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Research article

Functional and histological evaluation of bone marrow stem cell-derived exosomes therapy on the submandibular salivary gland of diabetic Albino rats through TGFβ/ Smad3 signaling pathway

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ABSTRACT

Background: To prevail over diabetes mellitus and its numerous complications, researchers are seeking new therapies. Exosomes are natural cargo of functional proteins and can be used as a therapeutic delivery of these molecules.

Objective: The aim of this study was to evaluate the effect of exosomes derived from bone marrow mesenchymal stem cells (BM-MSCs) as a therapeutic intervention in salivary gland diabetic complications.

Methods: Ten adult healthy male Albino rats, weighing about 150–200 g were grouped into 2 groups. Diabetic group I: consisted of 5 streptozotocin (STZ)-induced diabetic rats. Exosomes treated group II: consisted of 5 STZ-induced diabetic rats, each animal received a single injection of exosomes (100 μg/kg/dose suspended in 0.2 ml PBS) through the tail vein. All animals were sacrificed after 5 weeks from the beginning of the experiment. Submandibular salivary gland samples were excised and processed for histological, ultrastructural examination and PCR for TGFβ, Smad2 and Smad3. Blood glucose level was monitored weekly, salivary IgA and serum amylase were evaluated before and after diabetes induction and at the end of the experiment.

Results: Histological and ultrastructural results of the exosomes treated group were promising regarding the glandular and ductal elements with less fibrosis observed. Results of PCR supported the role of exosomes to inhibit the diabetic sequalae in salivary gland and its complications through inhibiting TGFβ and its related pathway via Smad2 and Smad3. Blood glucose levels were reduced. In addition, salivary glands' function was improved as evidenced by reduction in serum amylase and salivary IgA.

Conclusion: BM-MSC-derived exosomes could be a novel therapeutic strategy for diabetic complications involving salivary glands.

1. Introduction

Diabetes mellitus (DM) is a worldwide metabolic disease. More than 400 million people are affected globally. By 2045, it is assumed to reach 693 million. Type 2 diabetes mellitus (T2DM) is the common type of the disease, representing over 90% of diabetic people [1]. It is identified by impaired insulin effect on tissues and/or defect in insulin secretion, provoking a continuous state of hyperglycemia, promoting inflammation and producing excessive amount of reactive oxygen species (ROS). All these complications are responsible for the elevated death rates in T2DM [2].

In T2DM, systemic and organ integrity are compromised causing blindness, renal diseases, neuropathy, arteriosclerosis, infections such as candidiasis and dysfunction of various glands including the salivary glands. Salivary gland damage in T2DM patients can cause qualitative and quantitative changes in the saliva. Xerostomia is also more common in these patients than in non-diabetic ones [3].

Currently, anti-diabetic drugs and daily insulin injections are the main medications for T2DM. However, these treatments can reverse hyperglycemia temporarily and possess numerous side effects. Moreover, exogenous insulin admission can impair insulin formation and secretion in β-cells [4].

Over the past years, mesenchymal stem cells (MSCs) have attained great concern as a new regenerative therapy. Bone marrow (BM) derived MSCs were proved to control hyperglycemia in T2DM rat models [5]. However, the inadequate amount of donated tissues, reduced endurance

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of stem cells in vivo, in addition to their tumorigenic potentiality made these methods unsatisfying [6].

MSCs derived factors and culture medium from BM-MSCs were proved to regulate blood glucose level in T2DM animal models [5]. This proved that the therapeutic effect of MSCs largely depends on the paracrine actions of released factors, including exosomes [7].

Exosomes are extracellular vesicles. Their size rages from 30–100 nm in diameter. They are released in the microenvironment by various cell types, including stem cells and their precursors. Exosomes mediate the paracrine actions of their releasing cells [8]. They are present in various body fluids, such as urine, blood, ascetic fluid, breast milk and cerebrospinal fluid. They comprise proteins, mRNAs and microRNAs (miRNAs) that could be delivered to target cells activating genetic and epigenetic alterations [9].

In diabetes, hyperglycemia induces cellular hypoxia. Hypoxia is responsible for the progression of many diabetic complications [10]. Upregulation of BM-MSCs-derived exosomal miR-29b-3p was proved to protect cardiomyocytes against hypoxia-induced injury through down-regulation of TNF receptor-associated factor 5 (TRAF5) suggesting that miR-29b-3p could inhibit apoptosis through controlling other genes [11]. It was also demonstrated that miR-29b-3p play a major role in insulin resistance [12]. In another study, human MSCs-derived exosomes improved T2DM by reversing peripheral insulin resistance and alleviating beta-cell destruction [13]. Exosomes can be absorbed easily. They can cross blood brain barrier and be incorporated by target cells without being degraded by the endosomal-lysosomal system providing long-lasting releasing effect [14]. This proved exosomes to possess more advantages than other nano-particles and may be the upcoming smart nanomedicine for treating diabetes [13].

In view of this fact, the current study aimed to evaluate the therapeutic potentials of BM-MSCs-derived exosomes on the submandibular salivary gland dysfunctions as one of diabetic complications.

2. Materials and methods

2.1. Ethical statement

This experiment was conducted in the animal house at the faculty of Medicine, Cairo university, Egypt, according to the guidance and approval of the institutional animal care and use committee, Cairo university (CU-IACUC) (approval no. CU III F 53 19).

2.2. Isolation and culture of BM-MSCs

BM cells were obtained from the tibia of white female Albino rat (Cux1: HEL1) weighing about 150–200 g. Cells were flushed with Phosphate-Buffered Saline (PBS). The flushed cells (15ml) were placed on 15 ml Ficoll-Paque (Gibco-Invitrogen, Grand Island, NY) and were centrifuged for 35 min at 400 $\times g$ 5 °C. The upper layer was aspirated and the mono nuclear cell (MNC) layer was left at the interphase. The MNC layer was aspirated and washed twice in PBS with 2 mM ethylene diamine tetra acetic acid (EDTA). It was then centrifuged for 10 min at 200 $\times g$ 5 °C. The isolated BM-MSCs were then grown and spread on 25 ml culture flasks in Roswell Park Memorial Institute (RPMI)-1640 medium consisting of 0.5% penicillin, streptomycin and 10% Fetal Bovine Serum (FBS). The cells were incubated at 37 °C and 5% CO_2 ending up at 80~90% confluence in a period of 7 days [15]. This was performed at the Biochemistry department at faculty of Medicine, Cairo university.

2.3. Identification of BM-MSCs in culture

The cultured BM-MSCs were identified by their morphology and by using Fluorescent Activated Cell Sorting (FACS). The positivity of cluster of differentiation $CD105^+$, $CD90^+$ and negativity of $CD34^-$, $CD45^-$ were assessed [16]. This was performed at the Biochemistry department at faculty of Medicine, Cairo university.

2.4. Preparation of exosomes derived from BM-MSCs

Exosomes were obtained from the supernatants of BM-MSCs grown overnight in RPMI free of FBS. To obtain exosomes, cell-free supernatants were centrifuged at 10,000 $\times g$ 4 °C for 20 min for removal of debris. Centrifugation was then performed at 100,000 $\times g$ 4 °C (Beckman Coulter Optima L-90K ultracentrifuge) for an hour at 4 °C. Cell-free supernatants were then washed in serum-free medium 199 comprising N-2-Hydroxy Ethyl Piperazine-N′-2-Ethane Sulfonic acid 25 mM (Sigma) and exposed to a second ultracentrifugation in similar conditions [17]. This was performed at the Biochemistry department at faculty of Medicine, Cairo university.

2.5. Characterization of BM-MSCs-derived exosomes

2.5.1. Transmission electron microscope (TEM) characterization of exosomes

Exosomes were fixed with 2.5% glutaraldehyde for two hours. They were washed then ultra-centrifuged and suspended in 100 μL human serum albumin. A total of 20 μL of exosomes were loaded onto a formvar/carbon-coated grid, negatively stained with 3% aqueous phosphor-tungstic acid for one minute. Exosomes were then checked by TEM (HITACHI, H-7650, Japan), which demonstrated their spheroidal shape and confirmed their diameter [17]. This was performed at Ain Shams university at faculty of Sciences at the transmission electron microscope unit.

2.5.2. Western blot characterization of exosomes

The antibodies utilized were antigen affinity-purified polyclonal sheep IgG anti-rabbit CD81 (Catalog no. 0349509; BioLegend, San Diego, California, USA) and antigen affinity-purified polyclonal IgG anti-rabbit CD83 (Catalog no. MBS127731, MyBioSource, Inc., San Diego, California, USA). Protein was extracted from isolated exosomes by utilizing radio-immuno-precipitation buffer's composition. Twenty nanograms of protein were loaded and separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis on 4~20% polyacrylamide gradient gels. After being incubated in 5% non-fat dry milk, Tris hydrochloride, 0.1% Tween 20 for an hour, primary antibodies (1:500 dilution factor for all target proteins) were added to one of the membranes including specimen samples and incubated at 4 °C overnight. HRP-conjugated secondary antibody (Goat anti-rabbit IgG-HRP-1mg Goat mab -Novus Biologicals) solution was incubated against the blotted target protein for two hours at room temperature. Following washing for six times in 1 X TBS-T, densitometric analysis of the immunoblots was done to quantify the amounts of CD83 and CD81 against housekeeping protein β-actin by image analysis software on the Chemi Doc MP imaging system (version 3) generated by Bio-Rad [18]. This was performed at the Biochemistry department at faculty of Medicine, Cairo university.

2.6. Animals

Ten adult healthy male Albino rats, weighing about 150–200 g were bred into sterile, controlled environment (temperature 23 ± 5° and 12 h dark/light cycles). They were fed with standard pellets diet and water ad lib. Each rat was housed in a steel cage at the animal house at faculty of Medicine, Cairo university. Animals were divided randomly into 2 groups of 5 rats each for a randomized control trial, as follows:

Diabetic group (GP I): consisted of 5 streptozotocin-induced diabetic rats.

Exosomes (Exo) treated group (GP II): consisted of 5 streptozotocin-induced diabetic rats, each animal received a single injection of exosomes (100 μg/kg/dose suspended in 0.2 ml PBS) through the tail vein [19].

2.7. Study design

2.7.1. Diabetes induction

Streptozotocin (STZ) (provided by Sigma Chemical Co., St. Louis, MO, USA) was used to induce diabetes in rats. It was injected intra-

peritoneally at a single dose of 50 mg/kg of body weight after being freshly prepared in ice-cold citrate buffer [20].

2.7.2. Confirmation of diabetes induction

Diabetes was permitted to stabilize in those STZ treated rats over a period of one-week Animals with glucose levels above 200 mg/dL were involved in the study [20]. All animals were sacrificed 5 weeks from the beginning of the experiment. Submandibular salivary glands were dissected and processed for the following:

2.7.2.1. Histopathological examination. Serial 3–5 μ sections of the salivary glands were stained with hematoxylin and eosin for routine histopathological examination using a light microscope with a digital camera and software at the Oral Biology department, faculty of Dentistry, Cairo university.

2.7.2.2. Ultrastructural examination. Ultrastructural changes were examined by transmission electron microscope at Ain Shams university at faculty of Sciences at the transmission electron microscope unit.

2.7.2.3. Quantitative real time polymerase chain reaction (qRT-PCR) assessment. Total RNA containing genes were identified by RT-PCR. RNAs were separated by QIAZOL (QIAGEN). cDNA was created by cDNA synthesis kit (Applied bio system) and RT-PCR was performed by RT-PCR kit (Applied bio system) on Step One Plus instrument (Applied Biosystems) using standard protocols. The RQ of each target gene was quantified according to the calculation of $\Delta\Delta$Ct. Calculation of the RQ for the studied genes was done by $2^{-\Delta\Delta Ct}$ normalization to housekeeping gene. The studied primers are shown in Table 1. This was performed at the Biochemistry department at faculty of Medicine, Cairo university.

2.7.3. Evaluation of blood glucose level

Analysis of blood sugar levels was done weekly from all animals till the end of the experiment.

2.7.4. Biochemical analysis of submandibular salivary gland functions

Amylase alpha in serum and immunoglobulin A (IgA) in saliva were measured from all animals at day 0, 1-week post induction and at the 4th week after treatment. They were assessed according to manual instructions. The amylase kit was provided by (Novus Biologicals USA, CAT NO: NBP2-68205), while IgA kit was provided by (ARP American Research Products, Inc. ™ Catalog #: KTE100261). They were assessed by Colorimetric, Endpoint method at the Biochemistry department at faculty of Medicine, Cairo university.

2.8. Statistical analysis

Data were sum up using mean and standard deviation for all variables. Comparisons between groups were done using analysis of variance (ANOVA) with multiple comparisons between groups using T-test. P-values less than 0.05 were considered as statistically significant.

Table 1. Primers' sequence specific for each gene.

Smad2	F: GCCCCAACTGTAACCAGAGA
	R: GCCAGAAGAGCAGCAAATTC
	(#Accession number: XM_006254945.3)
Smad3	F: GGCTTTGAGGCTGTCTACCA
	R: GGTGCTGGTCACTGTCTGTC
	(#Accession number: XM_008766216.2)
TGFβ	F: TGGGGCAGCTGTACATTGAC
	R: GGCAGAAGTTGGCGTGGTA
	(#Accession number: NM_021578.2)
GADPH	F: CACCCTGTTGCTGTAGCCATATTC
	R: GACATCAAGAAGGTGGTGAAGCAG
	(#Accession number: XR_598347.1)

3. Results

3.1. Isolation and identification of BM-MSCs

BM-MSCs were isolated and identified by their spindle-fusiform shape and formation of colonies (Figure 1A, B & C). In addition, FACS analysis demonstrated that BM-MSCs were positive for CD90 and CD105 surface markers and negative for CD34 and CD45 (Figure 1D).

3.2. Identification of BM-MSCs-derived exosomes

BM-MSCs-derived exosomes were identified by utilizing TEM, exosomes were characterized by their homologous size (30–100 nm) and their spherical morphology. In addition, by their exhibition of specialized markers CD83 and CD81 as demonstrated by western blot (See supplementary material).

3.3. Histopathological results

Histopathological examination of the submandibular salivary glands of the diabetic group showed severe atrophic changes in the acini, intralobar ducts and granular convoluted tubules. Loss of normal gland architecture was noted with multiple intra acinar and intra ductal vacuoles (Figure 2A). Blood vessels associated with connective tissue (C.T.) stroma showed thickened lining, dilatation and congestion. Increase in the fibrous C.T. septum was noted. Fatty degeneration was also observed in some of the seromucous acini (Figure 2B). Excretory ducts revealed retained secretion (Figure 2C). However, the glandular elements of the exosomes treated group showed normal acinar size and architecture. The granular convoluted tubules showed normal eosinophilic granular content (Figure 2D). Intra-lobar ducts' lining showed properly arranged cells. Few dilated and congested blood vessels were noted. Less fibrosis was observed surrounding the excretory duct (Figure 2E, F). Mucous transformation of acini was observed (Figure 2E).

3.4. Ultrastructural results

Ultrastructural examination of the submandibular glands of diabetic rats revealed the acinar seromucous cells with different electron density secretory granules. Concentric membranes inside the secretory granules like a myelinic figure were present. Desmosomal junctions between the acinar cells were observed. Intercellular canaliculi were also evident (Figure 3A). Degenerated rough endoplasmic reticulum and dilated saccules of Golgi complex were noticed. Intracellular membrane bounded vacuoles were present. Pyknotic dark shrunken nuclei as an evidence of apoptosis were observed in between the acinar cells. Coalescence of seromucous granules with pooling of some seromucous granules was observed (Figure 3B). Some granular convoluted tubules showed few, irregular and disrupted secretory granules with some of the ductal cells devoid of secretory granules (Figure 3C). Striated duct showed few basal infoldings and few radially arranged mitochondria with its lumen showing retained granulated secretion (Figure 3D).

The exosomes treated rats revealed the acinar seromucous cell with more or less homogenous electron density of the secretory granules. Normal basally situated nucleus with prominent nucleoli (Figure 4A). Convoluted tubules showed increased, regular and large electron dense secretory granules (Figure 4B). Intercalated duct showed short columnar cells with central nucleus, many mitochondria and desmosomal junctions in between the cells. Myoepithelial cell was normally situated around the intercalated duct (Figure 4C). Striated duct showed basal infoldings and radially arranged mitochondria with clear lumen (Figure 4D).

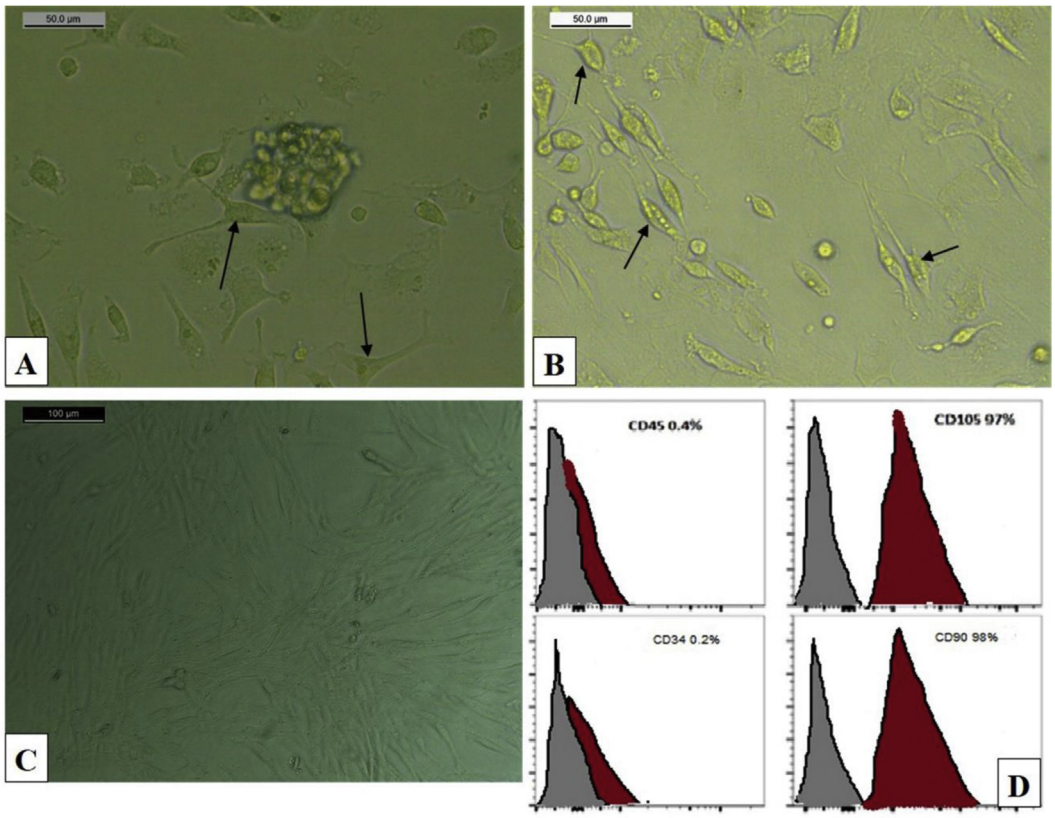

Figure 1. (A) BM-MSCs in culture, after 24 h with black arrows to identify MSCs as fibroblast like cell (50×) (B) after 72 h (50×) (C) after 14 days to show 80% confluence proliferating MSCs (100×) (D) Graph of FACS analysis for cultured MSCs. They were negative for $CD34^{-}$ (0.2%), $CD45^{-}$ (0.4%) & positive for $CD105^{+}$ (97%) & $CD90^{+}$ (98%).

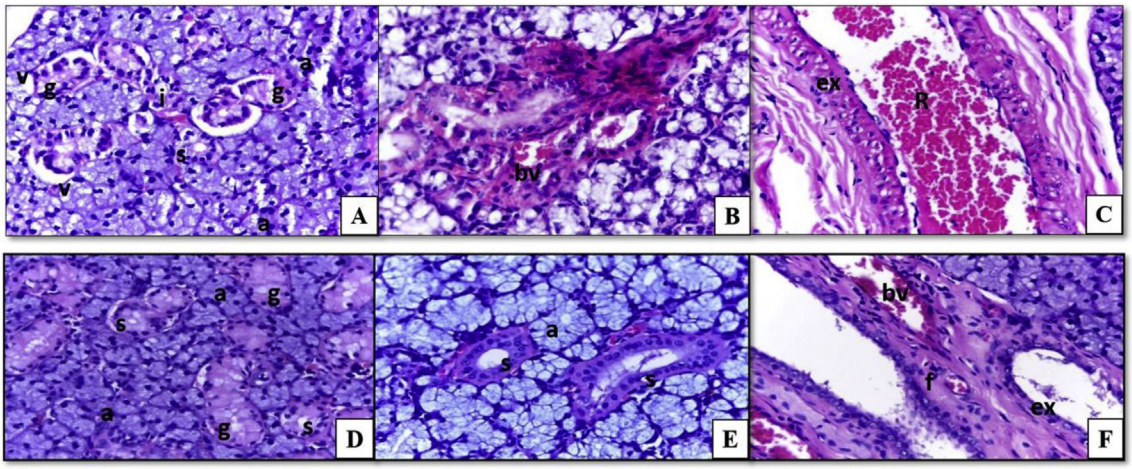

Figure 2. A photomicrograph of the diabetic submandibular salivary gland (A, B, C) and exosomes treated gland (D, E, F) showing: (A) atrophy in: acini (a) granular convoluted tubules (g), striated duct (s) & intercalated duct (i) Intracellular vacuoles (v) were noted. (B) blood vessel (bv) with thickened lining, dilatation and congestion. (C) excretory duct (ex) with intracellular vacuoles and retained secretion (R). (D) normal acinar size and architecture (a), granular convoluted tubules (g) with normal eosinophilic granular content & normal striated ducts (s). (E) striated ducts' lining with properly arranged cells (s) & mucous acinar transformation (a). (F) few dilated and congested blood vessels (bv). Less fibrosis (f) was observed surrounding the excretory duct (ex).

3.5. Real time PCR (q RT-PCR)

T-test revealed a significant upregulation ($p < 0.05$) in the quantitative gene expression of TGFβ, Smad2 and Smad3 in diabetic rats. Conversely, there was a significant reduction ($p < 0.05$) in the exosomes treated rats (Figure 5).

Different letters indicate significant difference between each two groups using T-test as p value <0.05.

3.6. Blood glucose level

The mean values of blood glucose levels revealed a reduction in the exosomes treated group than that in the diabetic group at days 14, 21, 28 & 35 (Figure 6).

3.7. Biochemical analysis of salivary gland functions

Serum amylase and salivary IgA levels revealed a significant rise in the diabetic group compared to the normal control group ($p < 0.05$).

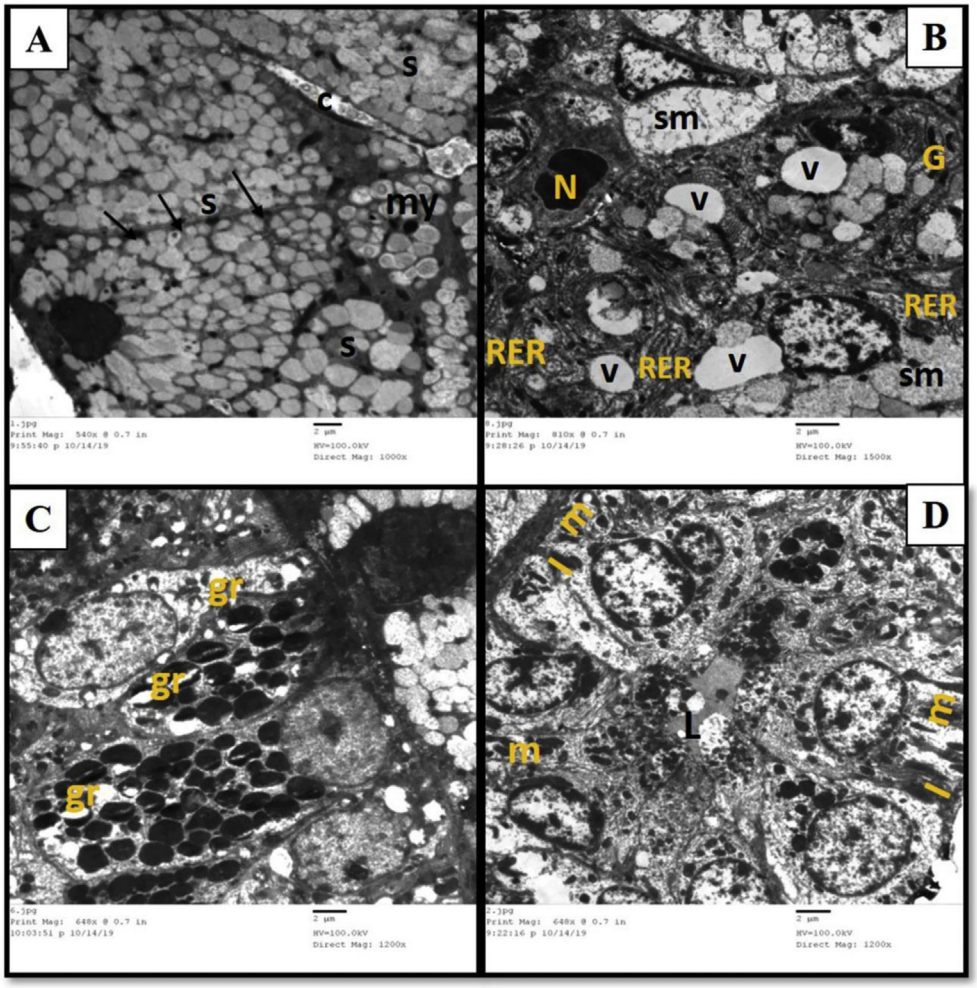

Figure 3. An electron micrograph of the submandibular salivary gland of the diabetic group showing: **(A)** acinar seromucous cells with different electron density secretory granules (s), myelinic figure (my), desmosomal junctions (arrows), intercellular canaliculi (c). **(B)** coalescence of seromucous granules (sm), intracellular vacuoles (v), dilated rough endoplasmic reticulum (RER) & Golgi saccules (G), pyknotic dark shrunken nuclei (N). **(C)** granular convoluted tubules with disrupted secretory granules (gr). **(D)** striated duct with few basal infoldings (I) & few radially arranged mitochondria (m) with its lumen (L) showing retained secretion.

However, the exosomes treated group revealed a significant reduction compared to the diabetic group ($p < 0.05$), but was still significantly elevated than the normal control group ($p < 0.05$) (Figure 7).

4. Discussion

To prevail over DM and its numerous complications, researchers are seeking new therapies. Exosomes are natural cargo of functional DNA, RNA and proteins. They can therapeutically deliver these molecules [21]. Therefore, the current study aimed to evaluate the therapeutic effect of BM-MSC-derived exosomes on salivary glands of diabetic rats.

In the present work, histological and ultrastructural examination of the salivary glands of diabetic rats showed signs of fibrosis, degeneration and apoptosis. Disturbances in the amount and composition of extracellular matrix (ECM) are noted in all diabetic complications. Various growth factors contribute to these complications. Disturbed growth factor signaling adversely influences tissue criteria and affects the ECM [22]. Macrophages are the principle inflammatory cells mediating tissue inflammation in experimental and human diabetes. Activated macrophages result in proinflammatory, profibrotic and antiangiogenic factors release. These factors include TGFβ, platelet-derived growth factor, angiotensin II, TNFα, IL-1, IL-6, ROS and matrix metalloproteinases [23].

TGFβ is a multi-functional cytokine. It regulates tissue regeneration and fibrosis in the kidney, liver and salivary glands. TGFβ upregulation led to salivary gland hypofunction as normal glandular parenchyma was substituted with interstitial fibrous tissue [24].

In our work, TGFβ and the transcription factors Smad2 and Smad3 levels were significantly upregulated in the diabetic group. This clarifies the degenerative changes that occurred in the salivary glands. These findings coincide with previous studies where TGFβ was significantly upregulated in STZ induced diabetic rats [19, 25]. Elevated TGFβ level was proved to promote glucose intolerance and was associated with T2DM. Increased insulin resistance in mice was accompanied by a significant 5–7fold increase in TGFβ levels. Moreover, the Smad3 gene was identified in T2DM [26].

TGFβ signaling is initiated by binding to TGFβ receptor II which activates TGFβ receptor I kinase, leading to phosphorylation of Smad2 and Smad3. The activated Smads form oligomeric complexes. These complexes move into the nucleus where they engage coactivators and repressors to control target genes' expression [27].

TGFβ/Smad3 pathway regulates energy homeostasis and glucose tolerance by controlling insulin gene transcription in the pancreatic islet β cells [28]. In DM, many fibro-genic factors as TGFβ, angiotensin II and advanced glycation end products can activate Smad2 and Smad3. In fibrotic kidney, it was proved that activated Smad2 and Smad3 were accompanied with loss of the inhibitory Smad7 [29]. Smad7 has a protective role in diabetic nephropathy as its inhibition promotes NFκB driven renal inflammation and Smad3 mediated renal fibrosis [30].

In the exosomes treated group, our histological and ultrastructural results were promising, showing normal acinar and ductal elements. Exosomes are active nanocomponents that mediate the paracrine actions of their producing cells [8]. Several studies supported the therapeutic

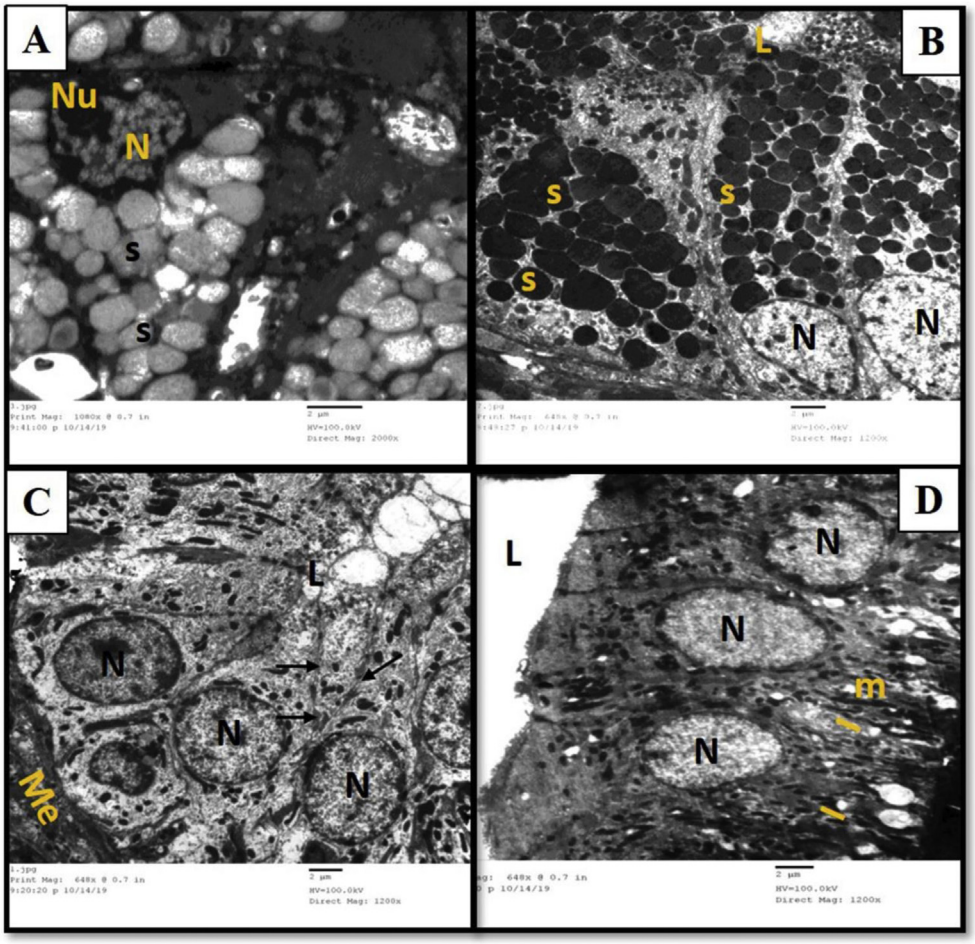

Figure 4. An electron micrograph of the submandibular salivary gland of the exosomes treated group showing: **(A)** acinar cell with open faced nucleus (N) & prominent nucleoli (Nu), homogenous secretory granules (s). **(B)** convoluted tubules with increased secretory granules (s), central nucleus (N), lumen with few secretion (L). **(C)** intercalated duct with short columnar cells & central nucleus (N), desmosomal junctions (arrows), lumen with few secretion (L), myoepithelial cell (Me). **(D)** striated duct with basal infoldings (I) & radially arranged mitochondria (m), central nucleus (N), clear lumen (L).

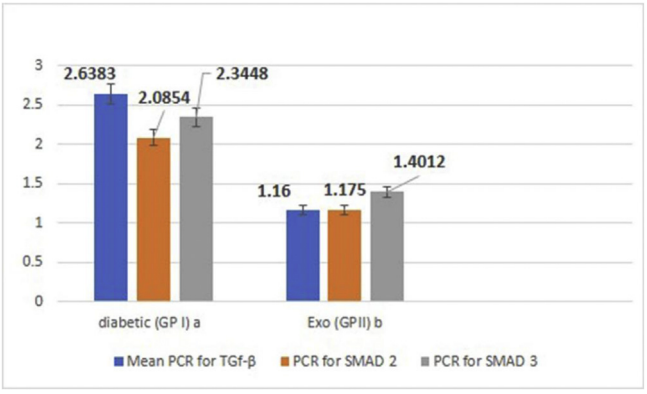

Figure 5. A graph showing the PCR results regarding TGFβ, Smad2 & Smad3.

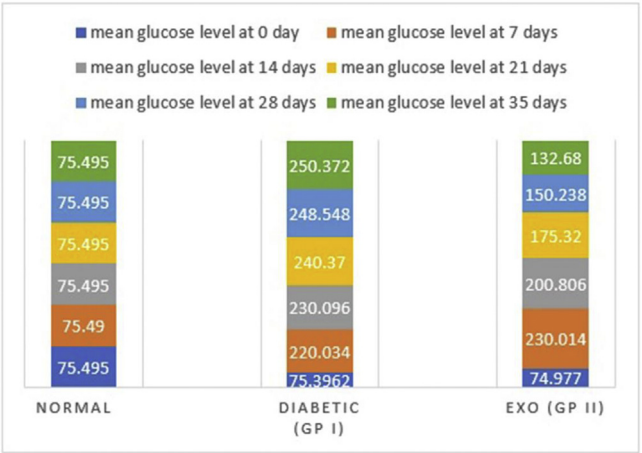

Figure 6. A graph showing the mean values of blood glucose levels at day 0, 7, 14, 21, 28 & 35.

potential of exosomes in DM. When MSC-exosomes were cocultured with high glucose affected human retinal endothelial cells in vitro and were injected into diabetic rats in vivo, levels of caspase-1, interleukin-1β and IL-18 were significantly downregulated [31].

Intravenous injection of human umbilical cord-MSC-derived exosomes into STZ induced rats with T2DM reduced hyperglycemia and insulin resistance, promoted insulin secretion and islet regeneration by inhibiting STZ-induced β cell apoptosis [13]. Moreover, transplantation of β cells derived exosomes in STZ induced diabetic mice improved glucose tolerance, increased insulin content, preserved pancreatic islets' architecture and induced islet angiogenesis [32].

In the current research, injection of BM-MSC-derived exosomes significantly downregulated TGFβ, Smad2 and Smad3 levels. This supports our former histological and ultrastructural results. This finding is also in accordance with a previous study where treatment of diabetic rats with BM-MSC-derived exosomes significantly reduced TGFβ levels and alleviated diabetic nephropathy [19]. It was also shown that removal of Smad3 reduces fibrosis while deletion of Smad2 upregulates it. This proved Smad2 to be protective and Smad3 to be pathogenic [30].

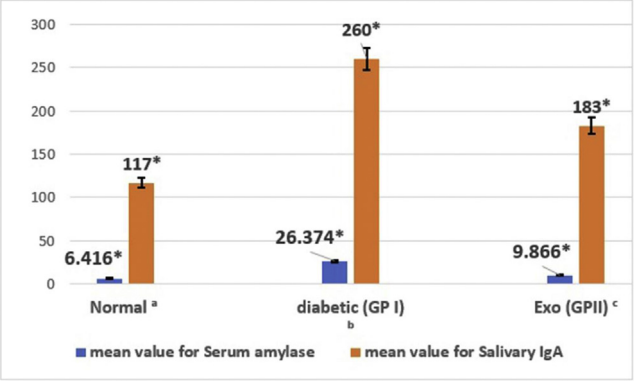

Figure 7. A graph showing the biochemical analysis results regarding serum amylase & salivary IgA. Different letters indicate significant difference between each two groups using T-test as p value <0.05. *Using ANOVA, results are statistically significant as p value <0.05.

Many studies showed a useful effect of suppressing TGF-β/Smad3 signals on glucose tolerance and overall improvement of metabolic profile. In DM, anti-TGFβ neutralizing antibody reduced phosphorylated Smad3 levels, enhanced insulin and glucose tolerance, suppressed hyperglycemia and hyperinsulinemia. Moreover, Smad3 deletion resulted in an improved pancreatic islet β cell function, glucose tolerance and insulin sensitivity [26, 33].

This supports the notion of TGF-β/Smad3 pathway as a potential target in treatment of diabetes and obesity. This is also in accordance with our study design where we assumed that BM-MSC-derived exosomes can exert their effect through suppressing TGFβ/Smad3 signaling pathway.

In this study, assessment of salivary glands' function was done by measuring salivary IgA and serum amylase levels. Gland damage can be monitored by elevated gland-specialized enzymes that are released in serum indicating functional loss or altered architecture [34].

As for serum amylase, our findings are in accordance with previous studies where increased levels of alpha-amylase were also reported in diabetics [35, 36]. However, other studies showed a significant decrease in alpha-amylase levels in diabetic groups compared to normal ones [37, 38]. These altered serum amylase levels in DM may be attributed to hyperglycemia, decreased stimulating insulin effect on pancreatic cells and increased destruction processes of pancreatic tissue [39].

Regarding IgA levels, our findings coincide with previous studies where T2DM was positively correlated to IgA and increased IgA concentrations were associated with hyperglycemia [40]. However, in another study, levels of salivary IgA in type II diabetic patients compared to their healthy controls did not show any significant difference [41].

It was concluded that high amylase and IgA concentrations may reflect oro-dental manifestations in T2DM. In addition, it was suggested that amylase and IgA may serve as a complementary and alternative fluid in screening for DM [42].

Our results demonstrated that BM-MSC-derived exosomes reduced serum amylase and IgA levels. When managing DM, many studies proved that downregulation of enzymes such as α-glucosidase and α-amylase which are involved in the breakdown of carbohydrates resulted in reducing starch hydrolysis, thus, regulating postprandial glycemic level [43]. Other study proved that administration of BM-MSC-derived exosomes to activated B cells significantly inhibited the production of IgM, as tested by the ELISA assay, while no significant change was observed in the IgG and IgA levels [44].

The molecular mechanisms underlying the effects of exosomes on diabetic alleviation might be attributed to its miRNAs content. Over 150 miRNAs are distinguished in MSC-derived exosomes [45]. miRNAs are non-coding, small RNAs that control vital cell functions as proliferation, differentiation and death. Therefore, we assumed that miRNAs present in exosomes mediate the mechanism underlying their effects. BM-derived exosomes were proved to contain miR-106b-5p and miR-222-3p. Intravenous administration of these miRNAs ameliorated hyperglycemia in mice with insulin-deficient diabetes by mimicking post-injury β-cell proliferation through Cip/Kip family down-regulation [46].

The most abundant miRNAs present in BM-MSC-derived exosomes are miR-21-5p, miR-486-5p, miR-10b-5p, miR-22-3p and miR-143-3p. They represent 43–59 % of the total miRNA reads present in BM-MSC-derived exosomes [47]. It was proved that miR-486 regulates stem cell senescence [48], miR-10b promotes BMSCs' migration [49], miR-22 controls MSC differentiation [50] and miR-143 modulate immune functions of MSCs [51]. miR-191, miR-222, miR-21, let-7a and let-7f are also present in BM-MSC-derived exosomes. They play an important role in controlling cell cycle proliferation and progression and in modulating angiogenesis [52, 53].

MSCexosomes were also proved to have an immunomodulatory effect through repression of M1 macrophage polarization and improvement of M2 macrophage activation. MSC exosomes accomplish this change in M1/M2 ratios by targeting signaling pathways affecting macrophage polarization. MSC exosomes can also modulate the function of B cells, natural killer cells, T cells and dendritic cells of the immune system [54].

5. Conclusions

From the results of the current study, it was concluded that BM-MSC-derived exosomes has a therapeutic effect on T2DM through the dramatic improvement in blood glucose levels where the mean values of blood glucose revealed a reduction in the exosomes treated group compared to the diabetic group. BM-MSC-derived exosomes also promoted salivary glands' function as evidenced by a significant reduction ($p < 0.05$) in serum amylase and salivary IgA levels compared to the diabetic group. In addition, salivary gland architecture was preserved as demonstrated histologically, ultra-structurally and confirmed by a significant down-regulation ($p < 0.05$) in the quantitative gene expression of TGFβ, Smad2 and Smad3 in the exosomes treated group. All these findings implicated exosomes as a novel therapeutic strategy for treating DM.

6. Recommendations

The entire data available greatly support the assumption that exosomes possess therapeutic potential and that they have an important role in the treatment of DM. However, further investigations are required to study the exact mechanism of action of exosomes. A comprehensive assessment of the types, amounts and functions of miRNAs present in exosomes has to be performed. Understanding how exosomes function would expand our knowledge of their therapeutic usage in systemic diseases. These investigations need to be carried out before exosomes can be clinically applied. In the future, comprehensive characterization is needed to identify the numerous subpopulations of exosomes. Providing these novel insights will help in curing various systemic diseases using exosomes. In addition, various sources of exosomes need to be discovered.

Declarations

Author contribution statement

N. AbuBakr and Z. Salem: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

T. Haggag: Conceived and designed the experiments; Analyzed and interpreted the data.

D. Sabry: Conceived and designed the experiments; Contributed reagents, materials, analysis tools or data.

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The authors declare no conflict of interest.

Additional information

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