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Systemic Versus Locally Delivered Mesenchymal Stem Cells Effect on Lingual Mucosa of STZ-Induced Diabetic Rats (Histological and Ultrastructural Study)

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Abstract

The purpose of the present study is the comparison of the therapeutic potentiality of systemically versus locally administrated bone marrow mesenchymal stem cells (BMSCs) on lingual mucosa after diabetes induction in rats. Forty adult male Sprague-Dawley rats were classified into four groups of ten each. Control group (GI), Diabetic group (GII) in which the diabetes was induced via streptozotocin (STZ) intra-peritoneal single injection, diabetic with systemically stem cells treated group (GIII) which received a single injection of (1×10^6) bone marrow mesenchymal cells (BMMCs) per rat intravenously, and diabetic with locally stem cells treated group (GIV) which received a single injection of 300,000 BM-MSCs directly in the tongue after confirmation of diabetes. Four weeks after BMSCs injection, the rats in all groups were sacrificed and the papillary part of the tongue was dissected and cut equally into two halves. For each animal, both halves were processed for histological and ultrastructural investigations. Lingual mucosa of GII showed significant diabetic deterioration including mainly keratin fragmentation, perinuclear vacuolations and reduced intercellular junctions. The administration of BM-MSCs, systemic and local, showed marked improvements and significant but not complete regaining of normal mucosal architecture, with relative advantage of systemic group. There was a significant decrease in fasting blood glucose level in systemic- and local-injected BM-MSCs in comparison to diabetic group. Diabetes mellites (DM) had many degenerative effects on lingual mucosa that reduced by BM-MSCs administration. Systemically administrated caused more obvious improvements in lingual mucosa of diabetic rats than locally administrated BM-MSCs.

Keywords: Stem cells, diabetes, streptozotocin, lingual mucosa, tissue regeneration

Full-length article

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1. Introduction

Diabetes mellitus (DM) is a chronic metabolic condition accompanied with hyperglycemia and glucose intolerance, it supposed as a major reason for early death and disability [1,2]. A non-enzymatic glycation interaction involving the carbonyl groups of reducing sugars, proteins, nucleic acids, and lipids forms molecules that break down into a variety of reactive free radicals when prolonged hyperglycemia persists. Advanced glycation end products (AGEs) are irreversible molecules created at the late stage of the glycation reaction [3]. The insoluble cross-linkers known as AGEs build up in a variety of organs, causing damage to tissue either by creating cross-links with lipids, proteins, and nucleic acids that alter their function and structure, or by activating particular AGE cell surface receptors that alter intracellular events, resulting in oxidative stress, apoptosis, and inflammation [4,5]. Prolonged hyperglycemia has also been linked to changes in the mouth, including mucosal changes, burning mouth, decreased salivary flow and altered saliva composition, increased susceptibility to oral infections with a delayed healing period, and mucosal alterations [6].

Regarding the lingual mucosa, earlier research revealed that the filiform papillae's typical conical morphology was changed in diabetic rats, resulting in their tips pointing backward and having an uneven form. Furthermore, a few irregular bundles have crossed the other bundles in different directions, causing some of the filiform papillae to get close to one another. There was desquamation of epithelial cells and an enlargement of the basal portion of the filiform papillae that protruded from the lingual mucosa [7]**.** On the other hand, other investigation revealed that the diabetic rats' epithelium thickened less, and their filiform papillae's length and width significantly shrank in

comparison to their normal counterparts. [8]. Furthermore, another study has reported that the fungi Candida species and blastospores on the filiform papillae of diabetic rats proliferate on their tongues. Among the filliform papillae, the fungiform papillae became larger and took on the shape of a cauliflower. The cauliflower's shape is uneven due to its round laminar configurations. In most papillae, there is evidence of desquamation of the epithelial cells [7].

Other investigations have revealed that while most papillae appeared short and had rounded tips, the filiform papillae had lost their typical thread-like appearance and were visibly deformed. The keratin layer covering the papillae was torn apart, revealing numerous intracellular vacuolations and some infiltration of inflammatory cells. A small number of papillae seemed to have atrophy and lost their keratin coating. There were dilated blood vessels, degraded regions, and loose connective tissue papillae [9]. Moreover, microbridge constructions with some bacterial colonies on the epithelial surfaces and noticeably thicker cell borders were seen by scanning electron microscopy. In diabetic rats, taste pores were shown to be dome-shaped on the surface of the papillae [7]. Streptozotocin (STZ) is D-glucopyranose derivative of N-methyl-N-nitrosourea that has high capability of cytotoxicity against the beta cells of pancreas. Thus, it is prevalently applied to induce type I diabetes in a variety of laboratory animals 48–72 h after injection [10,11].

It is possible to define stem cells as units in biological systems that are capable of the development and regeneration of tissues. These cells can also be thought of as units of natural selection-based evolution. Undifferentiated cells with the capability to differentiate into distinct cell lineages and self-renew are known as stem cells [12]. Bone marrow mesenchymal stem cells (BM-MSCs) have been shown in numerous studies to be effective in treating oral mucosal lesions, and this approach is employed in the treatment of numerous clinical cases [13]. The effective integration of cell migration and proliferation, extracellular matrix deposition, angiogenesis, and remodeling provides the foundation for the application of stem cells in the treatment of mouth ulcers and wound healing. The BM-MSCs are expandable, self-renewing stem cells that can engraft at the site of injury and facilitate wound healing and tissue regeneration by simultaneously downregulating proinflammatory cytokines and producing more soluble factors with anti-inflammatory, proangiogenic, and antioxidant characteristics [14]. . A rat tongue reconstruction model with a mucosa-sparing hemi-glossectomy pocket has been created in a prior study. Subsequently, either collagen or myoblast/collagen constructions were added to these pockets. After six weeks, there was less scarring and more muscle regeneration with the myoblast/collagen constructions [15].

2. Material and Methods

2.1. Animals

Forty adult male Sprague-Dawley rats, weighing between 160 and 180g, and five weeks old. The experimental animals were kept in typical cages with sawdust bedding in environments with regulated humidity (30–40%), temperature (20C), and light (12 hours of light and 12 hours of darkness). Every experimental animal was fed a regular meal and had access to water.

2.2. Ethical Approval

Ethical approval cleared by ethical committee of Faculty of Dental Medicine (Boys- Cairo), Al-Azhar University, Egypt (Ethical Code No. 590/1779). The procedures were followed in compliance with committee for the purpose of control and supervision on experiments on animals (CPCSEA Guidelines).

2.3. Experimental design

After one week of adaptation, the experimental animals were randomly divided into four main groups as follows.

Group I: ten rats were fed and kept in normal conditions and considered as the negative control to the other experimental groups as they received only the vehicle of STZ.

Group II: ten rats were subjected to diabetes induction via a single intra-peritoneal injection of (40mg/kg) STZ dissolved in 1ml of freshly prepared citrate buffer (0.1mol/l citric acid, 0.1mol/l sodium citrate; pH: 4.5) [16] and considered as the positive control. After 72 hours of STZ injection, blood samples were taken through tail vein puncture for the confirmation of diabetes. Animals were deemed to have diabetes if their fasting blood glucose (FBG) was greater than 250 mg/dl [17].

Group III: ten rats were subjected to diabetes induction via STZ as previous group, Thereafter, if diabetes was confirmed, they got a single dose of BM-MSCs (1×10^6 cells) suspended in 1ml phosphate-buffered saline (PBS) per rat via intravenous injection [18].

Group IV: ten rats were subjected to diabetes induction via STZ as previous group, Thereafter, if diabetes was confirmed, they got a single dose of 300,000 cells of BM-MSCs suspended in (0.3ml) PBS per rat directly in the tongue [19].

2.4. Isolation and culturing of BM-MSCs

BM-MSCs were extracted from the femur and tibia of male Wistar albino rats that were six weeks old. After cutting the ends of the long bones, the medulla was gently flushed using an 18-gauge needle and a syringe filled with 3 milliliters of L-DMEM supplemented with 10% FBS, 1% L-glutamine, and antibiotics such as penicillin and streptomycin. The same syringe and needle were gently dragged up and down multiple times to create a single cell suspension. The BM suspensions were then grown in polystyrene dishes. After two days, the adherent cells were expanded as monolayer cultures in 5% humidified CO2 at 37°C for 12–14 days as a primary culture or upon the establishment of large colonies. The non-adherent cells were removed from the culture by a series of washes in PBS. When the cells reached 80–90% confluence, they were separated using 0.01% EDTA and 0.25% trypsin, and they were then sub-cultured on fresh culture dishes—a process known as first-passage cultures. In the passage 3 sub-culture, the MSCs were distinguished by their ability to differentiate into osteogenic and adipogenic tissues, their adhesiveness, their fibroblastic shape, and their expression of CD29 and CD90 surface markers but not CD45 [20].

2.5. Sample collection and preparation

After four weeks of BMSCs injection, the rats in all groups were anesthetized, sacrificed by euthanasia. The papillary part of the tongue was dissected and cut equally into two halves. For each animal, both halves were processed

microtechnically for histological investigation of one half, while the other half processed for ultrastructural investigations.

2.6. Histopathological examinations

Tissue sections 4 µm thick were cut from paraffin blocks using a rotary microtome, processed, put on glass slides, and stained with hematoxylin and eosin (H&E) for light microscopic examination.

2.7. Ultrastructural investigations

2.7.1. Transmission electron microscopy

Small sized (1 mm^3) samples were taken from the tongue of the animals of different groups and prepared for transmission electron microscope examination as follows:

The samples were washed in the same solution, post-fixed in 1% osmium tetroxide for 2 hours at room temperature, then fixed for 2 hours in 3% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.0). The samples were dried using a succession of ethanol dilutions, from 10% to 90%, for 15 minutes at a time, and then with 100% ethanol for 30 minutes. Using a graded succession of epoxy resin and acetone infiltrations, samples were eventually filled with pure resin. On copper grids coated with Formvar, ultrathin sections were gathered. After that, sections were doubly stained with lead citrate and uranyl acetate. A JEOL JEM 1010 transmission electron microscope operating at 70 kV was used to examine stained sections at the Regional Center for Mycology and Biotechnology (RCMB) at Al-Azhar University in Cairo, Egypt [21].

2.8. Statistical analysis

After statistical analysis of the data, the mean and standard deviation (SD) were determined. The software SPSS version 17.0 for Windows was used to do a one-way analysis of variance (ANOVA). ANOVA was used with parametric distribution and quantitative data to distinguish between more than two distinct groups. Post hoc analysis using the LSD test was also conducted. The appropriate p-values were used to determine significance: p 0.05 indicates significant, $p > 0.05$ indicates non-significant, and $p < 0.001$ indicates extremely significant.

3. Results

3.1. Histological findings

Control group (GI): The tongue's dorsal surface was covered in many papillae. The filiform papillae were tightly packed together and had an elongated, thin shape with pointy and rounded tips. The numerous filliform papillae were mixed with the fungiform papillae, which had a dome-like structure. The dorsal surface of the tongue was covered by keratinized stratified squamous epithelium, according to a microscopic inspection. The basal lamina separated the connective tissue from the epithelial layer, which was situated beneath it. The many arteries and nerves found in the lamina propria were embedded in loose connective tissue (*Fig. 1A).*

Diabetic group (GII): The diabetic group's dorsal surface of the tongue revealed shallow epithelial ridges, regions of flattening and loss of the filiform papillae's distinctive conical shape, and an apparent shortening of the tongue papillae. Additionally, there were patches of hyperkeratosis alternated with localized loss of certain filiform papillae. Along with some epithelial cells vacuolating, there was focal dissociation of the keratin from their underneath keratinocytes. The nuclear alterations that the epithelial cells displayed included chromatin that was marginated toward the nuclear membrane, pyknosis, and condensation. The basal cell showed degeneration as well as nuclear pyknosis with frequent fragmentation (Fig. 1B).

Systemically stem cells treated group (GIII): The mucosa of the dorsal surface of the tongue showed significant improvement in the histological features after systemic administration of stem cells. The tongue mucosa was partially regained its normal configuration. However, some degenerative manifestations were still apparent. The filiform papillae appeared threaded in shape with few intracellular vacuolations. The detached keratin layer was noticed in some areas (Fig. 1C).

Locally stem cells treated group (**GIV):** The mucosa of the dorsal surface of the tongue revealed improvement in the histological architecture of both epithelium and lamina propria of the dorsal surface of tongue in comparison to GII animals. The epithelial layer showed almost normal structure of filliform papillae including their covering epithelium and keratin. The basal and parabasal cells showed open faced nucleus in the vast majority, however, some cells revealed perinuclear vacuolation particularly in the upper layers (Fig. 1D).

3.2. Ultrastructural findings

3.2.1. Transmission electron microscopy

Control group (GI): The transmission electron microscopic examination revealed that the basal and supra-basal cells cytoplasm was filled with delicate filamentous structures. There were some intercellular spaces, but plasma membranes of cells were in contact with one another via desmosomes. The interface between basal epithelial cells and lamina propria appeared smooth with frequent infiltration of leukocytes into the intercellular spaces in the superficial layer of lamina propria. The intermediate cells were relatively larger in size with irregular nuclear membrane. The cells contained many keratohyaline granules of different sizes. The keratohyaline granules in this layer were very minute in size and scattered in the cytoplasm. Because of numerous keratin-filament, epithelial cells in this layer have a high electron density (*Fig. 2A).*

Diabetic group (GII): The lingual mucosa showed diverse changes in all mucosal components. The keratinocytes demonstrated marked reduction in desmosomal junctions with distorted structure of the appeared junctions. The epithelial cells revealed marked increase in the intercellular spaces with variable degrees. The keratohyaline granules appeared pleomorphic with irregular configuration. Additionally, the basal lamina appeared relatively thickened in relation to that of control group, with regional difference of electro-density of lamina densa with small sized vacuoles *(Fig. 2B).*

Systemically stem cells treated group (GIII): The components of lingual mucosa revealed significant restoration of normal tissue architecture. The epithelial cells showed slight or even absence of inter cellular spaces with typical interdigitation of adjacent cells membrane. The desmosomal attachment between keratinocytes were almost average in frequency and nearly intact in configuration. The basal lamina showed diverse changes. Although the lamina lucida appeared narrow with uniform thickness, the lamina densa appeared significantly thick with heterogenous electro-density (*Fig. 2C).*

Locally stem cells treated group (GIV): The components of lingual mucosa showed marked relief of the diabetic degenerative changes in response to local administration of stem cells. The keratinocytes revealed obvious intercellular junctions including desmosomes, however, some intercellular spaces were still apparent. The basal lamina appeared almost intact and closely resemble that of control group, however, some areas showed regional thickening of lamina lucida with decreased number of cell-matrix junctions (Fig. 2D).

3.3. Statistical findings

Fasting blood glucose level(mg/dl) (Table 1)

4. Discussion

Diabetes mellitus (DM) is a serious chronic health disease that leads to hyperglycaemia. Numerous bodily organs are impacted by DM, which is the cause of numerous difficulties [(22)]. Patients with type I diabetes must receive insulin shots on a regular basis to survive; however, this does not reverse the disease. The ability of the BM-MSCs to regenerate and repair tissue has been thoroughly studied [23]. Both experimental research and clinical practice have shown a connection between diabetes mellitus and changes in the oral mucosa. Rats were selected for the current study for a variety of reasons, the primary one being that they are mammals whose physiological processes are most similar to those of humans. In addition, they were simple to get, care for, breed, handle, regulate, and test [24]. It has been established that the tongue mucosa could be represented as a mirror that reflects the individual general health status [25]. In previous literatures, the streptozotocin (STZ) has demonstrated higher percentage of successful DM induction with manifestations that closely resemble to that develop in human. Additionally, the STZ showed lower mortality rate [26].

4.1. Effect of STZ on lingual mucosa

The administration of STZ evoked many degenerative changes that involved all layers of lingual mucosa. Concerning the epithelial layer, hallmark changes were detachment and torn out keratin layer on the epithelial surface, intra cellular vacuolations as well as cellular degeneration. The detachment of keratin from the underlying epithelium was detected in previous investigations, which regarded this change to the elevated blood glucose level which has negative impact on cellular junctions. The elevated blood glucose was previously stated to leads to junctional protein degradation [(27,28)]. This interpretation is in a line with the ultrastructural investigations of the present study. The TEM figures revealed significant reduction or even regional absence of desmosomal junctions between epithelial keratinocytes. The lingual mucosal keratinocytes showed numerous intracellular vacuolations in the current investigation. These vacuolations were detected in previous research in lingual mucosa and in diverse tissues as well [16,29]. It was stated that intracellular vacuolations in diabetic tissues could be regarded to diabetic induced dysfunction of mitochondria that gives rise to more accumulation of reactive oxygen species (ROS) and oxidation stress [30,31].

The findings of the current study were coincident with the previous interpretation, as the TEM figures revealed significant disturbance in intercellular junctions which in turn negatively affect the structure of sarcoplasmic protein that ultimately impact the mitochondrial functions.

4.2. Effects of systemic BM-MSCs injection on diabetic rat`s tongue

The systemic administration of BM-MSCs has led to significant improvement in the architecture of diabetic lingual mucosa of rats. This improvement was represented by the lowered glycaemic status of rats as well as by the histological and ultrastructural figures of lingual mucosa. However, the blood glucose levels did not completely regain normal values and so the figures of lingual mucosa that did not completely restored to normal configuration. The marked enhancement of blood glucose levels that were close to normal values, would confirm the anti-diabetic effect of systemically administrated BM-MSCs, which could be regarded to the direct regenerative effect of BM-MSCs on the β-cells of pancreas. The improved diabetic status of rats was reflected on the histological features of lingual mucosa including both epithelial and connective tissue components. The detachment and breakdown of keratin layer were markedly reduced in comparison to diabetic animals (GII). Additionally, the perinuclear vacuolations in keratinocytes were obviously diminished in relation to that noted in diabetic rats (GII). Concerning connective tissue components, the lamina propria showed minimal degenerative features, whereas the muscular layer revealed relatively intact configuration with occasional atrophy.

These effects of systemically delivered BM-MSCs on lingual mucosa could be attributed to two different pathways. The first one is the supposed regeneration of the β-cells of pancreas, that in turn improved the glycaemic status and reduced the ROS release which ultimately has led to cease of degenerative manifestation and attempts of regeneration of mucosal components. This effect is bolstered by the direct effect of BM-MSCs populations that have reached the lingual tissue via blood stream. These direct and indirect pathways were reported in previous investigations which claimed that the paracrine effects of extrinsic stem cells on lingual mucosal cells which in turn regulate and improve cellular proliferation and regeneration could be a proposed mechanism of anti-diabetic effect of BM-MSCs [32-34]. The TEM manifestations revealed significant improvement of cellular constructure, whereas the hallmark findings were the increased intercellular junctions compared to diabetic group (GII), as well as the noticeable restoration of basal lamina to nearly intact and typical configuration. Taken together, these findings could confirm the anti-diabetic effect of systemically delivered BM-MSCs. The supposed direct anti-diabetic effect of BM-MSCs could be emphasized by the previous investigations that declared the homing of small amount of allogenic MSCs to the injured pancreas in STZ-induced diabetic rats. These cells survived and were capable to regenerate the damaged pancreas through trans-differentiation to the β-cells of pancreatic islets. Furthermore, they stated the MSCs delivered systemically via intravenous route could migrate to the injured extra-pancreatic organ or tissue leading to apparent regeneration of that injured tissue [35,36].

4.3. Effects of locally delivered BM-MSCs on tongue of diabetic rats

The local administration of BM-MSCs in diabetic tongue of rats showed relative improvement in the architecture of lingual mucosa in comparison to both diabetic group (GII) and systemically delivered BM-MSCs group (GIII).

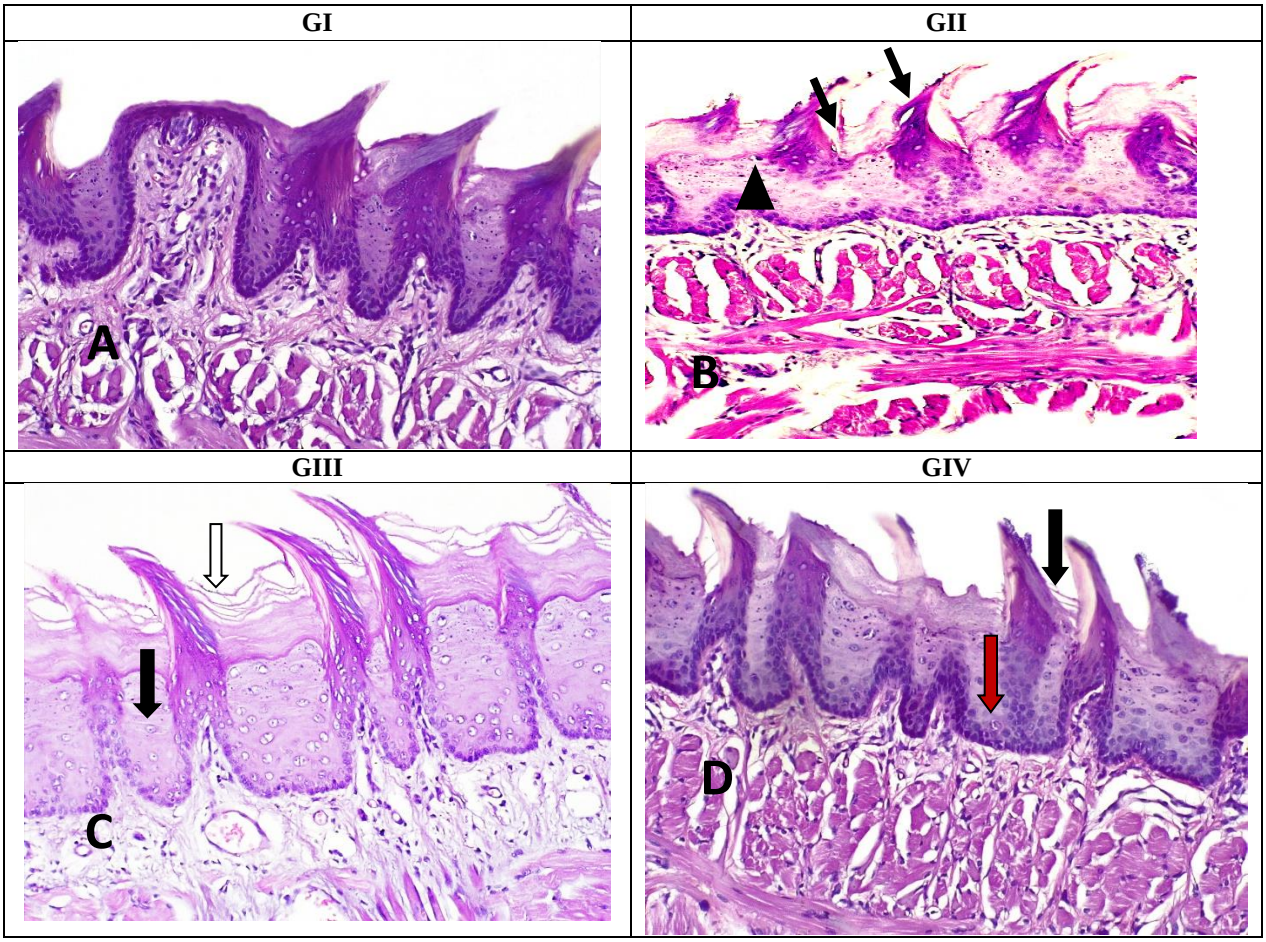
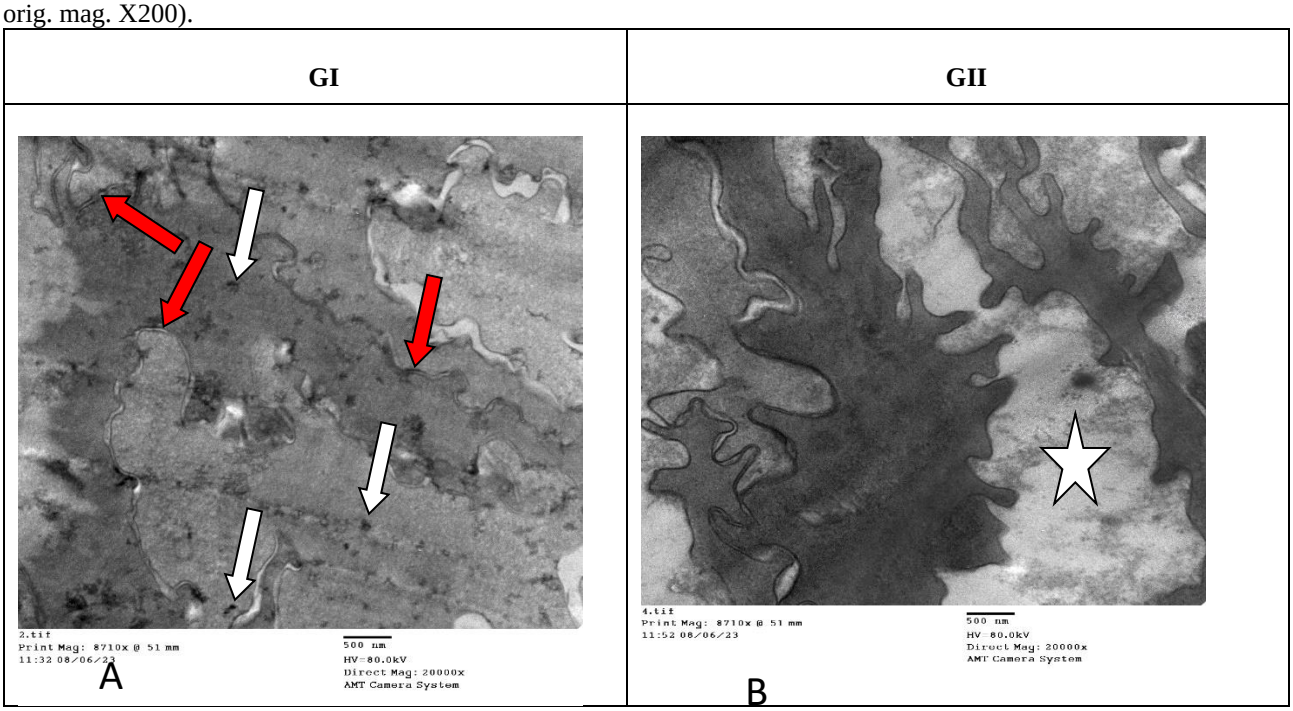


Figure 1. A- Photomicrograph of (GI) showing intact tongue papillae and sound architecture of underlying lamina propria and closely packed muscle fascicles. (H & E stain, orig. mag. X 200). **B-** Photomicrograph of (GII) showing area of detached keratin (arrow), perinuclear vacuolations in parabasal epithelial cells (arrowhead) as well as atrophy of muscle fibers with large inter-fascicular fibers. (H & E stain, mag. X 200). **C-** Photomicrograph of (GIII) showing thread like filiform papillae with detached keratin in some areas (white arrow), some intracellular vacuolations (black arrow). The lamina propria appeared loose and areolar with many blood capillaries. (H & E stain, orig. mag. X 200). **D-** Photomicrograph of (GIV) showing filiform papillae appeared in relatively normal architecture with few keratin fragmentation (black arrow) and perinuclear vacuolations (red arrow) (H & E stain, orig. mag. X200).



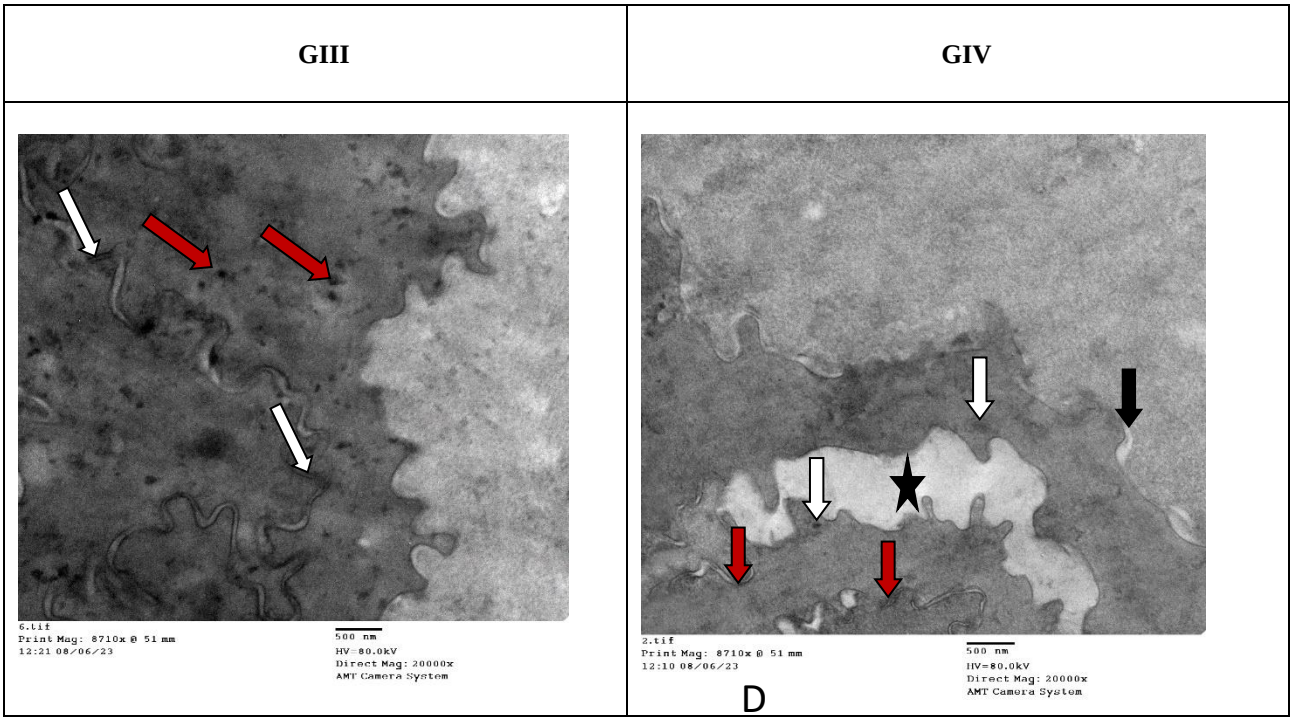


Figure 2. A- Transmission electron micrograph of tongue papillae in control group (GI) showing the cells were in contact via desmosomes (*red arrow)* with few inter cellular spaces *(star)* and many keratohyaline granules of different sizes and irregular shapes *(white arrow)*. (Mag. X 20000). **B-** Transmission electron micrograph of tongue papillae in diabetic group (GII) showing significant intercellular spaces *(star)* with prominent keratinocytes atrophy as well as marked reduction of desmosomal attachment. (Mag. X 20000). **C-** Transmission electron micrograph of tongue lamina propria in (GIII) showing nearly intact keratinocytes with few intercellular spaces as well as many typical desmosomal junctions (*white arrow)* and many pleomorphic keratohyaline granules (*red arrow)*. (Mag. X 20000). **D-** Transmission electron microscopic photomicrograph of lingual mucosa in (G IV) showing moderate intercellular spaces *(star)* and some intercellular junctions *(red arrow)* with some keratohyalin granules of different sizes related to cell membrane *(white arrow).* The basal lamina showing regional thickening in the lamina lucida *(black arrow).* (Mag. X 20000).

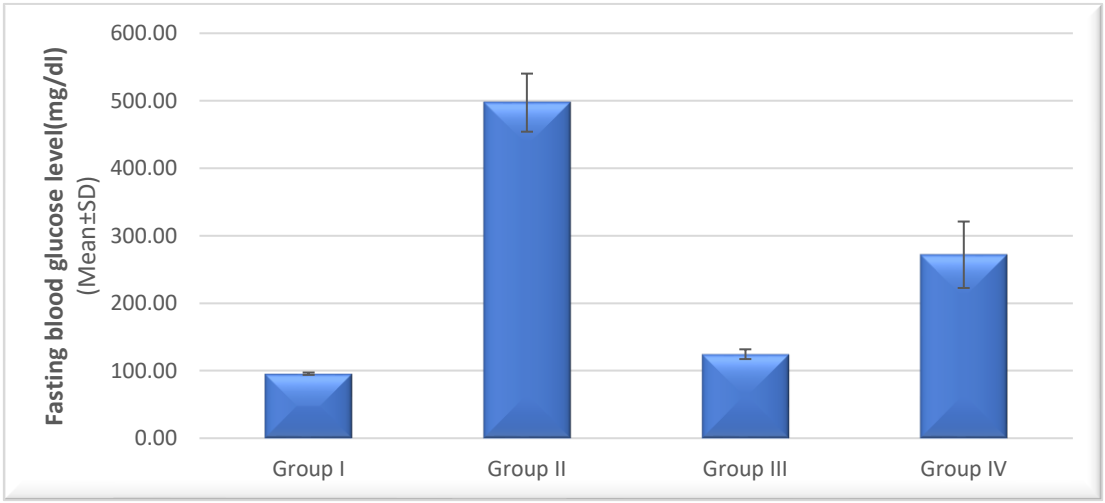


Figure 3. Mean ±SD of Fasting blood glucose level(mg/dl) between the different groups

Table 1. Comparison of Fasting blood glucose level(mg/dl) between the different groups

	Group I	Group II	Group III	Group IV	ANOVA P value
Fasting blood glucose level(mg/dl)	**95.60±1.78 C**	**497.20±43.04A**	**124.50±7.25 C**	**271.80±49.24 B**	**<0.001***

Data expressed as mean &SD.
P: Probability *: significance ≤0.05
Test used: One way ANOVA followed by post-hoc Tukey's.
Different superscript alphabetical letters indicate significance between different groups in the same row

Although the manifestations recorded in the lingual mucosa of locally delivered BM-MSCs group (GIV) revealed obvious relief of diabetic features, the anti-diabetic effects of local BM-MSCs were not as potent as the effects of systemically delivered BM-MSCs (GIII). Concerning the glycaemic status, the blood glucose levels showed apparent decrease following local administration of BM-MSCs, however, it was still higher than the control group (GI) level and even higher than systemically delivered BM-MSCs group (GIII). This finding could throw the light on the ability of BM-MSCs locally delivered to peripheral injured organ to reach other organs particularly the internal organs. It seems that the amount, if any, stem cells migrate from the peripheral injured organ to the internal organs like pancreas through blood stream, was minimum in amount which in turn diminished the level of pancreatic regeneration that ultimately negatively affect the glycaemic status of animals.

The relatively less potent anti-diabetic effect of locally delivered BM-MSCs was reflected on the histological picture of lingual mucosa that revealed relative relief of diabetic features with persistence of some diabetic manifestations as keratin layer fragmentation and detachment, intracellular vacuolation. The previous investigations have proposed many mechanisms related to the action of locally injected stem cells. These mechanisms include the ability of injected BM-MSCs to secrete a variety of cytokines, chemokines and growth factors that are considered as potential degeneration modifying factors [37]. These factors could have significant potential in enhancing angiogenesis, relief of inflammation, prohibiting apoptosis as well as improving intrinsic repair via stimulating dormant stem cells for proliferation [37,38]. The dual regenerative effects of BM-MSCs in both pancreas and lingual mucosa has bolstered the anti-diabetic potential in lingual mucosa. On the other hand, the locally delivered BM-MSCs have failed to restore the glycaemic state back to near normal levels which in turn did not inhibit the production of free radicals and ROS. This means that the locally injected BM-MSCs have lunched regeneration processes, however it was struggled by the ROS due to relatively persistent diabetic status. So, the persistent diabetic processes hindered the regenerative potential of locally injected BM-MSCs, however it has not eliminated the beneficial effects of BM-MSCs.

5. Conclusions

The present study, as well as the previous investigations have emphasized the harmful histological and ultra-structural effects of STZ-induced diabetes on lingual mucosa. The BM-MSCs have the capacity for differentiation into multiple cell lineages, so that the BM-MSCs can initiate and propagate repairing and regeneration of injured or diseased tissues. The BM-MSCs injection either systemically or locally has reduced the STZ-induced diabetic effects on lingual mucosa of albino rats. The systemic administration of BM-MSCs showed relative advantage over the locally delivered ones. However, complete amelioration was not established in either treated groups.

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7. Conflict of interest

There are no conflicts of interest.

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