

Preventive Effects of Exosome-Rich Conditioned Medium From Amniotic Membrane-Derived Mesenchymal Stem Cells for Diabetic Retinopathy in Rats

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Purpose: Diabetic retinopathy (DR) is an important disease that causes vision loss in many diabetic patients. Stem cell therapy has been attempted for treatment of this disease; however, it has some limitations. This study aimed to evaluate the preventive efficacy of exosome-rich conditioned medium (ERCM) derived from amniotic membrane stem cells for DR in rats.

Methods: Twenty-eight 8-week-old male Sprague-Dawley rats were divided into three groups: group 1, normal control (Con) group; group 2, diabetes mellitus (DM) group; and group 3, DM with ERCM-treated (DM-ERCM) group. DM was induced by intraperitoneal injection of streptozotocin. The DM-ERCM group received ERCM containing 1.2×10^9 exosomes into subconjunctival a total of four times every 2 weeks.

Results: On electroretinogram, the DM-ERCM group had significantly higher b-wave and flicker amplitudes than those in the DM group. In funduscopy, retinal vascular attenuation was found in both the DM and DM-ERCM groups; however, was more severe in the DM group. On histology, the ganglion cell and nerve fiber layer rates of the total retinal layer significantly increased in the DM group compared with the Con group, whereas the DM-ERCM group showed no significant difference compared with the Con group. Cataracts progressed significantly more in the DM group than that in the DM-ERCM group and there was no uveitis in the DM-ERCM group.

Conclusions: Subconjunctival ERCM delayed the progression of DR and cataracts and significantly reduced the incidence of uveitis.

Translational Relevance: Our study shows the clinical potential of minimally invasive exosome-rich conditioned medium treatment to prevent diabetic retinopathy.

Introduction

Diabetic retinopathy (DR) is an irreversible microvascular complication of diabetes mellitus (DM) that causes vision loss in many diabetic patients. One-third of diabetic patients have DR, and the World Health Organization has reported that DR accounts for 15% to 17% of total blindness in Europe and the United States.^{1,2} Therefore, DR is considered an important ocular disease in human society.

DR can be classified into two stages based on the degree of progression.³ The first stage is nonproliferative DR; when it advances, the second stage is called proliferative DR. The features of the fundus in DR include microaneurysms, venous dilation and tortuosity, and intraretinal hemorrhage. DR progression is related to abnormalities of the vasculature, permeability of the blood–retina barrier, microvascular injury with vascular endothelial cell and pericyte loss, impediment of capillaries, thickening of the vascular basement

membrane, and immoderate retinal neuronal and glial abnormalities.

Because DR is an irreversible disease, preventative therapy is needed; however, disappointingly, there are few therapeutic options that have many limitations. The treatment available to date is the intravitreal injection of vascular endothelial growth factor inhibitors and steroids; however, up to 50% of patients with DR fail to respond to this treatment.³ Another treatment for patients with proliferative DR is laser photocoagulation, which is a destructive procedure with many side effects. These therapeutic options are focused on the end stage of DR and have many complications, such as endophthalmitis, retinal detachment, ocular hemorrhages, cataract, and glaucoma.⁴

Because advanced treatment and preventative therapy for DR are needed, several studies have evaluated the therapeutic efficacy of intravitreal mesenchymal stem cell (MSC) therapy for DR and have shown optimistic results.^{5–7} In a previous study, intravitreal administration of multipotent stromal cells of mesenchymal origin showed therapeutic effects, such as improved neuronal function and alleviated vascular leakage, inflammation, and apoptosis in nonproliferative DR, the early stage of DR in streptozotocin (STZ)-induced diabetic rats.⁸ In another study, mouse adipose-derived stem cells reduced retinal ganglion cell (RGC) loss and retinal oxidative damage in STZ-injected mice.⁹

However, stem cell therapy has limitations owing to the difficulty of diffusion through biological barriers and its *in vivo* survivability and functionality.¹⁰ One study reported that intravitreal injection of MSCs induced negative effects such as cataracts, loss of pericytes, increased formation of acellular capillaries, retinal vasoregression, activation of retinal glial cells, and an inflammatory response.¹¹

To overcome these limitations, exosome-derived MSCs have recently been studied for the treatment of DR.^{12,13} Global attention on exosomes, the actual mediator of the biological effects of stem cells, is increasing owing to their efficacy and safety. Exosomes have a smaller particle size than stem cells, so they can pass through the blood–retinal barrier more easily without limitations of stem cells, such as allogenic and xenogenic immune rejection, malignant transformation, vitreoretinal proliferation, or obstruction of small blood vessels.^{14,15}

Several studies have demonstrated the protective effects of the intravitreal administration of MSC-derived exosomes in retinal ischemia and injury.^{16,17} In line with these findings, intravitreal MSC-derived exosomes may be beneficial for retinal diseases.

This study aimed to evaluate the preventive efficacy of subconjunctivally injected exosome-rich conditioned medium (ERCM) from amniotic membrane-derived MSCs for DR in rat models.

Methods

Origin of ERCM

ERCM was obtained from Designed Cells Co., Ltd. (Cheongju, Korea). It was obtained from human amniotic membrane-derived MSCs cultivated under hypoxic oxygen conditions (2% O₂, 5% CO₂). ERCM was harvested according to a previous protocol.^{18,19} ERCM contained 4×10^8 exosomes/ μ L. The characteristics of ERCM are described in previously published paper and U.S. Patent No. 18/046,719, European Patent No. EP22199777.8, and Korean Patent No. 10-2022-0110631.¹⁹

Animal Model and Grouping

Twenty-eight 8-week-old male Sprague-Dawley rats were obtained from Samtako Bio Korea Corp. (Osan, Korea). Rats were housed under standard laboratory conditions in the conventional area of the Laboratory Animal Research Center of Chungbuk National University. All procedures complied with the Institutional Animal Care and Use Committee of Chungbuk National University (CBNUA-165R-21-01) and the ARVO statement for the Use of Animals in Ophthalmic and Vision Research.

The rats were divided into three groups: group 1, normal control group (Con) (9 rats, 18 eyes); group 2, DM group (9 rats, 18 eyes); and group 3, DM with ERCM-treated group (DM-ERCM) (10 rats, 20 eyes). Before STZ injection, the body weight (BW) and blood glucose (BG) levels were recorded. All BW and BG data in this study were obtained after more than 6 h of fasting. Intraperitoneal injections of STZ were administered 4 days after housing was stabilized. DM (>250 mg/dL BG levels) was induced by intraperitoneal injection of STZ (55 mg/kg) in sodium citrate buffer (0.1 M, pH 4.5), whereas the normal Con group was injected with the same volume of buffer. BG levels in all groups were recorded 12 days after STZ injection. Hyperglycemic rats were divided randomly into DM and DM-ERCM groups.

Subconjunctival ERCM Injection

Three weeks after STZ injection, the first subconjunctival injection was performed under sedation with a

mixture of 0.17 mg/kg medetomidine (Domitor, Orion Pharma, Exton, PA) and 5 mg/kg alfaxalone (Alfaxan, Jurox Pty. Ltd., Rutherford, New South Wales, Australia) via intraperitoneal injection. This anesthesia protocol was applied during electroretinography (ERG), slit-lamp biomicroscopy, and fundoscopy. The subconjunctival injection was repeated every 2 weeks for a total of four times since then. DM-ERCM group received 30 μ L (1.2×10^9 exosomes) of ERCM via subconjunctival administration, whereas the other groups received the same volume of phosphate-buffered saline. Before the subconjunctival injection, the eyes were washed with povidone-iodine 0.05% for ophthalmic disinfection, and topical anesthesia was applied with 0.5% proparacaine hydrochloride (Alcaine, Alcon, Puurs, Belgium).

ERG Evaluation

The ERG was examined 2 months after STZ injections using the RETevet device after dark adaptation in all groups. Flash ERG was presented at a frequency of 2 Hz at 8.0 cd·s/m² with a background at 30 cd·s/m², and flicker ERG was presented at a frequency of 11 Hz at 8.0 cd·s/m² with a background at 30 cd·s/m².

Slit-Lamp and Fundoscopy Examinations

Before sacrificing the rats, at 9 weeks after STZ injections, slit-lamp biomicroscopy (MW50D; Grand Seiko Co. Ltd., Hiroshima, Japan) was performed. The fundus was also examined using an indirect ophthalmoscope (Vantage Plus, Keeler Ltd., Windsor, UK) with a 28-diopter lens (Volk Optical, Mentor, Ohio). Slit-lamp biomicroscopy and fundoscopy were performed after topical application of 0.5% tropicamide and 0.5% phenylephrine hydrochloride (Mydrin-P; Santen, Osaka, Japan) to induce mydriasis. The progression of diabetic cataract was assessed, and the cataract grade was classified into one to four stages: no cataract, stage 0; incipient cataract, stage 1; early immature cataract, stage 2; late immature cataract, stage 2.5; mature cataract, stage 3; and hypermature cataract, stage 4. After euthanasia, the eyes and pancreas were harvested from the rats.

Histological Examination

All eyes were fixed in Biofix HD (BioGnost Ltd., Zagreb, Croatia), and all pancreases were fixed in 10% formalin for one day, followed by tissue processing and paraffin embedding. All tissues were analyzed histologically using hematoxylin and eosin (H&E) staining. The ratio of the entire retinal layer was compared

among groups to evaluate the degree of degeneration of the retinal structure. For terminal uridine nick-end labeling staining, In Situ Cell Death Detection Kit (Roche, Base, Switzerland) were used according to the company's protocol.

Statistical Analysis

GraphPad Prism 9 was used for data analysis. Results are presented as mean $\pm$ standard deviation. Statistical significance between groups was determined by ordinary one-way analysis of variance and unpaired *t*-test. *P* values of less than 0.05 were considered statistically significant, and *P* values of less than 0.01 were considered statistically very significant.

Results

Assessment of BW and BG Levels

All data were recorded after 6 h of fasting. The first recordings of BW and BG levels were obtained after 4 days of housing before STZ injections. In the second measurement, significant changes in BW and BG were observed in the Con and STZ injection groups (both DM and DM-ERCM groups). Two months after STZ injection, BW and BG data were compared between all groups. The average of BW in the Con, DM, and DM-ERCM groups was 471.3 ± 14.0 g, 202.3 ± 33.0 g, and 217.0 ± 45.1 g, respectively (Fig. 1A). The average of BG levels in the Con, DM, and DM-ERCM groups was 92.9 ± 17.7 mg/dL, 495.4 ± 80.9 mg/dL, and 503.9 ± 71.1 mg/dL, respectively (Fig. 1B). The average BW and BG levels in the Con and DM groups were significantly different. In the Con group, the average BW increased compared with that 2 months back, whereas there was no increase in the DM and DM-ERCM groups. There were no statistically significant differences in BW and BG between the DM and DM-ERCM groups, indicating that subconjunctival injection of ERCM did not show systemic treatment effects of BW and BG on DM.

Assessment of Attenuation of Retinal Vessels With Fundoscopy

The fundus was examined before sacrificing the rats 9 weeks after STZ injections. In the Con group, the retinal blood vessels were thick and well-stretched with no bending of the veins and microaneurysms. In the DM and DM-ERCM groups, it was difficult to assess the fundus in the case of immature to hypermature cataracts; therefore, the fundus could be assessed only

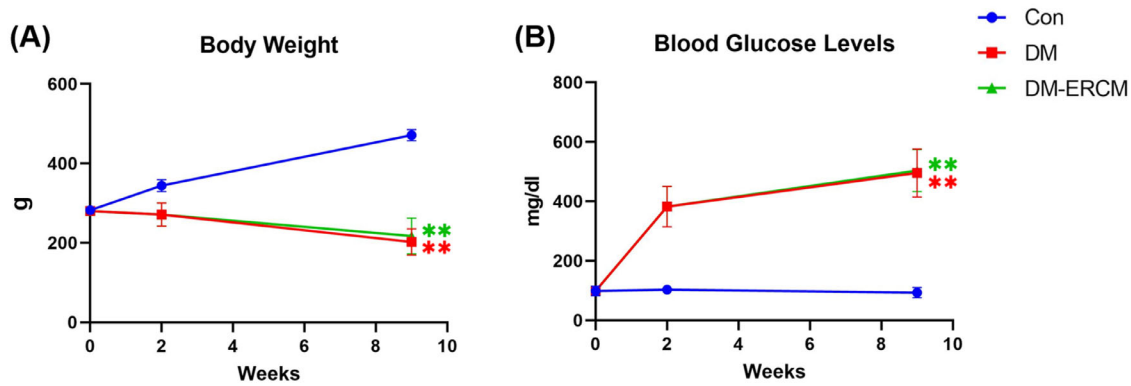


Figure 1. (A) A graph showing the change in body weight (BW) over time. (B) A graph showing the change in blood glucose (BG) levels over time. These graphs represent that the subconjunctival injection of exosome-rich conditioned medium (ERCM) does not show systemic effects because there are no significant differences in BW and BG levels between the diabetes mellitus (DM) and DM-ERCM groups. $**P < 0.01$. Control (Con), $n = 9$ rats, 18 eyes; DM, $n = 9$ rats, 18 eyes; DM-ERCM, $n = 10$ rats, 20 eyes.

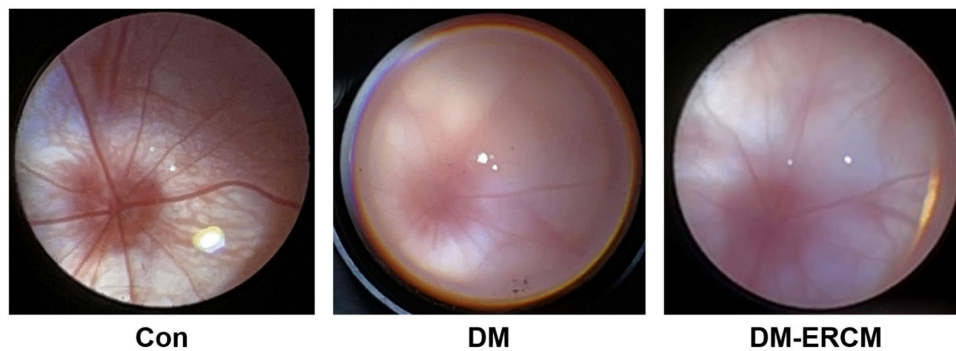


Figure 2. The fundus is examined using a 28-diopter lens, and these are representative images in each group. The retinal blood vessels are thick and well-stretched in the control (Con) group. However, there are mild attenuations of retinal arterioles and venules in the diabetes mellitus (DM) and DM with exosome-rich conditioned medium (ERCM) treated groups (DM-ERCM), and especially more advanced atrophy is observed in the DM group.

with incipient cataracts. Mild attenuation of retinal arterioles and venules was observed in the DM and DM-ERCM groups, and more advanced atrophy was observed in the DM group (Fig. 2). These findings indicate that subconjunctival ERCM injection delayed the progression of attenuation of retinal vessels. There were no retinal hemorrhages or hard exudates in any of the DM rats, indicating that all diabetic rats in our study were in an early stage of DR.

Evaluation of Retinal Function With ERG

The ERG was examined 2 months after STZ was injected to diagnose the progression of DR. The DM-ERCM group had statistically significantly higher b-wave amplitude (DM: $54.8 \pm 32.0 \mu V$ vs. DM-ERCM: $98.3 \pm 30.8 \mu V$) and flicker amplitude (DM: $54.1 \pm 32.0 \mu V$ vs. DM-ERCM: $74.9 \pm 23.8 \mu V$) than the DM group (Figs. 3A and B). These findings indicated

that the DM-ERCM group had higher retinal function than the DM group, and the progression of the disease was delayed by ERCM. There were no statistically significant differences in b-wave implicit time or flicker implicit time among the groups (Figs. 3C and D). This might be because it is an early DR stage, and the DR has not yet progressed sufficiently to affect the implicit time response.

Histological Evaluation of the Retinal Layer

The histology of the retinas of all rats was investigated by H&E staining. To reduce the error in the tissue processing process, it was evaluated using the ratio rather than the actual thickness of the retinal layer. The ganglion cell layer and nerve fiber layer were evaluated among the layers of the retina. In early retinal damage, thickening of the ganglion cell layer and nerve fiber layer and vacuolar changes in

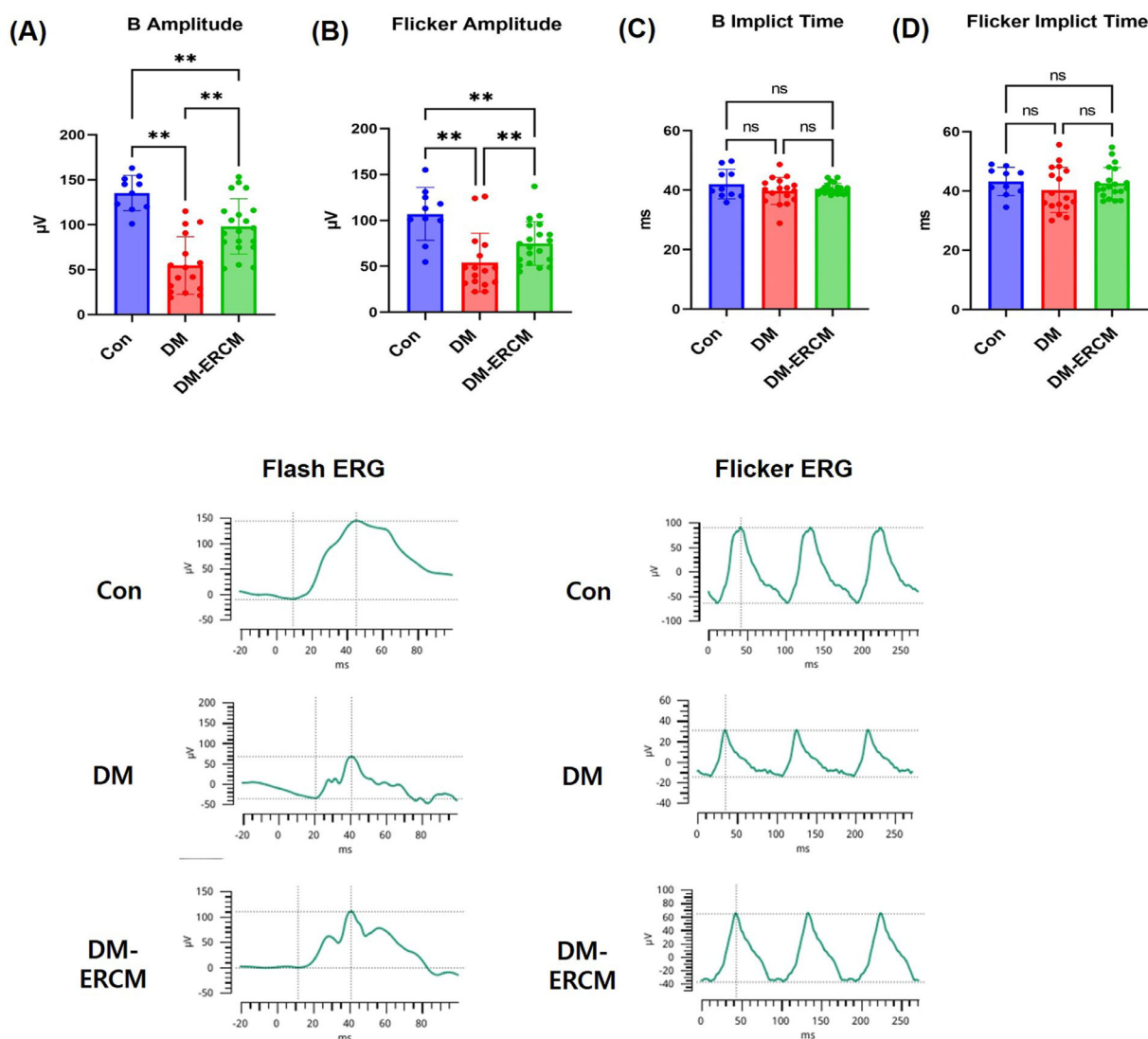


Figure 3. The diabetes mellitus (DM) with exosome-rich conditioned medium (ERCM) treated group (DM-ERCM) has statistically significantly higher b-wave and flicker amplitude than the DM group (**A, B**), whereas there are no significant differences in b-wave and flicker implicit time among the three groups (**C, D**). The ERG wave length of each group is shown at the bottom of the figure. $^{**}P < 0.01$. ns, no statistically significant difference. Control (Con), $n = 5$ rats, 10 eyes; DM, $n = 8$ rats, 16 eyes; DM-ERCM, $n = 10$ rats, 20 eyes.

ganglion cells occurred, as shown in the DM group (Fig. 4A). Therefore, the thickness of these layers indicates the extent of retinal damage. It was found that the thickness rates of the layers between the Con group ($10.4 \pm 2.3\%$) and DM-ERCM group ($13.6 \pm 3.8\%$) were not statistically significantly different, and there was a significant difference only between the Con and DM groups ($14.1 \pm 3.6\%$) (Fig. 4B). This finding indicates that retinal damage was less advanced in the DM-ERCM group than that in the DM group.

Terminal uridine nick-end labeling staining was performed. More apoptotic cells were observed in the RGC layers of the retina in DM group than in

other groups, although without statistical significance (Figs. 4C and D).

Histological Evaluation of the Pancreas

To evaluate the systemic effects of multiple subconjunctival injections of ERCM on DM, the pancreatic tissues of all the groups were assessed. In the H&E-stained pancreatic tissues, there were normal beta cells in the Con group; however, necrosis lesions of beta cells were observed in both the DM and DM-ERCM groups (Fig. 5). This finding indicates that subconjunctival injection of ERCM did not result in pancreatic tissue protect from STZ injection.

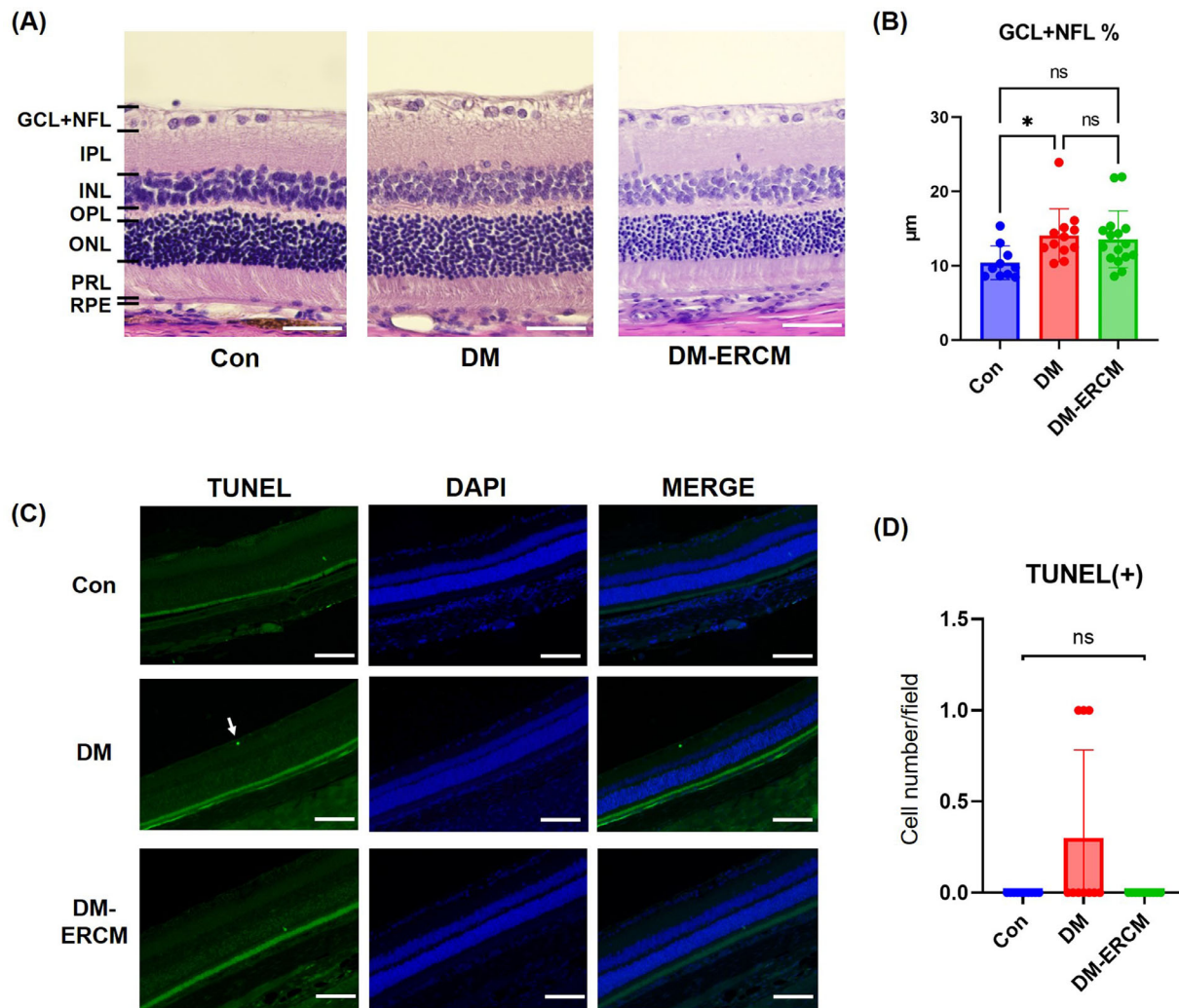


Figure 4. (A) Representative images of retinal layers in each group. The thickening of the NFL and retinal ganglion complex (RGC) in the DM group is notable. Scale bar, 50 μm (stain: hematoxylin and eosin, original magnification $\times 400$). (B) There is a significant difference only between the control and DM groups. * $P < 0.05$. ns, no statistically significant difference. Con, control; DM, diabetes mellitus; GCL, ganglion cell layer; IPL, inner plexiform layer; INL, inner nuclear layer; NFL, nerve fiber layer; OPL, outer plexiform layer; ONL, outer nuclear layer; PRL, photoreceptor layer; RPE, retinal pigment epithelium; ERCM, exosome-rich conditioned medium. Con, $n = 5$ rats, 10 eyes; DM, $n = 6$ rats, 12 eyes; DM-ERCM, 8 rats, $n = 16$ eyes. (C) Representative images of terminal uridine nick-end labeling staining in each group. Scale bar, 100 μm (original magnification $\times 200$). (D) More apoptotic cells (white arrow) were observed in the RGC layers of the retina in DM group than in other groups, although without statistical significance. $n = 10$ eyes.

Assessment of Other Ocular Complications Induced by DM

The day after the final fourth injection of ERCM, 2 months after STZ injections, the progression of diabetic cataract and uveitis was assessed using slit-lamp biomicroscopy (Fig. 6A). Diabetic cataracts were found in all rats in the DM group. One eye in the DM group had a mature stage, two eyes had a hypermature stage, and the others were in the incipient stage. Mature or hypermature cataracts were not detected in the DM-ERCM group. All the others were in the incip-

ient stage. Cataract grade was classified according to cataract stage, and the data were assessed. Cataract formation grade in the Con group was 0.0 ± 0.0 , whereas it was 1.9 ± 1.0 in the DM group and 1.2 ± 0.4 in the DM-ERCM group (Fig. 6B). The incidence of uveitis in the DM group was 12.5%, whereas in the Con and DM-ERCM groups, the incidence was 0%. There were statistically significant differences between the DM and DM-ERCM groups in cataract formation grades and uveitis incidence. The DM-ERCM group showed a significantly slower progression of diabetic cataracts. This finding indicated that ERCM was

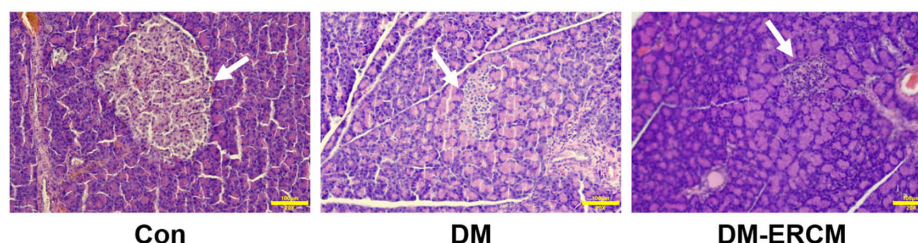


Figure 5. Representative images of pancreas histology in each group. Red arrows indicate beta cells. There are normal beta cells in the control group (Con); however, the necrosis lesions of beta cells are depicted in the diabetes mellitus (DM) groups. Scale bar, 100 μ m (stain: hematoxylin and eosin; original magnification $\times 200$).

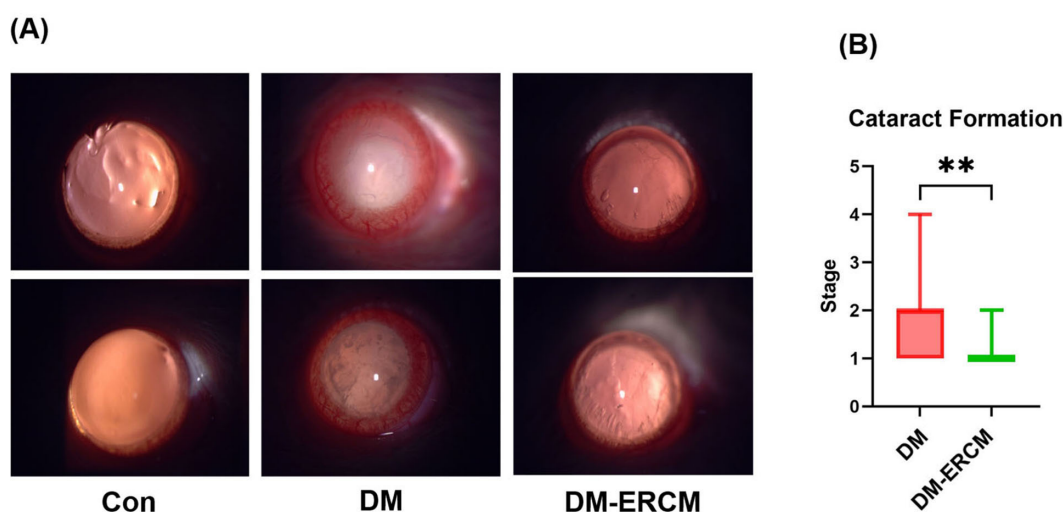


Figure 6. (A) Representation of the anterior segment of eyes in each group. There is no cataract in the control group (Con). In the diabetes mellitus (DM) group, hypermature cataract is found in two eyes, whereas in the DM with exosome-rich conditioned medium (ERCM) treated group (DM-ERCM), most of them are incipient cataracts. (B) The degree of cataracts is evaluated. There is a statistically significant difference between the DM and DM with ERCM groups (DM-ERCM) in cataract formation grades. $**P < 0.01$. DM, $n = 8$ rats, 16 eyes; DM-ERCM, $n = 10$ rats, 20 eyes.

effective in slowing the progression of cataracts and ocular inflammation.

Discussion

This study conducted repeated subconjunctival injections of ERCM in DR and evaluated the preventive effects of ERG, slit-lamp biomicroscopy, fundus examinations, and assessment of retinal tissues. Previous studies have demonstrated the therapeutic effect of retinal damage with a single intravitreal injection of exosomes.^{16,17} This study aimed to confirm the preventive effect of DR by subconjunctival injections, which is a less invasive and easier method than intravitreal injections, with multiple administrations.

Intravitreal administration has been used as a typical route to treat the retina because of the direct

action of drugs on retinal cells. However, there are risks such as cataract formation, endophthalmitis, retinal or vitreous hemorrhage, retinal tear and detachment, pain, and sudden increase in intraocular pressure.^{20–22} Huang et al.¹¹ demonstrated that intravitreal injection of MSC evokes retinal vascular regression, cataract, and inflammation in a rat model. In contrast, the subconjunctival injection is safe and a less invasive route than intravitreal, easier to perform, and required a lower depth of anesthesia.²³ Moreover, subconjunctival drug injection also show the efficacy for the ocular posterior segments.^{23,24}

One study reported that subconjunctival injections of exosomes have preventive efficacy, similar to intravitreal injections.²⁵ This study demonstrated that a single injection of rabbit adipose tissue-derived MSCs exosomes attenuated retinal degeneration and contributed to retinal repair in DR rabbits. Intravitreal and subconjunctival administration protected the

retinas of STZ-induced diabetic rabbits from retinal tissue and structural damage. A previous study administered a single injection; however, this study administered ERCM multiple times to obtain better efficacy. Thus, the subconjunctival route was chosen to evaluate the efficacy and safety of repeated ERCM injections. In addition, previous studies evaluated histology and gene expressions, but we focused more on functional aspects *in vivo*, including ERG, fundus evaluation, and clinical complications such as cataracts and uveitis. In previous studies, systemic BG levels decreased after the subconjunctival injection, possibly as an ocular improvement effect of systemic BG reduction. However, in the present study, systemic BG levels did not decrease, thereby confirming that ERCM exerted a local effect in the eye rather than a systemic effect.

In a study by Mead et al.,²⁶ bone marrow-derived MSC exosomes showed significant neuroprotective and neurogenic effects in an optic nerve injury model in rats. Mead et al. performed weekly and monthly intravitreal injections and found that weekly injections showed a better protective effect than monthly injections, and the experiment length was 21 to 56 days. Therefore, this study administered ERCM via the subconjunctival route every 2 weeks for 9 weeks to evaluate prophylactic efficacy over a longer period of time, by referring to the benefits and supplementing the limitations of the previous study.

Zhang et al.²⁷ demonstrated that MSC-derived exosomes successfully treated refractory and large macular holes in human patients. One patient ($n = 1/5$) who received a higher dose of MSC-derived exosomes presented with an inflammatory reaction. This result indicated that exosomes could also evoke inflammation with intravitreal injection. Therefore, to avoid side effects, the subconjunctival route with the injection of ERCM was chosen in this study.

Exosomes contain RNA, DNA, lipids, metabolites, and proteins.²⁸ They transport these materials and perform various functions, such as the disposal of unnecessary proteins, inflammation regulation, neuroprotection, regeneration processes, and apoptosis reduction.^{29,30} The efficacy of MSC-derived exosomes is the same as that of MSC because of the microRNAs (miRNAs) and proteins contained in exosomes.^{31,32} There is a 20S proteasome in MSC-derived exosomes, which exerts cytoprotective action by degrading intracellular proteins damaged by oxidative damage.^{31,33} The argonaut-2 protein, which is transported by exosomes, binds to miRNA and regulates biological functions. The argonaut-2 and miRNA have been shown to have neuroprotective and regenerative effects on retinal RGCs.²⁶ With these mechanisms, ERCM has

shown effectiveness in DR, cataracts, and uveitis in this study.

Our experiment focused on the clinical effects of ERCM delaying DR. Based on previous reports, the mechanism for these effects is thought to be the following. First, the platelet-derived growth factor, which was abundant in our ERCM, plays a role in neuroprotection and increases the viability and function of RGCs.^{19,34,35} This neuroprotective role of ERCM helps to maintain retinal function in the early stages of DR. Although not with statistical significance, more apoptotic RGCs were found in the diabetic group than in the Con and ERCM groups in our study. The anti-inflammatory effect of exosomes may have prevented cataracts and uveitis.^{36,37} Second, paracrine factors secreted from MSCs increase the function and proliferation of ocular progenitor cells.³⁸ Moreover, the conditioned medium exerts anti-inflammatory and regenerative effects, including biologically active secretomes.^{39–41} Therefore, the progression of DR was slower than that of the Con group, and the occurrence of complications was decreased.

We evaluated the effects of the ERCM rather than the effect of the exosome itself. In addition to exosomes, conditioned media contain biologically active soluble and insoluble secretomes, including growth factors, cytokines, and chemokines.^{39–42} Conditioned media contain many soluble factors and small extracellular vesicles of multiple particle sizes. Therefore, anti-inflammatory and regenerative actions have been reported.

This study has several limitations. First, the ERCM used in this study was not a purified exosome. Because it is a medium that cultivates amniotic membrane stem cells under hypoxic oxygen conditions, other factors may be involved that could affect the results. Second, in further studies, a comparison between multiple intravitreal and subconjunctival injections would be helpful. A preliminary study conducted with intravitreal injection of MSC showed that the progression of cataract and the incidence of inflammation increased.¹¹ Moreover, in the case of a diabetic cataract, because it would be an intumescent cataract, the probability of lens damage is increased during the intravitreal injection. Therefore, subconjunctival injections are expected to produce fewer side effects than intravitreal injections do. Third, the comparison of efficacy between stem cell and exosome therapy under various amount and between a single injection and multiple injections would also be needed in further studies.

In conclusion, this study found that subconjunctival injection of ERCM could delay the development of DR in rodents. In addition, other beneficial effects on the eyes were found, including delayed diabetic cataract

progression and inhibition of uveitis. Therefore, multiple subconjunctival ERCM injections are a minimally invasive and promising for preventative therapy for early DR.

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