form of; pleomorphism, pyknosis, karyorrhexis, karyolysis and marginated chromatin. These findings were in line with those of ***Nagy et al. (2000)*** and ***Caldeira et al. (2004)*** who stated that these changes are considered as pre-necrotic changes. The authors attributed these pre-necrotic changes to the persistent hyperglycemia since the β-cell destruction in rat models with type 1 DM results in loss of insulin secretion and therefore uncontrolled blood glucose levels. The persistent hyperglycemia causes increased production of

free radicals especially reactive oxygen species (ROS) from glucose auto-oxidation and protein glycosylation. Abnormally high levels of free radicals and simultaneous decline of antioxidant defense systems can lead to the damage of cellular organelles and enzymes, increased lipid peroxidation and development of complications of DM ***(Maritim et al., 2003; Robertson, 2004)***. Moreover, diabetes leads to atrophy in the epithelium of the oral cavity by decreasing the number of prickle cells. This could be explained according to the fact 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 might greatly affect the rate of cell division ***(Nagy et al., 2000)***. Additionally, in insulin deficiency decreased phosphorylation of Akt-mammalian target of rapamycin (Akt-mTOR) signaling pathway causes lower protein synthesis. Therefore, rate of cell proliferation in rat oral mucosal epithelium is reduced ***(Sricharoenvej et al., 2012)***.

The ill-defined cell membrane of the epithelial cells detected in Group II of this study could be attributed to the fact that among all biomolecules, lipids are the most sensitive to free radicals. Double bonds in fatty acids form peroxide products by reacting with free radicals and lipid radicals can be formed subsequently upon removal of electrons. Thus the free radicals produced in persistent hyperglycemia can elicit changes in the acyl fatty acids composition. Acyl fatty acids are the main components of the phospholipid bilayer thus they form the major constituents of the biological membranes. Changes in the phospholipid fatty acid composition of cell membranes will result in changes in the collective physicochemical properties of the bilayer, such as flexibility and integrity ***(Mahay et al., 2004; Leite et al., 2014)***.

Cytoplasmic vacuolations of epithelial cells observed in Group II of the current study could be explained according to ***Woolf, (2000)*** who stated that cytoplasmic vacuolation is indicative of necrosis and attributed this to the reduced activity of cellular adenosine triphosphatase (ATPase) resulting in failure of sodium pump mechanism that is responsible for water and electrolytes control. So, cells accumulate water and cellular degeneration develops. Moreover, ***Caldeira et al. (2004)*** attributed the cytoplasmic vacuolations to the accumulation of lipid droplets in the cell cytoplasm. Accumulation of lipid droplets in diabetic tissue could be related to carbohydrate and/or fat metabolism disorders that lead to damage of the cells which in turn initiates apoptosis ***(Mahay et al., 2004)***.

It should be mentioned that normally the epithelium of the buccal mucosa of albino rats is keratinized stratified squamous epithelium ***(Sultan et al., 2014)***.

In the current study, Group II revealed apparent thickening of stratum granulosum (hypergranulosis) with deeply stained and well defined keratohyaline granules. This was accompanied by hyperkeratosis of stratum corneum. Stratum granulosum, is a 3-5 sheets granular layer of keratinocytes producing keratohyaline granules that are involved in keratinization ***(Caldeira et al., 2004; Fahmy and Soliman, 2007)***. The hypergranulosis and hyperkeratosis in our study could be explained according to the fact that profilaggrin which is the precursor of filaggrin (filament-aggregating protein) is initially synthesized as a highly phosphorylated insoluble proprotein. The insoluble nature of the proprotein might facilitate its packing into the keratohyaline granules. During cornification profilaggrin is proteolytically cleaved in linker sites and dephosphorylated to release individual filaggrin monomers which decorate the surface of the keratin filaments, causing the filaments to aggregate ***(Denecker et al., 2007)***. Moreover, procaspase-3 activation is required for both profilaggrin and filaggrin processing ***(Weil et al., 1999)***. Since, STZ causes inhibition of the enzyme O-GlcNAcase (N-acetyl-D-glucosaminidase) which removes protein linked GlcNAc. Thus, an increase in the intracellular levels of proteins modified by GlcNAc

occurs and leads to activation of procaspase-3 and so enhancement of profilaggrin and filaggrin processing ***(Liu et al., 2000; Konrad et al., 2001; Szkudelski, 2001; Zafir et al., 2013)***. Additionally, hyperkeratosis was also demonstrated by previous investigators who attributed this finding to the fact that in DM there is decrease in salivary flow rate and pH value of saliva which may have an irritant effect. Furthermore, in DM the hyperkeratosis may be also a result of repeated infections of buccal cavity due to immunological injury ***(Li et al., 2000; Kadir et al., 2002)***. Moreover, it was also reported that in type 1 DM there is increased incidence of leukoplakia which is considered to be the most frequent precancerous state in the oral cavity ***(Kadir et al., 2002)***. The affection is not only in the keratin quantity but also quality. Only few studies have investigated whether filaggrin and filaggrin gene mutations may be involved in diabetes. There is a genetic relationship between filaggrin gene mutations and type 1 DM. Type 1 DM is associated with enhanced expression of proinflammatory cytokines such as tumor necrosis factor (TNF)-α, interleukin (IL)-1β, IL-18 and interferon (IFN)-γ which lead to mutations in filaggrin gene ***(Joussen et al., 2003; Saleh et al., 2011)***.

Group II in herein study showed lamina propria with differential orientation and arrangement of the collagen fiber bundles, areas of degeneration and hyalinization in addition to fibroblasts that showed signs of degeneration. Our findings could be attributed to the fact that the metabolic disturbances associated with diabetes can lead to the formation of advanced glycation end-products (AGEs) that induce fibroblast (the matrix producing cell) apoptosis, which is mediated through caspase-3 and signaled through both caspase-8 and caspase-9 activities leading to suppression of collagen synthesis and excessive collagenolytic activity ***(Caldeira et al., 2004; Riedl and Shi, 2004; Alikhani et al., 2005 a)***.

The histological results of Group II in this study revealed that the lamina propria was strongly infiltrated by inflammatory cells and invaded by multiple Russell bodies scattered throughout the C.T. The strong inflammatory cells infiltration could be - according to previous studies - due to what is known as "hyper-inflammatory trait" which occurs with diabetes. This is characterized by an exaggerated secretion of inflammatory mediators as TNF-α and IL-6 as well as systemic markers of inflammation which collectively act as chemotactic factors to the inflammatory cells ***(Page et al., 1997; Salvi et al., 1998)***. Russell bodies (Mott cells) are an uncommon finding that may be associated with chronic inflammatory conditions. T-cells might promote the formation of Russell bodies through the release of soluble factors, such as IL-2 ***(Shultz et al., 1984; Verheij et al., 2009)***. However, the precise mechanism by which T-cells affect Mott cells formation is presently unknown. It should be mentioned that the hyaline substance detected in the Russell bodies corresponds in particular to the accumulation of immunoglobulins in the cytoplasm of plasma cells within the endoplasmic reticulum cisternae and they may exist as smaller particles in extracellular locations as well ***(Paik et al., 2006)***.

In the current study, Group II showed dilated BVs; lined by swollen endothelial cells and engorged with RBCs. Our findings could be attributed to the fact that the dilatation and congestion of the BVs might be a part of inflammatory response to bring more blood to the areas of degeneration ***(Moubarak, 2008)***. Moreover, during hyperglycemia, increased ROS and diacylglycerol (DAG) stimulate protein kinase C (PKC) in the vascular tissue. Both PKC and ROS cause loss of permeability that leads to swelling of the endothelium ***(Evcimen and King, 2007)***.

Our imunohistochemical results confirm our histological findings observed in Group II of the current study, in which caspase-3 antibodies revealed intense positive reaction to activated caspase-3 in the keratin layer and in the nuclei and cytoplasm of the epithelial and C.T cells as well as in the Russell bodies. Our findings could be attributed - as previously mentioned - to the fact that the toxicity of STZ is related to the inhibition of the enzyme O-GlcNAcase (N-acetyl-D-glucosaminidase) which

removes protein linked GlcNAc ***(Liu et al., 2000; Konrad et al., 2001; Szkudelski, 2001)***. The increase in the intracellular levels of proteins modified by GlcNAc leads to cell death by apoptosis ***(Konrad et al., 2001)***. Furthermore, the metabolic disturbances associated with diabetes can lead to activation of the polyol pathway, high levels of the cytokine TNF-α, the formation of AGEs, high levels of PKC as well as activation and enhanced oxidative stress ***(Asnaghi et al., 2003)***. The activation of these pathways may be especially important in initiating events linked to inflammation and apoptosis ***(Dagher et al., 2004; Xu et al., 2004)***.

There are several mechanisms that could be responsible for the higher rate of apoptosis noted in Group II of the current study. One mechanism may be through the cytokine activation of receptors with 'death domains', such as TNF receptor-1 (TNFR-1) or fas ***(Alikhani et al., 2005 b)***. Diabetes is associated with both enhanced TNF and fas/fas-ligand expression ***(Joussen et al., 2003)***. IL-1 or IFN-γ may also promote apoptosis, even though their receptors lack death domains, by altering pro-apoptotic gene expression or enhancing production of oxygen radicals ***(Schroder et al., 2004)***. Another mechanism is through the increased oxidative stress which is one of the common pathogenic factors of diabetes complications as it leads to the formation of excess ROS which lead to severe oxidative damage of the cell's components like lipids, proteins and DNA by inhibiting many of the enzymes involved in DNA synthesis. These pathways lead to one result which is apoptosis through activation of caspases ***(Riedl and Shi, 2004)***.

Caspases are synthesized as catalytically inactive zymogens and must undergo proteolytic activation during apoptosis. Initiator caspases, such as caspases-8 and 9 are required to process the executioner caspases, such as caspase-3 leading to their activation. Active caspase-3 cleaves over 40 intracellular substrates that cause cell death ***(Boatright and Salvesen, 2003)***. Moreover, the active caspase-3 induces activation of caspase-activated deoxyribonuclease (CAD), also called DNA fragmentation factor-40 (DFF), that is involved in degrading DNA and apoptosis in different cells which in turn lead to organ atrophy and failure ***(Shredah and El-Sakhawy, 2014)***. It was reported that *in vivo* AGEs induce mitochondria apoptosis, which is mediated through caspase-3 and signaled through both caspase-8 and caspase-9 activities ***(Alikhani et al., 2005 a)***. However interestingly, AGEs stimulate nuclear factor-κβ (NF-κβ) activation, which is anti-apoptotic ***(Wang et al., 1998)***. It should be mentioned that one form of apoptosis in insulin producing cells is independent of NF-κβ mediated transcription but dependent on caspase-3 activity and poly adenosine diphosphate ribose (poly ADP-ribose) polymerase-1 cleavage ***(Saldeen and Welsh, 1998)***. In addition to modulating death-receptor-mediated apoptosis, keratins have a more general effect in apoptosis by controlling key stages of the caspase activation cascade. In epithelial cells receiving death stimuli that activate either the death-receptor or mitochondrial-dependent pathway, the death effector domain containing DNA binding protein (DEDD) directs procaspase-3 to keratin intermediate filaments at an early stage of apoptosis, preceding caspase activation and nuclear changes ***(Lee et al., 2002)***. Furthermore, the catalytically active caspase-9, cleaved at both Asp315 and Asp330, is similarly concentrated on keratin fibrils ***(Dinsdale et al., 2004)***. Emerging evidence supports a model in which keratin filaments and their associated DEDD provide a scaffold for accumulation and auto-activation of procaspase-9, which in turn cleaves procaspase-3 that is within close proximity. The active caspase-3 can further activate procaspase-9 through cleavage at Asp330, thereby facilitating a caspase amplification loop ***(Dinsdale et al., 2004)***.

In the present study, Group I revealed very mild immunohistochemical expression for caspase-3 in the keratin layer. This finding could be attributed -as previously mentioned- to the fact that during cornification profilaggrin is proteolytically cleaved in linker sites and dephosphorylated to release individual filaggrin monomers which decorate the surface of the keratin filaments, causing the

filaments to aggregate ***(Denecker et al., 2007)***. Moreover, procaspase-3 activation is required for both profilaggrin and filaggrin processing ***(Weil et al., 1999)***. This group also revealed mild nuclear and cytoplasmic immunohistochemical expressions for caspase-3 in few epithelial and C.T cells. This finding might be attributed to the physiological cell death ***(Shredah and El-Sakhawy, 2014)***.

In the present study, the development of Russell bodies in association with type 1 diabetes was confirmed through the use of anti-CD-138 antibody (syndecan-1) which was strongly expressed in the inflammatory cells and Russell bodies in Group II as compared to Group I which exhibited mild CD-138 immuno-expression in few inflammatory cells. Syndecan-1 (CD-138) is a member of the transmembrane heparan sulfate proteoglycan family that acts as an extracellular matrix receptor ***(Elenius et al., 1990; Mali et al., 1990)***. It is involved in many cellular functions, including cell-cell adhesion and cell-matrix adhesion. In addition, CD-138 modulates the activity of chemokines, cytokines, integrins, and other adhesion molecules which play important roles in the regulation of inflammation ***(Chilosi et al., 1999; Costes et al., 1999; Masouleh et al., 2009)***. Expression of syndecan-1 typically is observed on the surface of mature epithelial cells; however, stromal expression also might be seen in developing tissues. Within the hematopoietic system, syndecan-1 expression is thought to be restricted to normal and malignant plasma cells ***(Chilosi et al., 1999; Costes et al., 1999)***. As previously mentioned, Russell bodies are eosinophilic, large and homogenous immunoglobulin-containing cytoplasmic inclusions, usually found in plasma cells undergoing excessive synthesis of immunoglobulins. They are immunopositive for the CD-138 antibody. It was reported that Mott cells have been localized, although usually in small numbers, in the lymphoid organs of man and laboratory animals, and in increased numbers after hyperimmunization, lymphocytic malignancies and acquired immune deficiency syndrome (AIDS). Thus, Russell bodies are an uncommon finding that may be associated with chronic inflammatory conditions ***(Erbersdobler et al., 2004; Ensari et al., 2005; Pizzolitto et al., 2007; Verheij et al., 2009; De Petris and Leung, 2010; Habib et al., 2010; Del Gobbo et al., 2011)***.

In the current study, the insulin dose applied was based on the proposal that the best glycemic control was obtained with the dose 5 IU/kg body weight/ day ***(Pinheiro et al., 2011)***.

In this study the histological, immunohistochemical and statistical results of Group III were better than those of Group II. Our results could be attributed to the fact that insulin stimulates several processes such as carbohydrate transport, protein synthesis and regulation of lipid metabolism. Furthermore, insulin stimulates the synthesis of apolipoprotein E (ApoE) mRNA that leads to decrease in the level of cholesterol. Additionally, insulin has the ability to regulate the production/release of the pro-inflammatory cytokines, IL-1β and TNF-α as well as the expression of P- and E-selectin. Insulin also modulates leukocyte migration and improves anti-oxidant enzymes activity ***(Take et al., 2007; Aziz, 2009; Martins et al., 2010)***.

Our results showed that insulin did not completely inhibit the complications of diabetes. Our finding is in agreement with that of ***Lebovitz and Face, (2011)*** who stated that there are no better clinical outcomes in treatment with insulin compared to those that occur with other antihyperglycemic regimens. They attributed 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 also it is not stringent enough to prevent excessive protein glycation, which can increase tissue oxidative stress ***(Zobali et al., 2002; Jain et al., 2006)***.

The histological, immunohistochemical and statistical results of Group IV in the current study showed 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 Group III. Our results 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 ***(Greenbaum et al., 2009)***. Moreover, ω-3 fatty acids in CLO rather than other sources of ω-3 fatty acids significantly inhibit the production of arachidonic acid-derived eicosanoids, namely the prothrombotic thromboxane A2 by activated platelets and the proinflammatory leukotrienes B2 and C4 by activated leukocytes and considerably inhibit the elevation of inflammatory cytokines (TNF-α, IL-1 and IL-6) produced by infiltrating macrophages, lymphocytes and monocytes. Therefore ω-3 fatty acids inhibit the formation of oxygen free radicals, lipid peroxides as well as aldehydes which also cause damage to the pancreatic ß-cells ***(Rabinovitch et al., 1996 a; Hamdy et al., 2007; Tsitouras et al., 2008; Mayer-Davis et al., 2013)***. Consequently, ω-3 fatty acids in CLO may protect against further pancreatic ß-cell loss after onset of type 1 diabetes ***(Mayer-Davis et al., 2013)***. Additionally, ω-3 fatty acids maintain the antioxidant status in a normal range through significant increase in the activities of antioxidant enzymes such as superoxide dismutase (SOD) by up regulating gene expression of antioxidant enzymes and down regulating gene associated with production of ROS ***(Soltan, 2012; Hussein et al., 2014)***. As a result, after onset of type 1 diabetes ω-3 fatty acids may also play an important role in up regulation of insulin synthesis (by ß-cells) and its subsequent transport extracellularly through inhibiting oxidative stress formation ***(Soltan, 2012; Mayer-Davis et al., 2013)***.

C-peptide is a peptide composed of 31 amino acids; it is released from the pancreatic ß-cells during cleavage of insulin from proinsulin ***(Mayer-Davis et al., 2013)***. So monitoring of C-peptide concentration could be beneficial in the design of the future studies aiming to establish the possible effect of CLO intake in preventing the development of type 1 DM.

CONCLUSIONS

This study has demonstrated that diabetes has deleterious effect on the structure of the buccal mucosa. The raised levels of capase-3 in diabetic buccal mucosa are related to increased Russell bodies formation.

Insulin can't completely inhibit the complications of diabetes. However, it has been proved in this study that ω-3 fatty acids present in CLO can inhibit to a great extent the abnormalities caused by diabetes.

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