



Adipose mesenchymal stem cells–derived exosomes attenuate retina degeneration of streptozotocin-induced diabetes in rabbits

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Abstract

This study aimed to evaluate the effect of mesenchymal stem cells (MSCs)–derived exosomes in retina regeneration of experimentally induced diabetes mellitus (DM) in a rabbit model. Exosomes are extracellular vesicles that contain many microRNAs (micRNAs), mRNAs, and proteins from their cells of origin. DM was induced by intravenous (IV) injection of streptozotocin in rabbits. MSCs were isolated from adipose tissue of rabbits. Exosomes were extracted from MSCs by ultracentrifugation. Exosomes were injected by different routes (IV, subconjunctival (SC), and intraocular (IO)). Evaluation of the treatment was carried out by histopathological examination of retinal tissues and assessment of micRNA-222 expression level in retinal tissue by real-time polymerase chain reaction. Histologically, by 12 weeks following SC exosomal treatment, the cellular components of the retina were organized in well-defined layers, while IO exosomal injection showed well-defined retinal layers which were obviously similar to layers of the normal retina. However, the retina appeared after IV exosomal injection as irregular ganglionic layer with increased thickness. MicRNA-222 expression level was significantly reduced in diabetic controls when compared to each of healthy controls and other diabetic groups with IV, SC, and IO routes of injected exosomes (0.06 ± 0.02 vs. 0.51 ± 0.07 , 0.28 ± 0.08 , 0.48 ± 0.06 , and 0.42 ± 0.11 , respectively). We detected a significant negative correlation between serum glucose and retinal tissue micRNA-222 expression level ($r = -0.749$, $p = 0.001$). We can associate the increased expression of micRNA-222 with regenerative changes of retina following administration of MSCs-derived exosomes. The study demonstrates the potency of rabbit adipose tissue–derived MSCs exosomes in retinal repair. So, exosomes are considered as novel therapeutic vectors in MSCs-based therapy through its role in shuttling of many factors including micRNA-222.

Keywords

Exosomes, mesenchymal stem cells, micRNA, diabetes mellitus

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Introduction

Diabetic retinopathy (DR) is one of the serious complications of diabetes mellitus (DM),¹ which leads to severe visual impairment especially in late stages of the disease.² Hyperglycemia and defects in insulin signaling pathways result in many pathological processes which end by the development of DR.³ DR involves the presence of microvascular defects, neuroretinal dysfunction, and degeneration of the retina.⁴ Mesenchymal stem cells (MSCs) were isolated and purified from many sources, such as bone marrow, adipose tissue, fetal membranes, embryo, and cord blood, and could be further utilized in tissue repair including the retina.^{5–8} MSCs are multipotent cells which have the possibility to transform into many types of cells involving cardiovascular, neurogenic, endothelial cells, and adipocytes.⁹ MSCs have been utilized as therapeutic agents because of their direct role in tissue regeneration and their strong anti-inflammatory properties which were discovered more recently.¹⁰ Moreover, MSCs have been applied in many clinical attempts for management of retinal degeneration.¹¹ Exosomes are extracellular vesicles (EVs) of endosomal origin and are characterized from other vesicles derived from the cell by their origin, size, composition, and shape.¹² Accordingly, exosomes are present in supernatants of cell culture. CD81 and CD63 proteins have been utilized as specific markers of exosomes.^{13–16} It has been proved that exosomes may play a crucial role in transfer of mRNA, microRNAs (micRNAs), and proteins.^{13,14} It was found that presence of specific micRNAs, such as micRNA-17-5p, micRNA-126, micRNAs-221/222, and micRNA-296, involved in the regulation of angiogenesis.^{17,18} Angiogenesis, abnormal growth of retinal blood vessels, was associated with the severity of DR. MiR-221/miR-222 family sounds to negatively regulate angiogenesis by binding to the c-Kit receptor.¹⁹ The role of micRNA-222 in regulating the neovascularization observed in DR was detected by targeting signal transducer and activator of transcription 5A (STAT5A). Additionally, it is indicated that STAT5A is mostly regulated by micRNA-222 during inflammation-associated neovascularization.²⁰ Thus we focused on detecting the expression of micRNA-222. Recently, more research have investigated the functions of exosomes.²¹ Our present study investigated the therapeutic effect of micRNA-222-containing exosomes in treatment of retinal degeneration after their injection in experimental rabbit model with DM induced by streptozotocin (STZ).

Materials and method

MSCs isolation and expansion

The rabbit fat tissue was dissected and transported to 50 mL Falcon tubes and weighted. For enzymatic dissociation, an equal volume of 250 U/mL of collagenase type II was added to the fat tissue and incubated at 37°C for 90 min under agitation in shaking water bath.²² By using a sterile

strainer, cell suspensions were filtered. The filtered cells were centrifuged at $300 \times g$ for 5 min and resuspended in expansion medium (α -modified eagles medium [MEM] supplemented with 100 U/mL penicillin/streptomycin and 10% fetal calf serum) (R&D Systems, Abingdon, UK). Cells quantification was assessed by cell counter and cells were plated at 4000 cells/cm² for 7 days. On day 7, cells were trypsinized, counted, and replated in expansion medium at the density of 2000 cells/cm² for another period of 7 days (end of passage 1). The expansion was performed till reaching third passage.

Isolation of exosomes

Exosomes were isolated from supernatant of first, second, and third passages of MSCs cultured in α -MEM deprived of fetal bovine serum (FBS). After centrifugation at $2000 \times g$ for 20 min to remove debris, cell-free supernatant was centrifuged at $100,000 \times g$ (ultracentrifuge of Beckman Coulter Optima L 90 K) for 1 h at 4°C, washed in serum-free medium 199 containing HEPES 25 mM (Sigma, St Louis, Missouri, USA), and subjected in the same conditions to a second ultracentrifugation. The protein content of exosomes pellet was quantified by the Bradford method (Bio-Rad, Hercules, California, USA).²³

Exosomes labeling with PKH26

Exosomes were labeled with PKH26 dye for their in vitro tracing by fluorescence microscopy according to the manufacturer's recommendations (Sigma). Following ultracentrifugation, the exosomes pellet was diluted with PKH26 kit solution to 1 mL and then 2 μ L of fluorochrome was added to this suspension and incubated at 38.5°C for 15 min. After that, 7 mL of serum-free high glucose-modified eagles medium (HG-DMEM) was added to the suspension and then it was ultracentrifuged for second time at $100,000 \times g$ for 1 h at 4°C. The final pellet was resuspended rapidly in HG-DMEM and stored at -80°C for future injection in experimentally induced rabbit.²⁴

Western blot for characterization of exosomes

The antibody used was antigen affinity-purified polyclonal sheep IgG anti-rabbit CD81 (Catalog no. 0349509; BioLegend, San Diego, California, USA). Protein was isolated from isolated exosomes using radioimmunoprecipitation assay buffer. Twenty nanograms of protein were loaded and separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis on 4–20% polyacrylamide gradient gels. Following incubation in 5% nonfat dry milk, Tris hydrochloride, 0.1% Tween 20 for 1 h, CD81 polyclonal–monoclonal antibody was added to one of the membranes including specimen samples and incubated at 4°C overnight. Appropriate secondary antibody was incubated for 2 h at room temperature. After being washed twice n 1 \times

TBS-T, densitometric analysis of the immunoblots was performed to quantify the amounts of CD81 against house-keeping protein β -actin by image analysis software on the ChemiDoc MP imaging system (version 3) produced by Bio-Rad.

Experimental induction of diabetes

Animals were handled and cared for in accordance with the guide of Laboratory Animals published by the US National Institute of Health (NIH publications no. 8023, revised 1978) and approved by the ethics committee for animal experimentation at Faculty of Medicine, Cairo University. Male rabbits (1000–1200 gm) were injected by STZ at a dose of 60 mg/kg of the body weight intravenously. STZ induced diabetes within 3 days by damaging the beta cells. The experiment was carried out on rabbits and was divided as follows: group 1: rabbits received the standard diet, that is, control group ($n = 3$); group 2: rabbits were injected with STZ, that is, diabetic control ($n = 3$); group 3: diabetic rabbits injected systemically with exosomes (100 μ g protein/mL) labeled with PKH26 (intravenous (IV)) ($n = 3$); and group 4: diabetic rabbits injected locally with exosomes labeled with PKH26 (subconjunctival (SC) and intraocular (IO)) ($n = 3$ /each route of injection). At 4, 8, and 12 weeks from STZ injection and administration of exosomes, venous blood was collected from the retro-orbital vein to assess serum glucose and insulin. All rabbits were euthanized with carbon dioxide narcosis, and the retinal tissues were collected for histopathological examination and real-time polymerase chain reaction (RT-PCR) analysis.

Histopathological examination

Tissue specimens were collected from euthanized rabbits of all groups. Tissue sections (4–6 μ m) were prepared. Tissues were divided into two groups. The first group was examined for tracing of injected exosomes labeled with PKH26. The other group was washed with phosphate-buffered saline and fixed overnight in 10% buffered neutral formalin at 4°C. Serial micrometer sections of the dissected tissues were stained with hematoxylin and eosin.

RNA extraction

RNA was extracted from retinal tissues using miRNeasy extraction kit (Qiagen, Valencia, California, USA); manual instruction was available online at <http://asmlab.org/public/files/miRNeasy-Mini-Kit-EN.pdf>.

Real-time polymerase chain reaction

Conversion of individual micRNAs into the corresponding cDNA was done with a TaqMan MicroRNA real-time reverse transcriptase (RT) kit (assay ID 000397, catalog no. 4427975; Applied Biosystems, Foster City, California,

USA) according to the manual manufacturer's protocol; was accessible at https://tools.thermofisher.com/content/sfs/manuals/cms_042167.pdf. For micRNA detection reaction, a forward primer and a 3' universal reverse primer were provided by the RT kit. Rabbit U6 snRNA was amplified as an internal control. qPCR using SYBR Green Master Mix (Applied Biosystems) was performed employing the Applied Biosystems instrument with software version 2.1 (StepOne™). The amplification conditions were as follows: 1 cycle of 50°C for 2 min and 1 cycle of 95°C for 10 min, followed by 40 cycles of 95°C for 30 s and finally 60°C for 1 min. Specificity was verified by melt curve analysis. The data were expressed in cycle threshold (Ct). The PCR data sheet involves Ct values of assessed micRNA-222 and internal control (U6 snRNA). The relative quantitation of micRNA was normalized by $\Delta\Delta$ Ct method.

Statistical analysis of data

The obtained data were organized and statistically analyzed using SPSS software statistical computer package version 18 (SPSS Inc., Chicago, USA). For quantitative data, the mean and standard deviation were measured. One-way analysis of variance was used to test the difference about mean values of measured parameters among groups, multiple comparison between pairs of groups were performed using Tukey honest significant difference (post hoc range test). Pearson correlation was run to identify the relation of relative expression levels of micRNA-222 with serum glucose and insulin levels. Significance was adopted at $p \leq 0.05$.

Results

Serum glucose and insulin levels

We first investigated serum glucose concentrations (Table 1) and insulin levels (Table 2) in different groups of study. The diabetic controls had significantly higher concentrations of serum glucose than other groups involved in the study, healthy controls, diabetic with IV injected exosomes, diabetic with SC and IO administrated exosomes (322.9 ± 41.4 vs. 85.6 ± 10.7 , 164.8 ± 30.4 , 177.2 ± 12.3 , and 185.4 ± 29.0 , respectively). Serum insulin levels were significantly reduced in diabetic control group than each of healthy controls, diabetic with SC and diabetic with IO injected exosomes groups (7.0 ± 1.9 vs. 40.2 ± 10.5 , 30.8 ± 2.5 , and 29.3 ± 2.6 , respectively).

MSCs and exosomes characterization

Adipose tissue-derived MSCs were cultured through first, second, and third passages where they were characterized using inverted microscope by their specific spindle fusiform shape in different passages. Exosomes were analyzed by electron microscopy which appeared as micrographs for spheroidal exosomes with a formal size of less than 100 nm and contained homogeneously sized particles (Figure 1).

Table 1. Comparison of serum glucose levels between different studied groups.

Glucose (mg/dl)	Healthy control (n = 3)	Diabetic control (n = 3)	Diabetic + exosomes (IV) (n = 3)	Diabetic + exosomes (SC) (n = 3)	Diabetic + exosomes (IO) (n = 3)
Mean \pm SD	85.6 \pm 10.7	322.9 \pm 41.4	164.8 \pm 30.4	177.2 \pm 12.3	185.4 \pm 29.0
P1	—	0.00 ^a	0.00 ^a	0.00 ^a	0.00 ^a
P2	0.00 ^a	—	0.00 ^a	0.00 ^a	0.00 ^a
P3	0.00 ^a	0.00 ^a	—	0.29	0.41
P4	0.00 ^a	0.00 ^a	0.29	—	0.34
P5	0.00 ^a	0.00 ^a	0.41	0.34	—

SD: standard deviation; IV: intravenous; SC: subconjunctival; IO: intraocular; P1: comparison with control group; P2: comparison with diabetic group; P3: comparison with diabetic + exosomes (IV) group; P4: comparison with diabetic + exosomes (SC) group; P5: comparison with diabetic + exosomes (IO) group.

^a $p \leq 0.05$ is considered significant.

Table 2. Comparison of serum insulin levels between different studied groups.

Insulin (μ U/ml)	Healthy control (n = 3)	Diabetic control (n = 3)	Diabetic + exosomes (IV) (n = 3)	Diabetic + exosomes (SC) (n = 3)	Diabetic + exosomes (IO) (n = 3)
Mean \pm SD	40.2 \pm 10.5	7.0 \pm 1.9	34.5 \pm 10.9	30.8 \pm 2.5	29.3 \pm 2.6
P1	—	0.00 ^a	0.16	0.013 ^a	0.00 ^a
P2	0.00 ^a	—	0.00 ^a	0.00 ^a	0.00 ^a
P3	0.16	0.00 ^a	—	0.23	0.25
P4	0.013 ^a	0.00 ^a	0.23	—	0.47
P5	0.00 ^a	0.00 ^a	0.25	0.47	—

SD: standard deviation; IV: intravenous; SC: subconjunctival; IO: intraocular; P1: comparison with control group; P2: comparison with diabetic group; P3: comparison with diabetic + exosomes (IV) group; P4: comparison with diabetic + exosomes (SC) group; P5: comparison with diabetic + exosomes (IO) group.

^a $p \leq 0.05$ is considered significant.

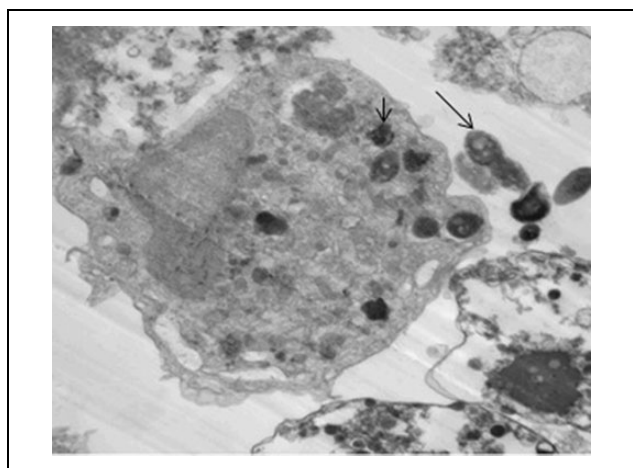


Figure 1. Ultrastructure of exosomes. Exosomes isolated from MSCs. They are heterogeneous in size, ranging from 30 nm to 100 nm. They are directly frozen and observed by cryo-electron microscopy without chemical fixation or contrasting. Exosomes appear round and are visualized with improved resolution (arrows). Scale bar 100 nm. MSCs: mesenchymal stem cells.

The isolated exosomes had specific membrane markers such as CD81 which was scanned versus β -actin in different passages by Western blot analysis (Figure 2(a)). The detected levels of CD81 showed a gradual increase in isolated exosomes while moving from one passage to another.

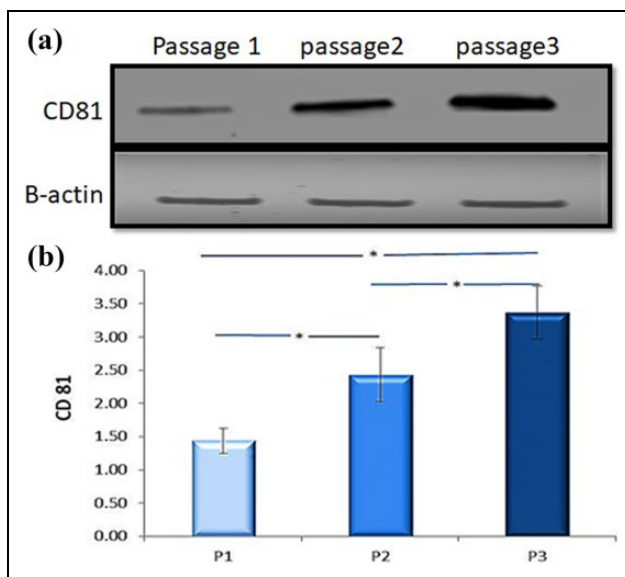


Figure 2. (a) Western blot scanning densitometry of CD81 versus β -actin protein levels in MSCs-derived exosomes in different passages. Lane 1: passage 1, Lane 2: passage 2, Lane 3: passage 3. (b) Mean of ratio of CD81 versus β -actin protein levels in MSCs-derived exosomes in different passages. CD81 showed a significant increased level in passage 2 when compared to passage 1 and significant elevated levels in passage 3 when compared to passage 2. P1: passage 1; P2: passage 2, and P3: passage 3. MSCs: mesenchymal stem cells.

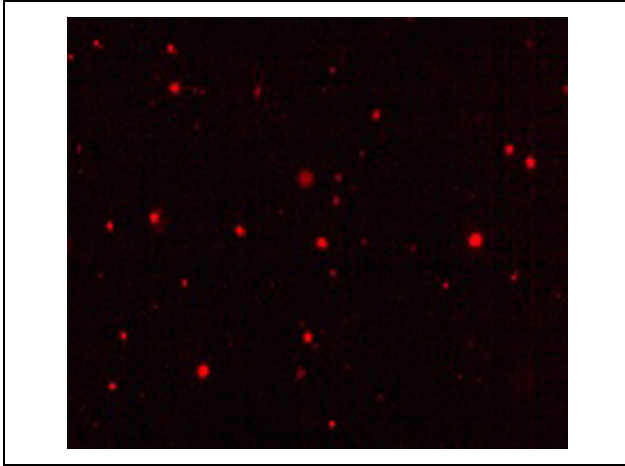


Figure 3. MSCs-derived exosomes labeled with PKH26 under fluorescent microscopy. MSCs: mesenchymal stem cells.

Quantitative expression of CD81 against β -actin showed a significant increase in passage 2 when compared to passage 1 (2.43 ± 0.41 vs. 1.44 ± 0.19 , $p < 0.0001$) and a significant increase in passage 3 when compared to passage 2 (3.37 ± 0.40 vs. 2.43 ± 0.41 , $p < 0.0001$) (Figure 2(b)).

Evaluation of exosomal uptake by retinal tissue

Exosomes labeled with PKH26 dye, red fluorescence chromophore inserting into the lipid bilayers of exosomes, were incorporated in degenerated retinal tissue which became clearly visible under fluorescent microscopy as shown in Figure 3.

Histopathological results

Histological examination of retina tissue of induced diabetic rabbit (positive control) demonstrated the presence of abnormal thickness of all retina layers (ganglionic layer

(GL), inner plexiform layer (IPL), outer plexiform layer (OPL), outer nuclear layer (ONL)) with well-observed degenerative areas in both GL and IPL (Figure 4(a)). Furthermore, other retina tissue showed well-observed hemorrhage in IPL and ONL (Figure 4(b)). Toward the end of experimental duration, retina tissue of that group showed complete damage of GL and IPL with well-observed hemorrhage and abnormal thickness of all other retinal layers (Figure 4(c)). Following SC microvesicles (MVs) injection (SC group), retina tissue showed well-organized retinal layers with well-observed vacuoles in ONL, GL, and IPL (Figure 5(a)). Moreover, at the end of experimental duration, retinal tissue showed normal architecture (Figure 5(b)).

Following IO MVs injection (IO group), retina tissue showed degeneration and vacuoles in GL and IPL (Figure 6(a)). These findings were markedly improved after the third dosage posttreatment (Figure 6(b)).

However, retina after being IV injected with exosomes (IV group) demonstrated irregular GL with increased thickness of all other layers (Figure 7).

Gene expression analysis of micRNA-222 level

To analyze the therapeutic effect of MSCs exosomes in regeneration of induced diabetic retinal tissue damage, relative gene expression levels of micRNA-222 in retina were investigated by RT-PCR in different groups of our study (Table 3 and Figure 8). It was found that micRNA-222 was significantly decreased in diabetic control group compared to each of healthy controls, diabetic with IV injected exosomes, diabetic with SC and diabetic with IO administrated exosomes groups (0.06 ± 0.02 vs. 0.51 ± 0.07 , 0.28 ± 0.08 , 0.48 ± 0.06 , and 0.42 ± 0.11 , respectively). p Values were <0.001 in relation of diabetic control group to each of the previous other groups, respectively. It was found that the level of micRNA-222 was significantly

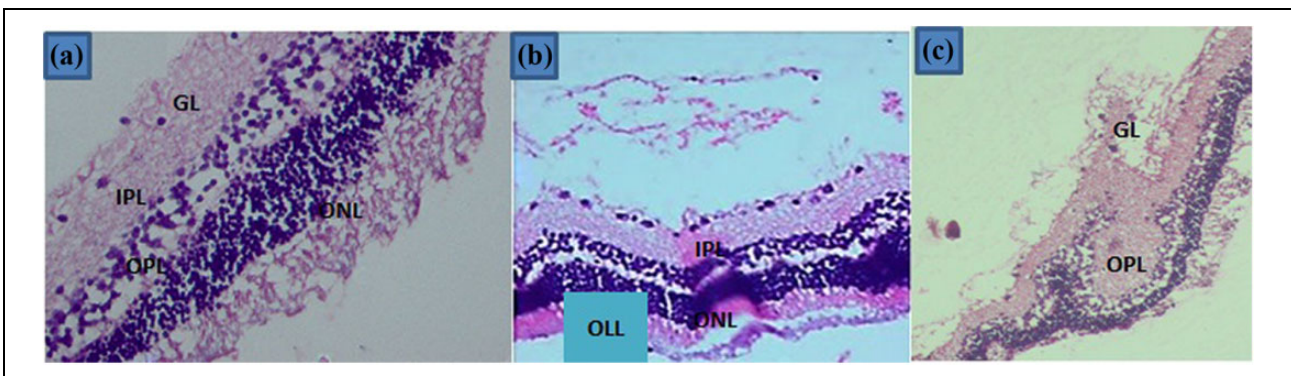


Figure 4. (a) Photomicrograph of retina tissue of positive control (diabetic eye) showing abnormal thickness of all retina layers (GL, IPL, OPL, ONL) with well-observed degenerative areas in both GL and IPL, H&E $\times 100$. (b) Photomicrograph of retina tissue of positive control (diabetic eye) showing hemorrhage in IPL and ONL, H&E $\times 100$. (c) Photomicrograph of retina tissue of positive control (diabetic eye) showing complete damage of GL and IPL layer with well-observed hemorrhage and abnormal thickness of all other retinal layers, H&E $\times 100$. GL: ganglionic layer; IPL: inner plexiform layer; OPL: outer plexiform layer; ONL: outer nuclear layer; OLL: outer limited layer rod and cone; H&E: hematoxylin and eosin.

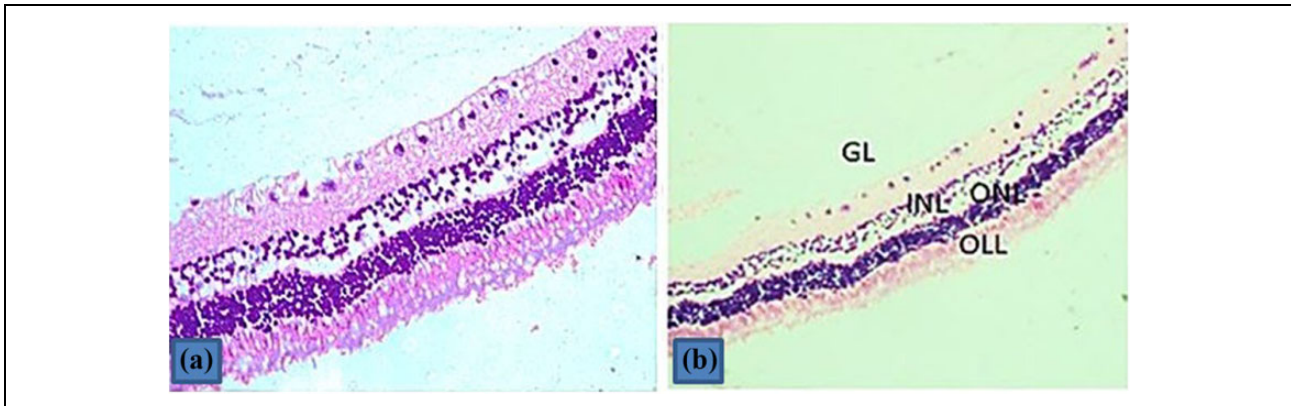


Figure 5. (a) Photomicrograph of retina tissue of SC MVs–injected groups showing well-organized retinal layers with well-observed vacuolation in ONL, GL, and IPL, H&E $\times 100$. (b) Photomicrograph of retina tissue of SC MVs–injected groups at the end of experimental duration with retinal tissue showing normal architecture, H&E $\times 100$. GL: ganglionic layer; IPL: inner plexiform layer; ONL: outer nuclear layer; OLL: outer limited layer rod and cone; INL: inner nuclear layer; H&E: hematoxylin and eosin; SC: subconjunctival; MVs: microvesicles.

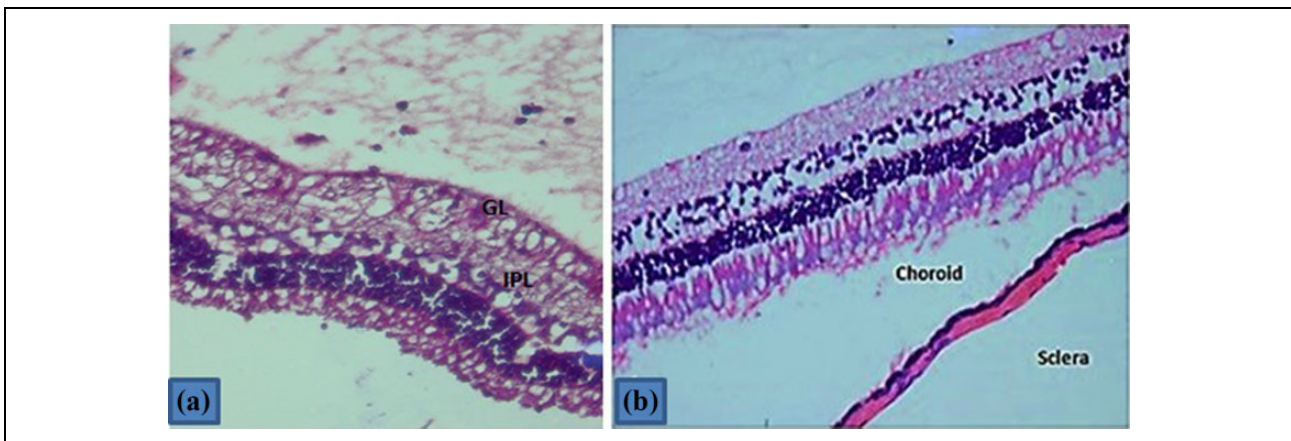


Figure 6. (a) Photomicrograph of retinal tissue after IO MVs injection showing degeneration and vacuoles in GL and IPL, H&E $\times 100$. (b) Photomicrograph of retinal tissue after IO MVs injection showing well-defined components of retinal layer, H&E $\times 100$. GL: ganglionic layer; IPL: inner plexiform layer; H&E: hematoxylin and eosin; MVs: microvesicles; IO: intraocular.

elevated in diabetic group with SC injected exosomes than diabetic IV injected exosomes group (0.48 ± 0.06 vs. 0.28 ± 0.08 , $p < 0.001$). Also, we detected that the level of micRNA-222 in diabetic group associated with IO exosomes was significantly increased than diabetes with IV exosomes (0.42 ± 0.11 vs. 0.28 ± 0.08 , $p < 0.001$). However, there was no increase in relative micRNA-222 level in diabetic group with IO exosomes than in diabetic group with SC route of injected exosomes (0.42 ± 0.11 vs. 0.48 ± 0.06). The increased expression level of retinal micRNA-222 in healthy controls in relation to other diabetic groups involved in the study (diabetic controls, diabetic with IV, SC, and IO routes of administrated exosomes) indicated the role of high glucose conditions in reducing micRNA-222 expression level. There is a significant negative correlation between serum glucose concentrations and gene expression levels of micRNA-222 ($r = -0.749$, $p = 0.001$) (Figure 9) and a significant

positive correlation between serum insulin and micRNA-222 levels ($r = 0.736$, $p = 0.002$) (Figure 10) in different groups of study.

Discussion

The reestablishment of damaged, lost, and aging tissues in the human body is the main target of regenerative medicine. Various types of stem cells have been tested in both animal models and human clinical studies for its utilization in tissue regeneration.²⁵ The current study demonstrates that systemic, SC and IO administration of rabbit adipose MSCs exosomes can induce repair of diabetic retinal degeneration in a model of rabbits.

DR is a common complication of diabetes which if left untreated can lead to loss of vision.^{26,27} Hyperglycemia impairs retinal microvasculature, which leads to increased permeability, blood leakage to the extravascular space,

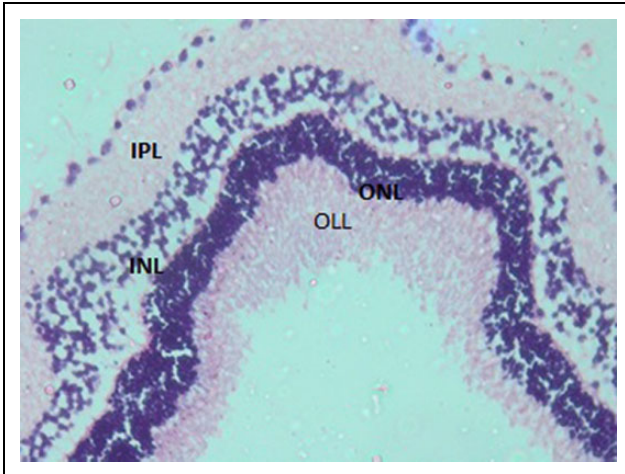


Figure 7. Photomicrograph of retinal tissue after IV MVs injection showing well-defined layers with increase in all layer thickness (IPL, INL, ONL, and OLL) and losing their regular form, H&E $\times 100$. IPL: inner plexiform layer; INL: inner nuclear layer; ONL: outer nuclear layer; OLL: outer limited layer rod and cone; H&E: hematoxylin and eosin; MVs: microvesicles; IV: intravenous.

continuous decrease in retinal blood flow, and closure of these microvessels.² Many studies are supporting the role of MSCs in promoting tissue regeneration involving retinal degenerative diseases¹¹ without the need of cellular differentiation. The effect of MSCs-dependent therapy is mostly due to the paracrine secretion of trophic factors, exosomes.^{28–30} Exosomes have therapeutic effects in mediating tissue repair, through its role in the transport of specific mRNA from one cell to other cells. This leads to the production of multiple proteins from each mRNA that is transported to these cells.³¹ Furthermore, it was detected that MSCs-derived exosomes contain other regulatory elements, micRNAs.³² Many studies demonstrated that the released micRNAs from exosomes between cells can directly inhibit or stimulate the transcription of target genes and lead to differential expression of these protein-coding genes which may be involved in different biological processes.^{20,33–37} Therefore, exosomes act as a very powerful tool by which the cells can transport messages to others.³¹

In our study, we proved the existence of exosomes through measurement of CD81 by Western blot assay. CD81 is one of tetraspan proteins family, CD9, CD63, CD81, and CD82. The membranes of exosomes are very rich in CD81 due to their endosomal origin.³⁸ As the expansion of MSCs was done until reaching third passage, we detected that CD81 showed a significant increased level in the second passage than in the first passage. Also, the relative quantitation of CD81 to β -actin protein was significantly elevated in the third passage compared to second and first passages, and these results confirm that the isolated molecules from MSCs-conditioned media are exosomes.

The current study showed the presence of well-observed edema, complete damage of retina with detached sclera from underlying muscles and extensive hemorrhage in retinal layers in diabetic controls by histological examination. By IV administration of rabbit adipose MSCs exosomes at 4, 8, and 12 weeks following STZ injection, induced irregular GLs with increased thickness of retina appeared. However, SC stem cell-derived exosomes injection restored the cellular components of the retina which were organized in well-defined layers. Also, our results showed that IO exosomes infusion encouraged the regeneration of retina which appeared as well-defined retinal layers similar to layers of normal retina, and that occurred over a period of 12 weeks. These results indicate the vital therapeutic role of MSCs-derived exosomes in regenerative changes in retinal tissue. However, the duration of up to 12 weeks was chosen in many studies where this period was found to be sufficient to detect a complete regenerative response in a model of rats.^{32,39} It is worth to be mentioned that human MSCs-derived exosomes do not have major histocompatibility complex proteins of classes I and II as it was detected by previous studies, so we can utilize exosomes derived from human MSCs in the future without the need of induction of immunosuppression.^{32–34}

In the current study, we revealed that micRNA-222 has a highly significant reduced expression level in retinal tissue of diabetic control group compared with other groups administrated with MSCs exosomes as well as the healthy controls. We detected a significant negative association between the expression level of micRNA-222 and serum glucose levels in the groups of our study. So we can suspect that hyperglycemia leads to decreased micRNA-222 expression. Previous studies showed that hyperglycemic conditions cause reduction of micRNA-222 expression in microparticles derived from endothelial cells in coronary artery disease.^{40,41} It has been detected that micRNA-222 acts as a critical modulator of vascular smooth muscle cells proliferation by targeting p27Kip1 and p57Kip2 genes.⁴² Moreover, micRNA-222 can inhibit neovascularization which may occur in advanced stages of DR by regulating STAT5A protein expression.²⁰ These effects of micRNA-222 appear to be specific to endothelial cells and vascular smooth muscle cells.⁴³ It was found that micRNA-222 is highly expressed in endothelial cells present in normal vessels, while decreased expression levels were detected in endothelial cells of atherosclerotic blood vessels exposed to inflammatory stimuli. The increased numbers of new vessels with low micRNA-222 expressing atherosclerotic plaques were determined.²⁰

So this study could demonstrate for the first time, to our knowledge, the important role of exosomal shuttled micRNA-222 in retinal tissue repair. Reduced micRNA-222 expression levels in retinal tissue of STZ-induced diabetes were associated with the development of severe retinal damage and extensive hemorrhage in different layers of retina of this group by histopathological

Table 3. Comparison of quantitative micRNA-222 gene expression between different groups.

micRNA-222	Healthy control (n = 3)	Diabetic control (n = 3)	Diabetic + exosomes (IV) (n = 3)	Diabetic + exosomes (SC) (n = 3)	Diabetic + exosomes (IO) (n = 3)
Mean \pm SD	0.51 \pm 0.07	0.06 \pm 0.02	0.28 \pm 0.08	0.48 \pm 0.06	0.42 \pm 0.11
P1	—	0.00 ^a	0.00 ^a	0.33	0.015 ^a
P2	0.00 ^a	—	0.00 ^a	0.00 ^a	0.00 ^a
P3	0.00 ^a	0.00 ^a	—	0.00 ^a	0.00 ^a
P4	0.33	0.00 ^a	0.00 ^a	—	0.12
P5	0.015 ^a	0.00 ^a	0.00 ^a	0.12	—

SD: standard deviation; IV: intravenous; SC: subconjunctival; IO: intraocular; P1: comparison with control group; P2: comparison with diabetic group; P3: comparison with diabetic + exosomes (IV) group; P4: comparison with diabetic + exosomes (SC) group; P5: comparison with diabetic + exosomes (IO) group.

^a $p \leq 0.05$ is considered significant.

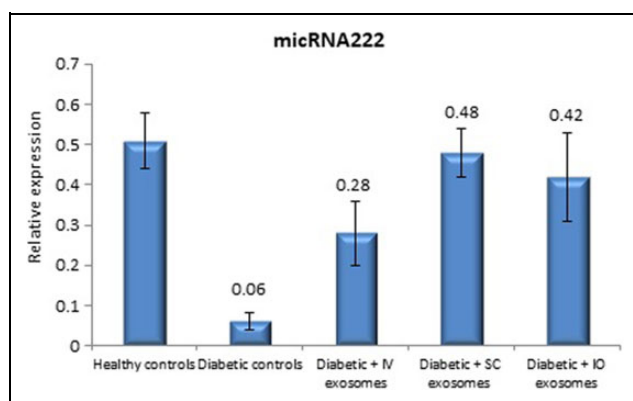


Figure 8. Relative quantitation of micRNA-222 expression levels in all studied groups. micRNA-222 showed marked decrease in diabetic group with retinal degenerative changes in comparison to other groups involved in the study.

examination. Furthermore, exosomal-mediated transfer of micRNA-222 by IV, SC, and IO routes caused increased expression level of micRNA-222 in retinal tissue of these groups and these results were associated with regenerative changes of retina we detected. Thus, we can provide evidence that micRNA-222 has a crucial role in retinal tissue regeneration which may occur via its effect in regulation of vascular cell biology.⁴⁰

In conclusion, adipose MSCs-derived exosomes act as biological mediators in retinal tissue repair through its role in delivering micRNA-222 to retinal cells. So exosomes containing micRNAs may provide a novel therapeutic procedure to prevent or delay the DR which is a major complication of DM and may lead to severe impairment of vision. However, further studies are needed to know the mechanism by which hyperglycemia caused decrease in expression level of micRNA-222 and how administration

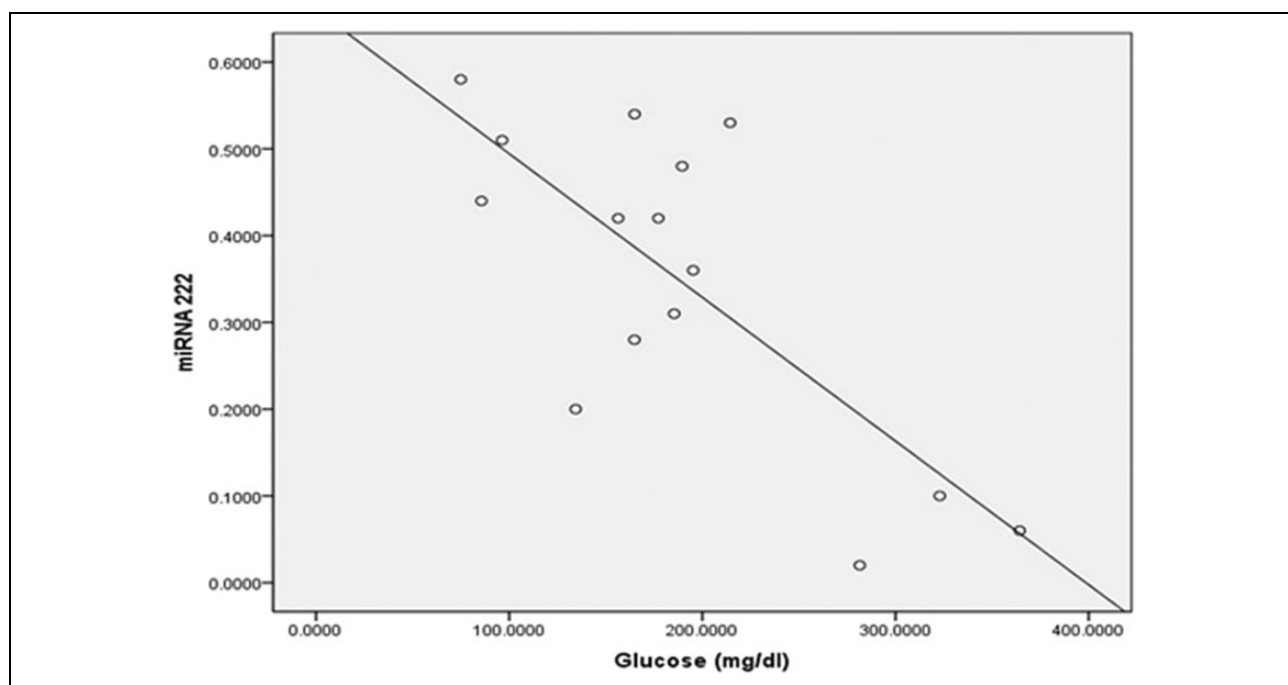


Figure 9. Correlation of plasma micRNA-222 expression level with serum glucose in all studied groups.

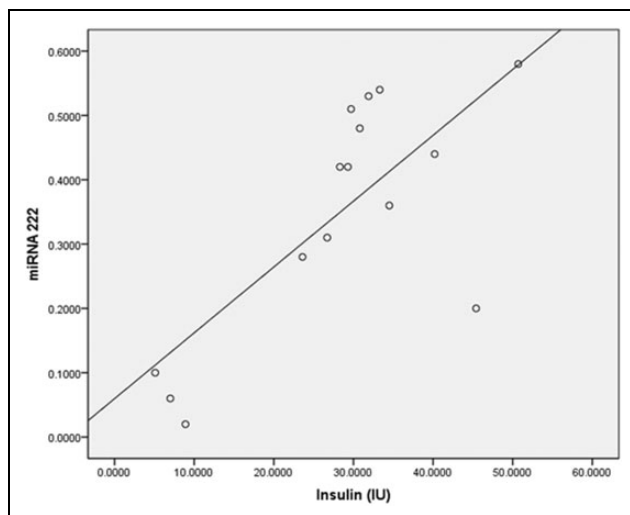


Figure 10. Correlation of serum micRNA-222 expression level with serum insulin in all studied groups.

of exosomes was associated with reduced serum glucose levels. Also, other components of EVs are needed to be explored in each of diabetic and healthy subjects to detect their mechanisms of action and to study the possibility of utilizing these components in cell-dependent therapies.

Declaration of Conflicting Interests

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