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Effect of Stem Cells versus Statins on the Mucous Membrane and Salivary Glands of the Tongue of Induced-Diabetic Rats

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Abstract

Background: **Diabetes Mellitus (DM) is one of the most prevalent metabolic diseases. Statins are the most commonly prescribed medications to lower plasma LDL-C levels. Statins also have been shown to reduce the progression of atherosclerotic plaques. Stem cell (SC) therapy is emerging as a potentially revolutionary way to treat disease and injury with wide ranging medical benefits.** ***Aim:*** **to evaluate the effect of SC versus simvastatin on the mucous membrane and salivary glands of the tongue of rats with streptozotocin-induced DM.** ***Material and Methods:*** **Seventy-five male albino rats (weight 150-180gr) were divided into the following groups: Group1.1 (15 rats) served as controls. Group 2.1 (15 rats) were subjected to a single intraperitoneal injection of streptozotocin (60mg/kg body weight) for induction of DM. Group 3.1 (15 rats) were subjected to induction of DM (as in group 2.1). One week later, the animals were treated with simvastatin in a daily intraperitoneal dose of 5mg/kg body weight. Group 4 (30 rats) were subdivided into: Subgroup.4.1 (15 rats) subjected to induction of DM (as in group 2.1). One week later they were subjected to single intravenous infusion of mesenchymal bone marrow SCs. Subgroup. 4.2 (15 rats) were used for isolation and culture of bone marrow SC. 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 diabetic rats' tongues revealed atrophic and degenerative changes on the tongue papillae. The examined tongues of the rats treated with simvastatin showed partial improvement in the histological picture while the tongue of rats treated with SC showed almost normal histology.** ***Conclusion*****: The anti- inflammatory effect of SMV on the diabetic tongue. Bone marrow-derived SC are responsible for repairing the tissues and replacing them when injured or exposed to tear, wear or diseases.**

Keywords: LDL-C- atherosclerotic- diabetic tongue- tongue papillae- light and SEM.

Introduction

Changes in human behavior and lifestyle over the last century have resulted in a dramatic increase in the incidence of diabetes. Diabetes is a serious illness that affects many people, and there are many new cases diagnosed every year in all populations around the world. Diabetes mellitus is a disorder characterized by elevated blood

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glucose levels caused by an absolute or relative lack of insulin; there can be a low output of insulin from the pancreas, or the peripheral tissues may resist insulin. Glucose homeostasis depends mainly on the pancreatic hormone insulin. Insulin facilitates glucose transport into cells and thus lowers blood sugar level. Diabetes mellitus affects the blood circulations and is associated with many complications such as retinopathy, ischemic heart disease, nephropathy, cerebrovascular disease, neuropathy and peripheral arterial diseases in the lower extremity, which can lead to foot ulcer[1,2]. But the most obviously apparent complication of diabetes happens to be periodontal disease which, in cases of uncontrolled diabetes, may eventually lead to tooth loss and total or partial edentulism[3-5]. Unfortunately, these Diabetics have always posed as a contraindication to dental implants[6]. Dysfunction of taste and burning mouth syndrome (glossodynia/stomatopyrosis) are reported in numerous diabetic patients that could result in hyperphagia and obesity. Dry, atrophic, and cracking oral mucosa is the result of inadequate production of saliva and leads to burning mouth syndrome. Mucositis, desquamation, ulcers, and a depapillated and inflamed tongue are also frequent problems in diabetic patient. These complications are considered as sensory dysfunction[7-9]. The statin drugs known as hypolipidemic drugs have recently been examined for their usefulness in the treatment of the conditions such as osteoporosis, Alzheimer's disease[10], cardiac diseases, organ transplantation, stroke and diabetes[11]. The synergism of statins with other drugs can also be useful in reducing the incidence of cardiovascular events. For example, synergism of simvastatin with losartan (angiotensin II type 1 receptor antagonists) prevents angiotensin II-induced cardiomyocyte apoptosis (which has an important role in the transition from compensatory cardiac remodeling to heart failure) *in vitro.* Stem cell transplantation is a promising regenerative therapy. The transplantation of various adult mesenchymal stem cells (MSCs) and their derivatives into damaged areas promotes tissue repair in humans and model animals, transplanted MSCs accelerate new bone formation in various preclinical animal models for bone defects[12].

Material and Methods

Seventy-five male albino rats with body weight ranging from 150-180gr.were used in the present investigation. They were divided into the following groups: *Group1.1:* consisted of 15 animals and served as controls. *Group 2.1*: consisted of 15 animals, they were subjected to a single intraperitoneal injection of streptozotocin in a dose of 60mg/kg body weight for induction of diabetes mellitus[13]. *Group 3.1:* consisted of 15 animals. They were subjected to induction of diabetes mellitus in the same way as group 2.1 animals. One week later, the animals were treated with simvastatin in a daily intraperitoneal dose of 5mg/kg body weight[14]. *Group 4:* consisted of 30 rats that were subdivided as follows: *Subgroup.4.1:* fifteen rats subjected to induction of diabetes mellitus, the same way as group 2.1 One week later they were subjected to single intravenous infusion of mesenchymal bone marrow SC (1.5×10^3 - 10^6) cells per rat in 0. 2ml phosphate buffer saline, slowly injected into the tail vein over 2 min period using a 22 gauge needle[15]. *Subgroup. 4.2:* fifteen rats were used for isolation and culture of bone marrow SC

Preparation of mesenchymal bone marrow-derived stem cells:

Bone marrow was harvested by flushing the tibiae and femurs of the rats (subgroup4.2) with Dulbecco's modified Ea-

gle's medium (DMEM, Gibco/BRL) supplemented with 10% fetal bovine serum (Gibco/BRL). Nucleated cells were isolated with a density gradient {Fico plaque (pharmacial)} and resuspended in complete culture medium supplemented with 1% penicillin – streptomycin (Gibco/BRL). Cells were incubated at 37°c in 5% humidified CO_2 for 12-14 days as primary culture or upon formation of larger colonies. When large colonies develop (80-90%confluence), cultures were washed twice with phosphate buffer saline and the cells were tripsinized with 0.25% trypsin in one mm EDTA for 5 minutes at 37°c. After centrifugation, cells were resuspended with phosphate buffer saline. At the end of the experiment, the animals of the different groups were sacrificed by cervical dislocation; their tongues were dissected out, fixed in 10% neutral buffered formalin, processed and embedded in paraffin. Six microns thick sections were cut to be stained.

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: Hematoxylin and Eosin (H&E) stain.

Preparation of the specimen for examination by scanning electron microscopy:
Representative tongue specimens from the rats of all groups were fixed in 2.5% glutraldhyde in 0.1M phosphate buffer (PH 7.4) for 4 hours. Then, the samples were washed with phosphate buffer and post fixed in 1% osmium tetroxide for 90 minutes then the samples were washed again with phosphate buffer and dehydrated through series of ascending concentrations of ethanol to 100% amyl acetate[16]. After that, samples were coated with gold under vacuum with sputter coater. After gold coating, tongue samples were examined and photographed with JEOL, JSM-53009 scanning electron microscope in EM unit, National Research Centre, Cairo.

Results

I. Histological findings
Hematoxylin and Eosin stain: The histological examination of hematoxylin and eosin stained sections of the mucous membrane of the tongue of the control rats showed the normal histological features of surface epithelium and underlying lamina propria.
The filliform papillae (fig.1 A) appeared sharp conical covered with stratified squamous epithelium with thin regular keratin layer. The fungiform papillae (fig.1 B) were seen in between filliform ones with broader surface and wide vascular connective tissue core and most often at the tip and lateral borders of the body of the tongue. Examination of the tongues of diabetic rats revealed atrophic and degenerative changes that involved the surface epithelium and lamina propria of both the dorsal and ventral surfaces of the tongue as well as the lingual salivary glands. The filliform papillae (fig.2) lost the normal appearance. They were markedly atrophic; their number and length were apparently much decreased when compared with those of the control animals. Most of them showed flattening, loss of their characteristic conical shape, with evident hyperkeratosis. Their epithelial covering showed marked thickening with many vacuolated cytoplasm and ill-defined CT papillae. The fungiform and circumvallate papillae showed signs of atrophy in their epithelium. Most of the examined fungiform papillae (fig.3&4A) appeared dome shaped. Taste buds appeared vacuolated with peripheral arrangement of the cells and empty center. The ventral surface of the tongue of diabetic rats showed marked atrophic and degenerative changes in its mucous membrane. The surface epithelium was so thin, atrophic with intracellular

cytoplasmic vacuolization and a decrease in the epithelial ridges. The collagen fibers of the lamina propria showed dissociation with dilatation of blood vessels and their engorgement with blood (fig.4B).

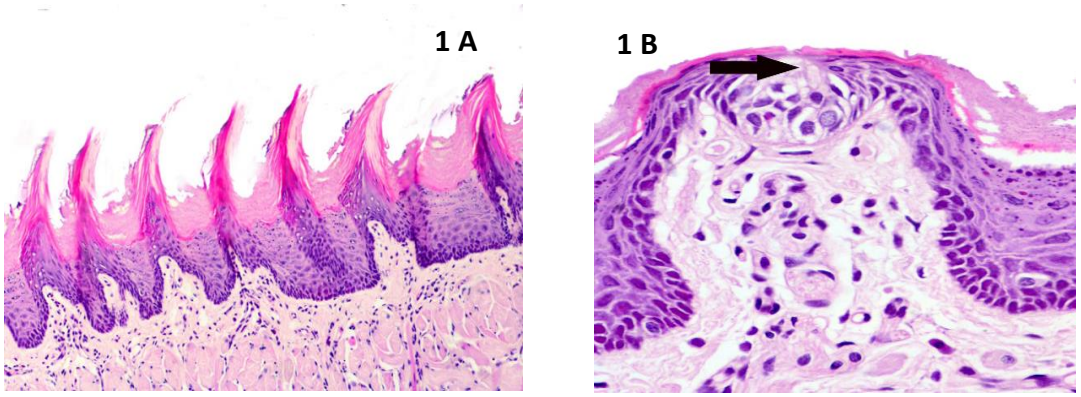


Figure 1:A Photomicrographs of the dorsal surface of the control tongue showing: A) sharp conical projections of filiform papillae with thin smooth keratinized epithelial covering and lamina propria. Skeletal muscle fibers running in different directions are seen underneath the papillae. B) A fungiform papilla showing normal barrel like intraepithelial taste bud (arrow). (H&E, orig. mag.A- 100 , B- 400)

The examined tongues of the rats treated with simvastatin showed partial improvement in the histological picture of the surface epithelium and lamina propria of the dorsal and ventral surfaces of their tongues as well as their lingual salivary glands when compared with group II animals. There was regain in the number of the filliform papillae in comparison with the diabetic rats (fig.5). The fungiform and circumvallate papillae appeared more or less normal with minor signs of shrinkage or degeneration. The taste buds of the fungiform showed improvement in their histology. The circumvallate papillae were nearly normal (fig.6). The examined tongues of the rats that received single intravenous injection of stem cells showed improvement in their histology when compared with group II animals (fig.7 & 8). The dorsal surface of the tongue of rats treated with stem cells showed almost normal structure of filliform papillae including their covering epithelium and keratin.

II. Immunohistochemical results

Proliferating Cell Nuclear Antigen (PCNA): The histological sections of tongues of the controls revealed moderately to strongly positive PCNA staining reaction mainly at the basal and parabasal cells of the surface epithelium indicating normal proliferation of the cells of the dorsal and ventral surfaces of the tongue. All the basal cells were positively stained (fig.9). The sections of the tongues of diabetic rats showed low expression of PCNA in the basal layer of the epithelium of the dorsal and ventral surfaces ranging from negative to weakly positive staining reaction which indicated a decrease in proliferation of basal and parabasal cell layers denoting marked decrease in the rate of turnover and cell renewal. Most of the basal cells were negatively stained (fig.10). The sections of the tongues of rats that treated with simvastatin showed moderately to strongly positive PCNA expression in the basal and parabasal cells of the dorsal and ventral surfaces of the tongue, however not all the basal cells were positively stained (fig.11). The sections of the tongues of rats that received IV injection of stem cells, showed moderately to strongly positive PCNA expression in the basal and parabasal cells of the dorsal and ventral surfaces (fig.12).

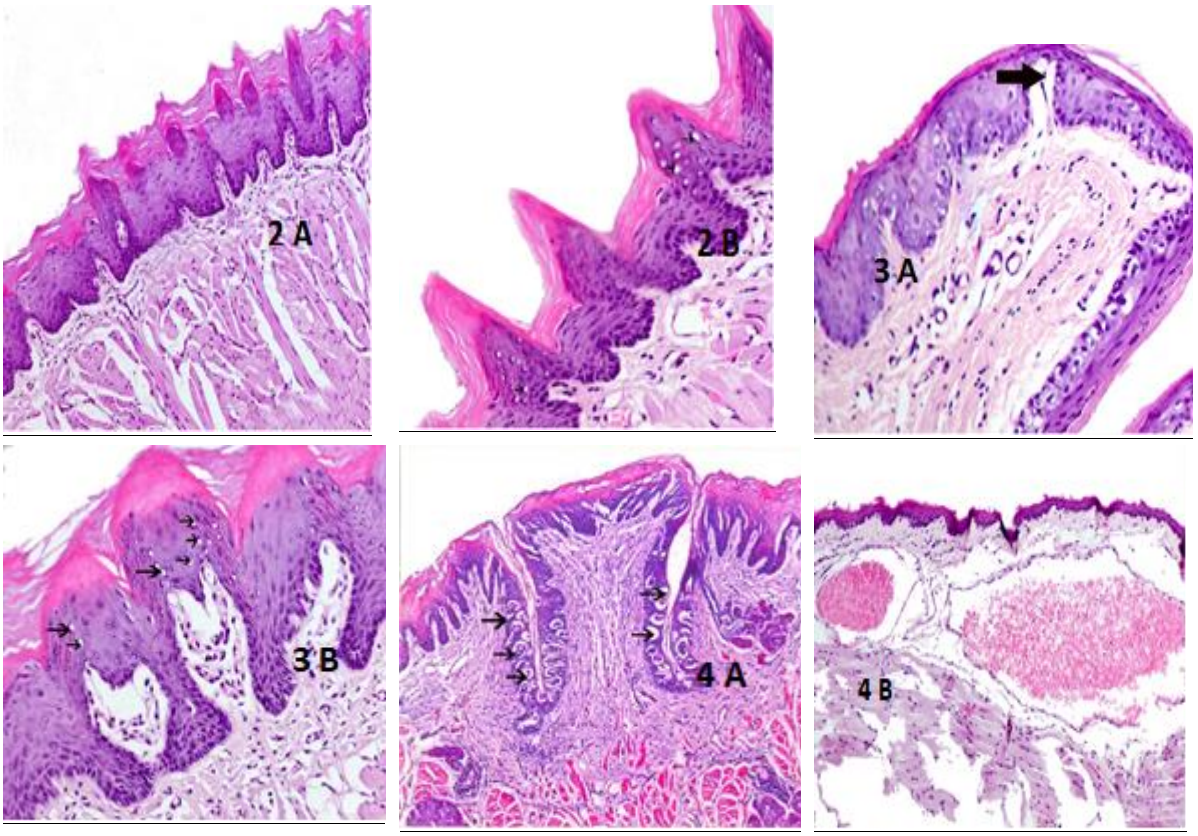


Figure 2: A photomicrograph of the dorsal surface of the tongue of group II animals showing: A) atrophy and apparent decrease in the length and number of filliform papillae. B) hyperkeratotic changes and evident cytoplasmic vacuolization of epithelial cells.(H&E, orig. mag.A- 200 , B- 250). **Figure 3**: A photomicrograph of the dorsal surface of the tongue of group II animals showing: A) mal-shaped fungiform papillae with atrophic epithelium, cytoplasmic vacuolization, degenerated connective tissue of lamina propria and atrophic taste buds. B) the epithelial lining of the fungiform papillae with many cytoplasmic vacuolizations (arrows). (H&E, orig. mag.A- 200 , B- 250). **Figure 4:** A photomicrograph of the dorsal surface of the tongue of group II animals showing: A) atrophy of the taste buds (arrows). B) A photomicrograph of the ventral surface of the tongue of group II animals showing extreme atrophy of the surface epithelium, dissociation of the collagen fibers of the lamina propria and dilatation of blood vessels engorged with blood.(H&E, orig. mag.A- 250 , B- 100)

III. Scanning Electron Microscopic results

Examination of the dorsal surface of the control rats' tongues showed numerous sharp conical projections of Filliform papillae with uniform keratinized tips arranged in parallel regular rows depicting constant antero-posterior direction towards the tongue root. Scattered fungiform papillae with broad apices were seen among numerous filliform ones, they were dome-shaped or mushroom shaped and wider in diameter than the filliform ones. Thin epithelial smooth cells were depicted around centrally located well defined regular gustatory pore surrounded by shallow indentation (fig.13). Examination of the dorsal surface of diabetic rats' tongues revealed numerous filliform papillae with evidently disturbed orientation and inclination. They showed an apparent decrease in number and change in distribution. Some of them depicted bifid or bifurcated tapering ends.

The degeneration of filliform papillae gave them appearance of accessory processes around the main protrusion. They became thinner having more slender shape than a conical shape. Others were covered by constricted keratin. Blunt eroded tips of some filliform papillae were also seen. Se verely destructed filliform papillae with desquamation of its epithelial covering were depicted (fig.14). The examined tongues of the rats treated with simvastatin showed some improvement under the (SEM) when compared to diabetic rat's tongue.

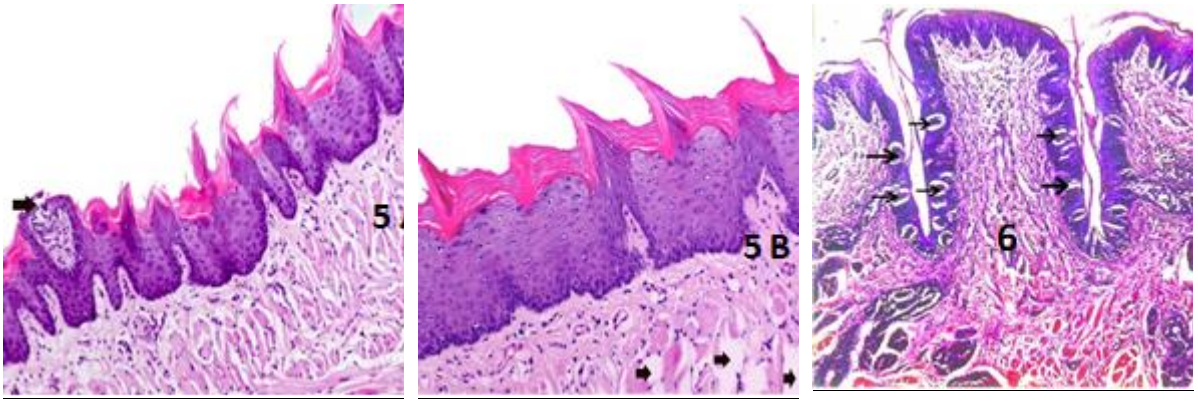


Figure 5: A photomicrograph of the dorsal surface of the tongue of group III animal showing: A) Partial regeneration of the filliform papillae and taste bud found on the top of fungiform papilla (arrow). B) Improvement in the structure of the filliform papilla and lamina propria but sill there is discontinuity of collagen fibers (arrows). (H&E, orig. mag. A- 100, B- 250). **Figure 6:** A photomicrograph of the dorsal surface of the tongue of group III animal showing the circumvallate papillae showing partial regeneration of the taste bud (arrows). (H&E, orig.mag.200).

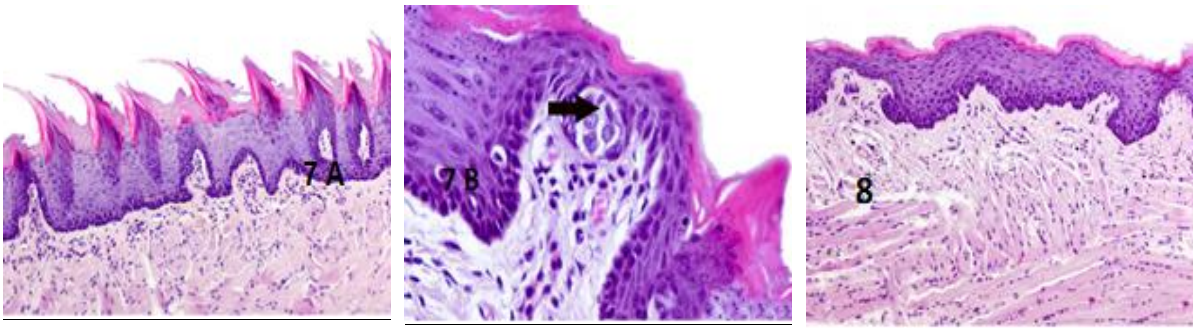
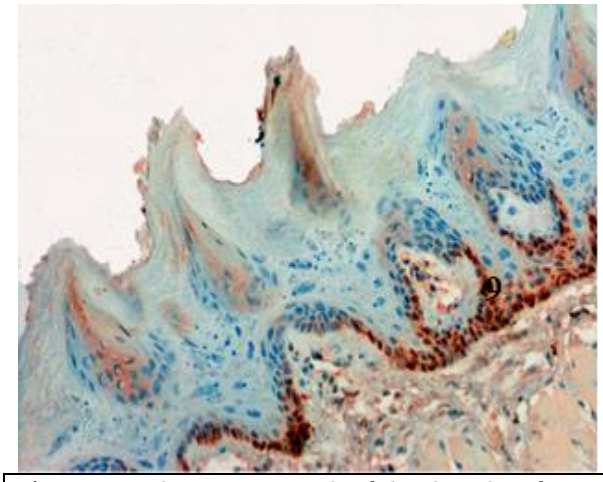


Figure 7: A photomicrograph of the dorsal surface of the tongue of group IV animal showing: A) Regeneration of the surface epithelium and lamina propria with slight apparent increase in number and length of filliform papillae. B) Fungiform papilla with normal taste bud with no signs of atrophy (arrow). (H&E, orig. mag. A- 100, B- 400). **Figure 8:** A photomicrograph of the ventral surface of the tongue of group IV animal showing normal epithelium covering and normal density of the collagen fibers of the lamina propria (H&E, orig.mag.200).

The overall morphology of the dorsal surface of the tongue showed normal distribution of the papillae. Filliform papillae facing same direction and fungiform papillae between them. However, some of the filliform papillae were still atrophic and fungiform papillae suffered invisible or obliterated taste pore. Examination of dorsal surface rats' tongues of the stem cell treated group revealed almost normal di-

rection and distribution of filliform papillae, however some papillae still suffered signs of destruction. They were conical in shape and long. The filliform papillae were numerous in number covering the dorsal surface and extend to the lateral borders (fig.15). The fungiform papillae showed al most normal appearance and size with well -defined taste buds. Taste pores were obvious on the surface of the fungiform papillae along with some scaling cells at higher magnification (fig.16).



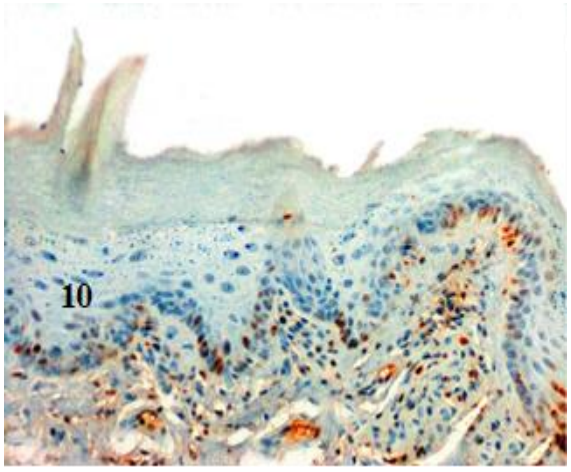
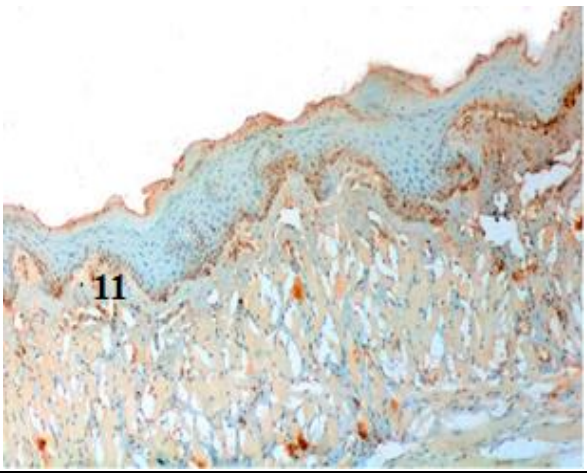


Figure 9: A photomicrograph of the dorsal surface of the tongue of group I animal showing strongly positive PCNA staining at the basal cells of the epithelium of filliform papillae (orig.mag.200). **Figure 10:** A photomicrograph showing the dorsal surface of the tongue of group II animal showing negative to weakly positive staining reaction of the basal cells to PCNA (orig.mag.200).



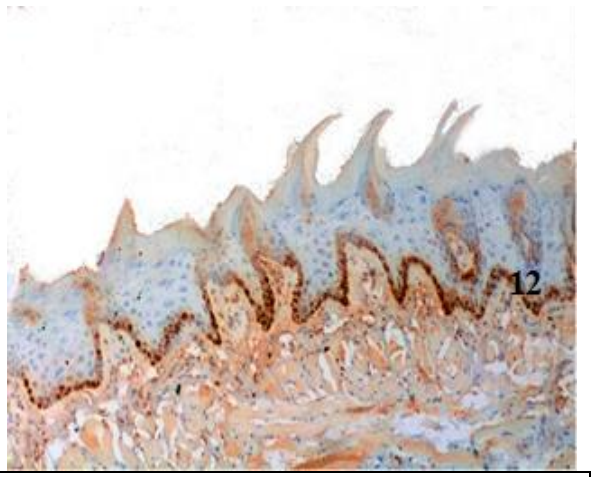
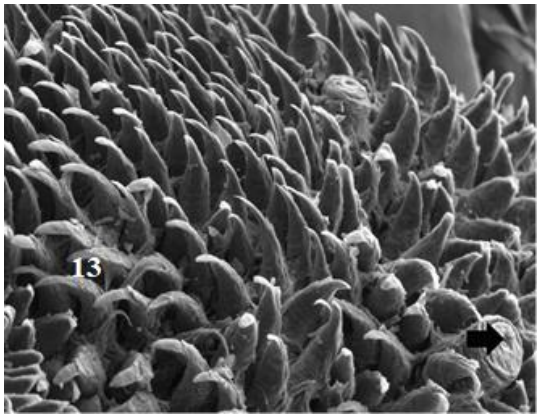


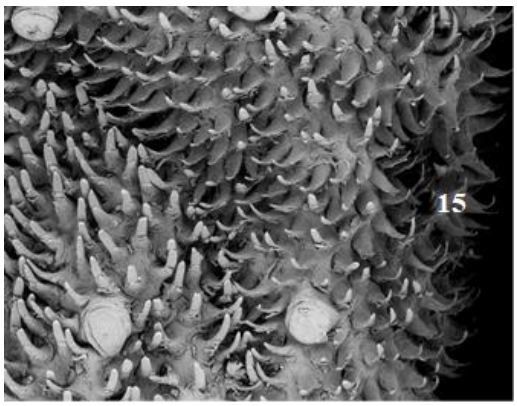
Figure 11: A photomicrograph showing PCNA expression in basal and parabasal layers of the ventral surface of the tongue of group III rats showing moderately to strongly positive staining reaction, however some of the basal cells are negatively stained (orig.mag.100). **Figure 12:** A photomicrograph of the dorsal surface of the tongue of group IV rats showing strongly positive PCNA staining of the basal and parabasal cells (orig.mag.100).

Discussion

In the present investigation streptozotocin was used to induce diabetes, the detected increased blood glucose level in diabetic rat after one week of streptozotocin injection 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 rose to 23.3 ± 1.9 mmol/l 15hr after STZ

administration[17]. Using STZ as diabetogenic agent is preferable due to the higher percentage of successful diabetes induction and lower mortality of experimental animals. STZ treatment causes diabetes in 95% of rats or more[18,19]. The present results reflected atrophic changes in the lingual papillae of diabetic rats in the form of distorted filliform papillae with alteration in their 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. attributed these complications to chronic inflammation, changes in innervations and microvasculature secondary to diabetes[20]. The observed flattening of the filliform papillae in the present study were also recorded by Naganuma et al. who observed flattening of the papillae with exfoliation and atrophic changes in a scanning electron microscopic study as an effect of aging on taste buds. The altered blood and nerve supply associated with diabetes could give effect like aging[21].





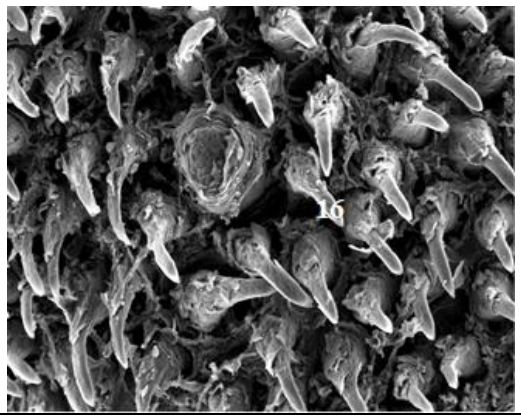


Figure 13: scanning electron micrograph of tongue of the control group showing the regular parallel rows of long conical filliform. Fungiform papillae appeared with central taste pore at their tops (arrow) (Mag. X500). **Figure 14:** Scanning electron micrograph of diabetic rat's tongue showing thin slender filliform papillae with bifurcated or trifurcated tapering ends (Mag. X600). **Figure 15**: Scanning electron micrographs of the dorsal surface of the tongue showing apparent increase in the length and number of the filliform papillae and fungiform papillae with indistinct taste pores (Mag. X300). **Figure 16:** Scanning electron micrograph of the dorsal surface of the tongue of group IV animal showing well organized filliform and fungiform papillae showing large taste pore (Mag. X500).

In the current work, the inflammatory cells that were detected in the lamina propria of diabetic rats, could induce production of inflammatory cytokines such as IL-β and TNFα that result in degenerative changes. West stated that circulating monocytes of diabetic patients show exaggerated inflammatory response to gram negative bacteria releasing large amount of pro-inflammatory cytokines[22]. Mucous and serous acini of the lingual salivary glands reflected apparent degeneration of the secretory cells with cytoplasmic vacuolizations. Oxford et al. stated that the changes of the lingual salivary glands that occur by diabetes could be related to decrease in the epidermal growth factor level (EGF)[23]. This concurs with a study suggested that hyperglycemia may affect EGF synthesis as well as its receptor binding[24]. In the present investigation simvastatin was used in a daily intraperitoneal dose of 5mg/kg body weight to study their effect on preventing or even delaying damaging that occurs by STZ induced diabetes on the tongue epithelium, connective tissue and lingual salivary glands. The examined tongues of the rats treated with simvastatin showed partial improvement in the histological picture of the surface epithelium and lamina propria of the dorsal and ventral surfaces of their tongues as well as their lingual salivary glands when compared with group II animals. Studies stated that the treatment with simvastatin improved alveolar bone loss, thus demonstrating anti-inflammatory and antioxidant activity. Simvastatin reduced expression of iNOS, MMP-1 and -8 RANK and RANKL and increased BMP-2 and OPG levels in the periodontal tissue. Systemic administration of simvastatin increased TAP activity on day 11 compared with topical administration[25]. Dalcico and colleagnes have shown that preventive treatment with simvastatin in induced periodontitis improved gingival oxidative stress and reduced cytokine levels (Il- 1β and tumor necrosis factor-α) and neutrophil influx in gingival tissue. Furthermore, simvastatin treatment reduced the expression of inducible nitric oxide synthase, receptor activator of nuclear factor Kappa-B and its ligands and increased bone morphogenetic protein-2 levels[26]. In the present investigation bone marrow derived stem cells was used to study their effect on preventing or even delaying damaging that occurs by STZ induced diabetes on the tongue epithelium, connective tissue and lingual salivary glands. By the administration of BMSCs in the present investigation, the surface epithelium of all types of papillae showed marked increase in its thickness, number of cells and length of epithelial ridges. These changes in results suggest regenerative effect of stem cells which migrate to injured tissue and participate in the regeneration of the tongue tissue due to their ability to differentiate and trans-differentiate into tissue. Specific cells by different mechanisms like regulating the immune response of the local microenvironment or by secreting factors to stimulate the tissue regeneration[27]. The effect of stem cells on the oral tissues in this investigation goes with what pittenger et al. had reported earlier. They reported that the stem cells have the capacity for renewing and repairing the tissues and replacing them when injured or exposed to tear, wear or disease[28]. The dorsal surface of the tongue showed an apparent increase in the number and the length of filiform papillae. There was regain in the number of the fungiform papillae in comparison with controls. The fungiform and circumvallate papillae appeared normal with improvement of the taste buds. The circumvallate papillae were nearly normal. Tanaka et al. reported that the stem cells were able to maintain and regenerate the keratinized epithelial cells. The authors stated that the

stem cells used in their study rapidly entered the cell cycle and regenerated tongue epithelium[29].

Conclusions

Diabetes mellitus has damaging effects on the tongue and pancreatic islands of Langerhans of the rats. The anti- inflammatory effect of SMV on the diabetic tongue. The effect of SMV on activating the proliferation of keratinocytes in addition to the organization of collagen fibers of lamina propria. Stem cells are capable of regenerating diseased and injured tissues in different organs by different mechanisms. Mesenchymal stem cells can be easily obtained from bone marrow and have the capacity for differentiation into multiple cell lineages. Bone marrow-derived stem cells are responsible for repairing the tissues and replacing them when injured or exposed to tear, wear or diseases.

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