

Exosomes Derived From Mesenchymal Stem Cells Modulate miR-126 to Ameliorate Hyperglycemia-Induced Retinal Inflammation Via Targeting HMGB1

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PURPOSE. In this study, we aim to investigate whether mesenchymal stem cell (MSC)-derived exosomes (MSC-Exos) could regulate hyperglycemia-induced retinal inflammation by transferring microRNA-126 (miR-126).

METHODS. MSC-Exos were isolated from the media of human umbilical cord-derived mesenchymal stem cells (hUCMSCs), and this isolation was followed by the transfer of miR-126. MSC-Exos or MSC-Exos overexpressing miR-126 were intravitreally injected into diabetic rats in vivo and were cocultured with high glucose-affected human retinal endothelial cells (HRECs) in vitro. Plasma samples were obtained from the vitreous of rats and from HREC cells after treatment for ELISA assay. Retinal sections were examined using immunohistochemistry. RT-PCR and Western blotting were conducted to assess the levels of high-mobility group box 1 (HMGB1), NLRP3 inflammasome, and NF-κB/P65 in retinas and HRECs.

RESULTS. Our results showed that hyperglycemia greatly increased inflammation in diabetic rats or HRECs exposed to high glucose, increasing the levels of caspase-1, interleukin-1β (IL-1β) and IL-18. The administration of MSC-Exos could effectively reverse this reaction. Compared to control MSC-Exos, MSC-Exos overexpressing miR-126 more successfully suppressed the HMGB1 signaling pathway and suppressed inflammation in diabetic rats. The administration of miR-126-expressing MSC-Exos significantly reduced high glucose-induced HMGB1 expression and the activity of the NLRP3 inflammasome in HRECs.

CONCLUSIONS. miR-126 expression in MSC-Exos reduces hyperglycemia-induced retinal inflammation by downregulating the HMGB1 signaling pathway.

Keywords: mesenchymal stem cell, exosome, miR-126, HMGB1c NLRP3 inflammasome

Diabetic retinopathy (DR) is one of the main causes of vision loss in the elderly population. Increased inflammatory cytokines in the retina are closely related to retinal pathologies in DR. High glucose levels can affect the retinal neuro-vascular unit, leading to gradual neuro-degeneration,¹ neuro-inflammation,² gliosis,³ vascular abnormalities involving vascular permeability,⁴ blood-retinal barrier (BRB) breakdown,⁵ inflammation,⁶ angiogenesis,⁷ and ultimately fibrosis.⁸ Vascular endothelial growth factor (VEGF) has been shown to stimulate the proliferation of retinal endothelial cells and lead to vascular leakage. Targeting VEGF has been shown to be very effective in the treatment of proliferative DR but can also damage vascular and neuronal survival and function.⁹ Thus, it is very important to develop new therapeutic treatments for DR.

There has been great interest in using MSC transplantation as a possible therapeutic strategy for the treatment of DR.¹⁰ The effects of bone marrow-derived MSC transplantation are partly due to the ability of these cells to reduce BRB breakdown. Recently, increasing evidence has shown that extracellular vesicles released by MSCs (termed “exosomes”) provide therapeutic benefits.¹¹ Exosomes represent a class of extracellular vesicles approximately 100 nm in size (diameter of 30–120 nm)^{12,13} and are released from many different types of cells.

Exosomes have been reported as the principal therapeutic agent that mediates the paracrine action of MSCs and supports the therapeutic abilities of MSCs to inhibit cell apoptosis, reduce damage, and promote receptor cell repair.¹⁴ Therefore, MSC-derived exosomes (MSC-Exos) may be involved in the regulation of inflammation in DR.

MSC-Exos carry a complex cargo of protein, nucleic acid and lipids, contain abundant miRNAs,¹⁵ and transmit these contents into the recipient cells. Accumulating evidence suggests that a miRNA transferred by exosomes could mediate inflammation through the regulation of target proteins in an inflammatory signaling pathway. MiR-126 is considered an endothelial cell-restricted miRNA that mediates inflammation and vascular development and has been reported as an important therapeutic treatment for various diseases involving aberrant inflammation and vascular leakage.¹⁶ A report from our laboratory has demonstrated that the miR-126 pathway may contribute to ameliorating DR through its ability to enhance vascular repair and reduce inflammatory effects.¹⁷ Previous research also showed that miR-126 plays a key role in modulating the expression of inflammatory factors after hyperglycemia-induced retinal inflammation.¹⁸



It was reported that DR can be improved by intravitreal injection of MSCs and that higher concentrations of MSCs confer a better protective effect in DR.¹⁹ We also found that hUCMSCs and neural stem cells originating from hUCMSCs conferred the protective effect by increasing the survival of RGCs and significantly reducing the progression of DR.²⁰ However, the mechanism underlying the therapeutic effects of MSCs in DR inflammation is still unknown. Therefore, we hypothesized that miR-126 transferred by MSC-Exos suppresses the hyperglycemia-induced inflammatory response via a specific signal pathway. In this study, we found that HMGB1 was a target of miR-126, and miR-126 could ameliorate hyperglycemia-induced retinal endothelial dysfunction and inflammation by modulating the expression of HMGB1.

MATERIALS AND METHODS

Isolation and Characterization of Exosomes

The current study was approved by the Tianjin Medical University Medical Ethics Committee and done in accordance with the Declaration of Helsinki; current revisions and experiments were carried out in accordance with the approved guidelines. The hUCMSCs (Tianjin Saier Biological Company, TEDA, Tianjin, China) were incubated in six-well plates and cultured for 48 hours. The cells were cultured in Dulbecco's modified Eagle's medium (DMEM)/F12 (Sigma-Aldrich Corp., St. Louis, MO, USA) containing 10% fetal bovine serum, 100 U/ml penicillin, and 100 µg/ml streptomycin at 37°C in humidified atmosphere with 5% CO₂. Then, the cells were seeded with antibodies against FITC-CD105, FITC-CD73, FITC-CD29, FITC-CD90, FITC-CD31, FITC-CD44, FITC-CD34, FITC-CD45, and PE-HLA-DR for 30 minutes at 37°C. After digestion with trypsin and centrifugation, the cells were collected and washed twice with PBS. The cells were preblocked with 5% bovine serum albumin for 30 minutes and incubated overnight with antibody at 4°C. Fluorescence was examined by flow cytometry (BD FACSAris; Beckman-Dickinson, San Jose, CA, USA). The dermal fibroblasts (Tianjin Saier Biological) were incubated in DMEM (Beyotime, Nantong, China) containing 100 U/ml penicillin, 10% fetal bovine serum, and 100 µg/ml streptomycin under conditions of 5% CO₂ and 37°C.

Exosome isolation was performed according to a previously published protocol.²¹ Briefly, hUCMSCs or dermal fibroblasts (passage 3–5) were cultured in FBS-free medium; the supernatant was then centrifuged (CP100WX/CR22N, HITA-CHD) at 4°C sequentially for 10 minutes at 300g, 20 minutes at 2000g, and 30 minutes at 10,000g. After each centrifugation step, the pellet was removed and discarded. Exosomes were then precipitated by ultracentrifugation of the supernatant (70 minutes at 100,000g). The pellet was resuspended in PBS after washing twice. The exosome preparation was passed through a 0.22 µm filter and stored at –20°C until use. The size distribution of exosomes was measured using a nanoparticle tracking and NanoSight analysis system (NS300; Malvern Instruments, Malvern, UK). The morphology of the exosomes was characterized by transmission electron microscopy. The surface markers of the exosomes were examined by Western blotting analysis. Total protein from exosomes was extracted in lysis buffer, and protein concentrations were measured with a protein BCA assay kit. Samples were boiled at 95°C for 5 minutes and loaded onto a sodium dodecyl sulfate polyacrylamide gel for electrophoresis, then transferred to a PVDF membrane. The primary antibodies included antibodies to CD63 (Invitrogen, Carlsbad, CA, USA), CD9 (Invitrogen), CD81 (Invitrogen), and GADPH (Invitrogen). The membranes were

blocked with 5% nonfat dried milk and incubated in the primary antibodies overnight at 4°C. The membranes were incubated with secondary antibodies for 2 hours.

miR-126 Pretreatment of MSC-Exosomes

The hUCMSCs were seeded in six-well plates and then treated with siPORT NeoFX (Thermo Fisher Scientific, Carlsbad, CA, USA) containing 100 nM negative control or miR-126 mimics (Sigma-Aldrich Corp.) for 24 hours. We then replaced the medium, cultured the cells for another 24 hours, and then collected the supernatants for further exosome experiments.

Induction of Diabetes and Treatment With Exosomes

Diabetes was induced by the injection of streptozotocin (STZ, 45 mg/kg; Sigma-Aldrich Corp.) into the tail vein of Wistar rats. One week after STZ injection, the rats showing hyperglycemia (blood glucose greater than 16.7 mmol/L) were recognized as diabetic and subjected to the outlined experiments. For treatment, 100 diabetic rats were randomly divided into different treatment groups that were treated via intravitreal injection in the right eye: The PBS treatment group was injected with 5 µl PBS; the hUCMSC-exosome (MSC-Exo) group was injected with 50 µg MSC-Exos (RNA concentration) suspended in 5 µl PBS; the fibroblast exosome (Fib-Exo) group was injected with 50 µg Fib-Exos suspended in 5 µl PBS; the miR-126-pretreated hUCMSC-exosome (miR-126-Exo) group was injected with 50 µg MSC-Exos transfected with miR-126 suspended in 5 µl PBS; and the negative control pretreated hUCMSC-exosomes (miR-NC-Exo) group was injected with 50 µg MSC-Exos transfected with miR-NC suspended in 5 µl PBS. Each group contained 20 rats, and the left eyes of the rats were regarded as normal controls.

Cell Culture and Treatment With Exosomes

Human retinal endothelial cells (HREC) were purchased from Tianjin Saier Biological Company. All cells were seeded in endothelial cell medium (Sigma-Aldrich Corp.). Upon reaching 60% to 70% confluence, HRECs were treated with 500 µg MSC-Exos (RNA concentration) suspended in 500 µl PBS, 500 µg hUCMSC-miR-126-derived exosomes suspended in 500 µl PBS, or 500 µg hUCMSC-miR-NC-derived exosomes suspended in 500 µl PBS. After treatment for 6 hours, the cells were treated with high glucose (25 nM) for 24 hours, then cells and supernatants were collected for the next experiment.

Analysis of Inflammation Factors From Rats and HRECs

Animal samples were obtained from the vitreous of rats at the third month after treatment. Media samples were collected from HREC cells at 24 hours after treatment. The levels of caspase-1 (Invitrogen, Carlsbad, CA, USA), interleukin-1β (IL-1β) (Invitrogen), and IL-18 (Invitrogen) were detected by a multidetection microplate reader using a double-antibody sandwich ELISA kit (Becton Dickinson, Bedford, MA, USA) according to the manufacturer's protocols.

Histologic and Immunohistochemical Measurements

The eyes of rats were removed at the third month after administration and fixed in 4% paraformaldehyde. The eyes were then placed into PBS at pH 7.4 for 2 hours before being dehydrated in a graded alcohol system and embedded in

paraffin wax. The thickness of the paraffin slices was 5 μm . The sections were stained with hematoxylin and eosin (HE). Immunohistochemical analysis was used to prepare eye slices from paraffin embedded tissues and incubate the slices with antibodies against ICAM-1 (1:400; Sigma-Aldrich Corp.), VCAM-1 (1:500; Sigma-Aldrich Corp.), and HMGB1 (1:500; Sigma-Aldrich). Then, the eye sections were stained with biotinylated anti-mouse and anti-rabbit IgG secondary antibodies (1:200; Sigma-Aldrich Corp.) for 2 hours and then incubated with horseradish peroxidase conjugated streptavidin for 1 hour. Images were taken with a Leica DMI4000B (Olympus Soft Imaging Solutions GmbH, Hamburg, Germany).

Western Blotting Analysis

Western blotting was performed as described previously.²² In short, total protein was extracted from HREC cells and retinal samples. Protein was quantified by a commercial bicinchoninic acid (BCA) kit. Equal amounts of protein were separated by SDS-PAGE. Antibodies specific to HMGB1 (1:1000; Thermo Fisher Scientific), NLRP3 (1:1000; Thermo Fisher Scientific), NF- κB /P65 (1:1000; Thermo Fisher Scientific), VCAM-1 (1:1000; Thermo Fisher Scientific), ICAM-1 (1:1000; Thermo Fisher Scientific) and β -actin (1:1000; Thermo Fisher Scientific) were purchased from Abcam (Cambridge, MA, USA). Target proteins were detected using an enhanced chemiluminescence kit (Amersham Pharmacia Biotech, Uppsala, Sweden).

Quantitative Real-Time Reverse Transcription PCR Assays (qRT-PCR)

Total RNA of HREC cells and retinal samples was isolated using Trizol reagent and was reverse transcribed into cDNA with a TransScript First-Strand cDNA Synthesis SuperMix (Sigma-Aldrich). The primer sequences were as follows: β -actin were Fwd 5'-AGCCATGTACGTAGCCATCC-3'; Rev 5'-ACCTCATA GATGGGCACAG-3'; HMGB1: Fwd 5'-ATCCAATGCACCCAA GAGG-3'; Rev 5'-CAATGGACAGGCCAGGATGT-3'; NLRP3: Fwd 5'-GACCATCGGCCCGACTAAAA-3'; Rev 5'-CTTGCACACT GGTGGGTTTG-3'. The relative amounts of HMGB1 and NLRP3 mRNA were detected by qRT-PCR with TransScript Top Green qPCR SuperMix (Sigma-Aldrich Corp.). For miRNA-126 detection, miRNA fractions were separated from retina samples and HREC cells using a small RNA separation kit (TransGen Biotech, Beijing, China); U6-rRNA was selected as the internal control. MiR-126 levels were quantified based on the ratio of microRNA/U6-rRNA using a formula. The PCR used the TaqMan PCR Reagent (TransGen Biotech). All procedures were performed according to the manufacturer's instructions. The relative mRNA expression was displayed using $2^{-\Delta\Delta\text{Ct}}$ method.

Measurement of Blood-Retinal Barrier (BRB) Breakdown Using Evans Blue (EB) Dye

Rats were deeply anesthetized and EB dye (30 mg/mL in saline; Sigma-Aldrich Corp.) was injected through the tail vein over 10 seconds at a dosage of 45 mg/kg. Retinal vascular permeability was quantified as described previously.²³ BRB breakdown was calculated according to the procedure of Qaum et al., and values were expressed as EB (ng) \times retinal weight (mg^{-1}).

Statistical Analysis

All data were analyzed using SPSS 16.0 (SPSS, Inc., Chicago, IL, USA).

Statistical analyses were performed using the Student's *t*-test for comparisons involving two sets of data and a 1-way analysis of variance (ANOVA) or 2-way ANOVA for comparisons involving three means. For each analysis, values of $P < 0.05$ were considered significant.

RESULTS

Isolation and Characterization of Exosomes

hUCMSCs were positive for the surface marker expression of CD73, CD90, and CD105 but weakly expressed CD34, CD45, CD11b, CD19, and HLA-D (Fig. 1A), as previously described.^{20,24} Electron microscopy examination showed that the vesicles derived from hUCMSCs were cup-shaped (Fig. 1B), with a peak size of 129 nm as shown by NanoSight analysis system (Fig. 1C). Western blot analysis demonstrated that the vesicles from both MSCs and human dermal fibroblasts expressed markers characteristic of exosomes, CD9, CD63, and CD81 (Fig. 1D).

MSC-Exosomes Alleviated the Inflammatory Response of Diabetic Rats

We established the rat model of STZ-induced diabetes to observe the effects on the inflammatory response and tight junction organization. Blood glucose levels were greatly increased in the diabetic group and the Exo-treated diabetic group compared with normal controls in our study ($P < 0.05$; Fig. 2A). Moreover, there were no significant differences in blood glucose level between the diabetic group and the Exo-treated diabetic group ($P > 0.05$), suggesting that Exo treatment did not affect the blood glucose level.

We examined the expression of IL-1 β , IL-18, and caspase-1 (three important markers of NLRP3 inflammasome activation) in the vitreous of rats using an ELISA and found that IL-1 β (Fig. 2B), IL-18 (Fig. 2C), and caspase-1 (Fig. 2D) levels were remarkably increased in the diabetic group compared to the control group. Treatment with MSC-Exos successfully reduced the levels of these markers. However, treatment with Fib-Exos did not reduce these levels. Immunohistochemical analysis and Western blots analysis revealed that the protein expression of ICAM-1, VCAM-1, and HMGB1 was much higher in diabetic rats than in normal controls, and these markers were mainly distributed in the ganglion cell layer and nerve fiber layer (Fig. 2E, 3A). Administration of MSC-Exos but not Fib-Exos significantly reduced the protein expression of ICAM-1 and VCAM-1 compared to diabetic rats. In general, MSC-Exo administration significantly suppressed inflammation and adhesion molecule expression after diabetic induction.

MSC-Exo Treatment Inhibited HMGB1 Signaling Pathway Activation in Diabetic Rats

The HMGB1 signaling pathway can regulate the inflammatory response. To investigate the role of MSC-Exos in alleviating inflammation, we detected the expression of HMGB1 and its downstream target proteins, the NLRP3 inflammasome, and NF- κB /P65 in diabetic rats after MSC-Exo administration. As shown in Figure 3A, HMGB1, the NLRP3 inflammasome, NF- κB /P65, ICAM-1, and VCAM-1 were significantly increased in the retinas of diabetic rats in comparison with those of normal controls ($P < 0.05$). This increase was reversed by MSC-Exo treatment ($P < 0.05$; Figs. 3B-F), but it could not be reversed by Fib-Exo treatment. We also examined HMGB1 mRNA levels in diabetic rats after different treatments by RT-PCR; this showed that HMGB1 mRNA levels (Fig. 3G) increased after

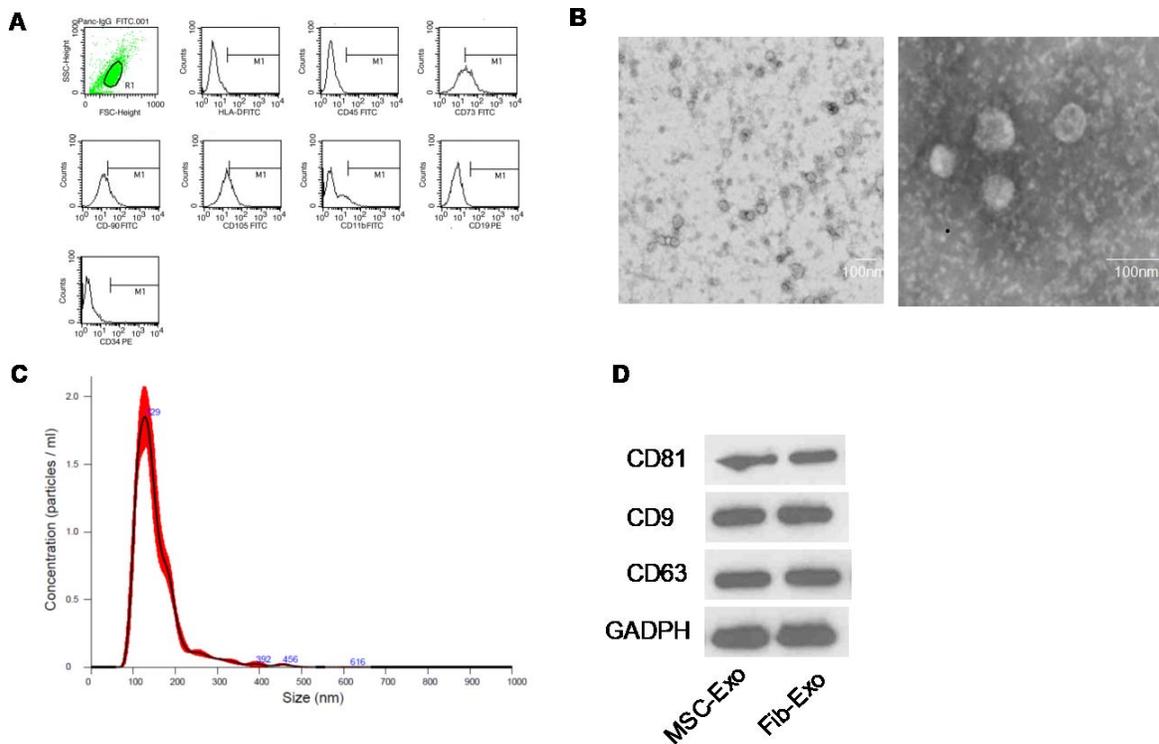


FIGURE 1. Identification of MSC-Exos. (A) Immunophenotypes of the cultured hMSCs were examined by flow cytometry. The vast majority of cells (>90%) were positive for CD29 and CD90, but a few cells (<0.5%) expressed CD34 and CD45. (B) Identification of the main morphological characteristics of hUCMSC-exosomes by transmission electron microscopy. Scale bar: 100 nm. (C) Analysis of the size distribution of exosomes with a NanoSight analysis system. (D) Western blot analysis demonstrated that the vesicles from both MSCs and human dermal fibroblasts expressed characteristic markers of exosomes: CD9, CD63, and CD81.

diabetes induction compared to normal controls. However, MSC-Exo or Fib-Exo administration did not affect HMGB1 mRNA expression ($P = 0.476$). These results indicate that MSC-Exos regulated HMGB1 protein levels but not mRNA levels.

MiR-126 is thought to be expressed in endothelial cells, which are closely related to the regulation of inflammation. Our results showed that the level of miR-126 in the retina of diabetic rats was decreased compared with levels in the control group ($P < 0.05$; Fig. 3H). However, treatment with MSC-Exos but not Fib-Exos significantly increased retinal miR-126 expression ($P < 0.05$; Fig. 3F). To further study the active component of MSC-Exos, we assessed miR-126 expression in hUCMSCs, MSC-Exos, Fibs, and Fib-Exos. Compared with expression in Fib-Exos, the expression of miR-126 in the MSC-Exos was significantly increased ($P < 0.05$; Fig. 3I). In summary, our results suggest that MSC-Exo administration remarkably enhances miR-126 expression in diabetic retinas, which may help regulate HMGB1 protein translation.

MSC-Exo Treatment Suppressed High Glucose-Induced Inflammation in Human Retinal Endothelial Cells

To identify the mechanisms of these anti-inflammatory effects, we used high glucose-stimulated HREC cells as an inflammatory cell model. The levels of HMGB1, NLRP3 inflammasome, and NF- κ B/P65 proteins were greatly increased in high glucose-stimulated HREC cells when compared with the control group (Fig. 4A), and MSC-Exo administration significantly downregulated high glucose-induced HMGB1, NLRP3 inflammasome, and NF- κ B/P65 protein expression (Figs. 4B–D). Compared to MSC-Exos, Fib-Exos had no effect on inflammatory marker expression. Consistent with the in vivo results, RT-PCR

measurement showed that HMGB1 mRNA levels were higher after high glucose stimulation when compared with the control group, however MSC-Exo administration did not affect HMGB1 mRNA expression ($P = 0.569$; Fig. 4E). Similarly, MSC-Exo but not Fib-Exo treatment greatly increased miR-126 expression in high glucose-stimulated HREC cells (Fig. 4F).

ELISAs were used to detect the inflammatory factors in the supernatant of cultured HREC cells. We found that the levels of IL-1 β (Fig. 4G), IL-18 (Fig. 4H), and caspase-1 (Fig. 4I) were much higher after high glucose stimulation when compared with levels in the control group, and MSC-Exos effectively reduced the levels of these proteins. As in vivo, treatment with Fib-Exos could not similarly reduce protein levels. Our results demonstrated that MSC-Exos suppressed inflammation in high glucose-induced HREC cells.

MiR-126 Reduced HMGB1 Expression and Inhibited the Inflammatory Response

We found that the immunomodulatory effect induced by high glucose involved miR-126. To prove the relationship between miR-126 expression and the inflammatory response, we treated diabetic rats with exosomes derived from miR-NC-transfected hUCMSCs or from miR-126-transfected hUCMSCs for 3 months. Our results showed that miR-126 levels increased upon miR-126 transfection (Fig. 5A). RT-PCR analysis showed that the mRNA levels of HMGB1 were not substantially different among the groups (Fig. 5B), while the miR-126 level was significantly higher in the diabetic rats of the miR-126-Exo group at the third month after administration (Fig. 5C). HMGB1, NLRP3 inflammasome, NF- κ B/P65, VCAM-1, and ICAM-1 protein levels were greatly decreased in the miR-126-Exo treatment group compared with the miR-NC-Exo treatment group (Figs. 5D–I).

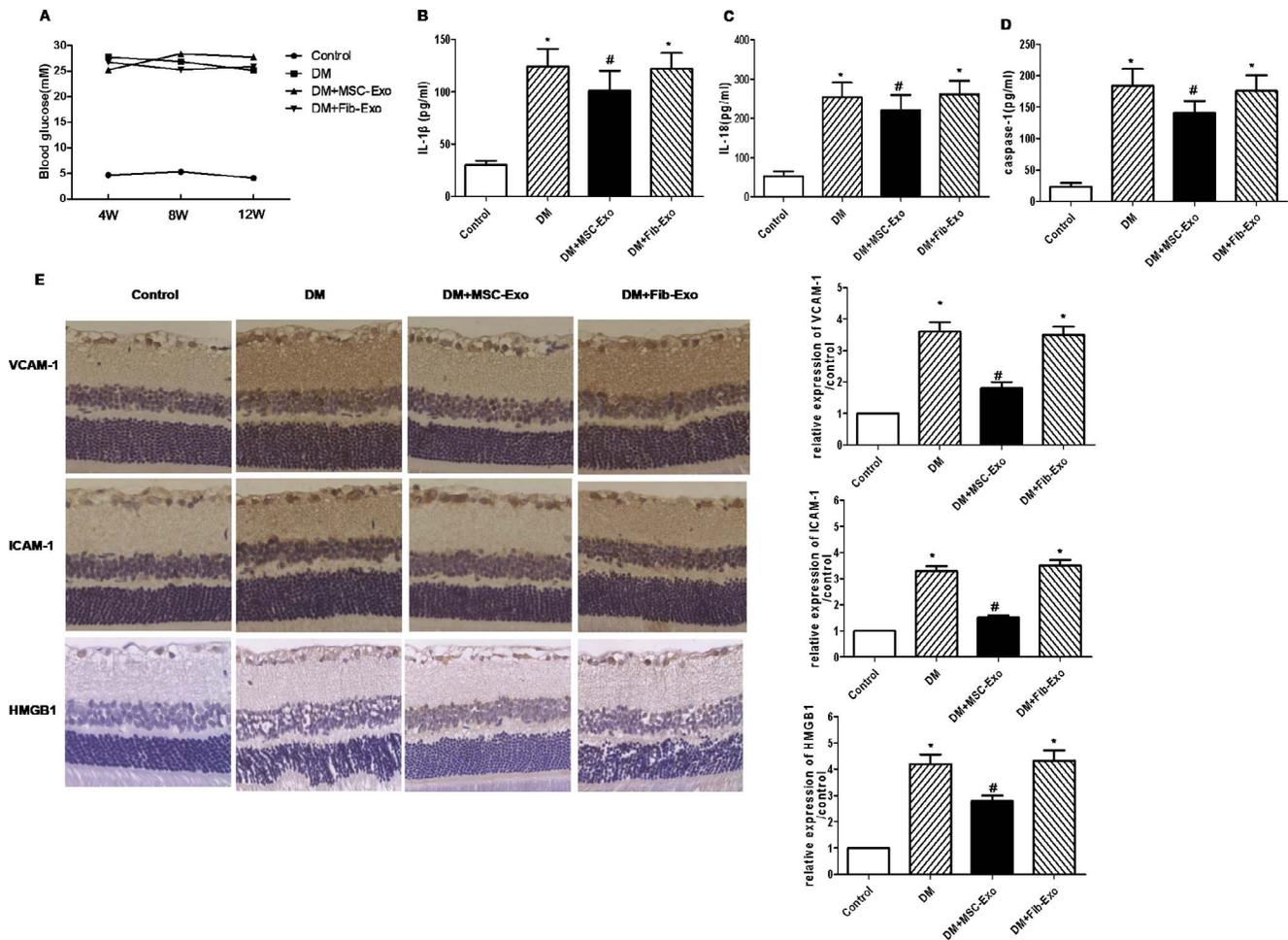


FIGURE 2. MSC-exosomes alleviated the inflammatory response of diabetic rats. (A) Blood glucose levels were significantly increased in the diabetic group and Exo-treated diabetic group compared with normal controls. Data are shown as the mean \pm SD, $n = 8$ for each treatment. (B–D) ELISA analysis of TNF- α , IL-1 β , and IL-18 levels in the vitreous of rats treated with hUCMSC-exosomes, hSFC-exosomes or PBS. Data are shown as the mean \pm SD, $n = 6$ for each treatment. (E) Representative positive ICAM-1, VCAM-1, and HMGB1 staining in the retinas from different groups were examined by immunohistochemistry. The quantitative assay was done using Image Pro Plus software. * $P < 0.05$ versus control group; # $P < 0.05$ versus DM group.

We used ELISAs to detect the inflammatory factors in the vitreous from treatment groups and demonstrated that IL-1 β (Fig. 5J), IL-18 (Fig. 5K), and caspase-1 levels (Fig. 5L) markedly decreased in the miR-126-Exo administration group compared to the miR-NC-Exo administration group. Immunohistochemical measurement found that ICAM-1, VCAM-1, and HMGB1 positive staining was much lower in the miR-126-Exo administration group than in the miR-NC-Exo administration group (Fig. 5M). Figure 5N shows that the retinal blood vessel leakage of diabetic rats was much lower in the miR-126-Exo administration group than in the miR-NC-Exo administration group. Overall, treatment with miR-126-Exos could significantly inhibit the occurrence of inflammation after diabetic induction. Our results indicate that miR-126 plays an important role in the regulation of diabetes induced inflammation through downregulating HMGB1 pathway activation.²⁵

MSC-Exo-Derived miR-126 Restricted High Glucose-Induced Inflammation by Targeting HMGB1

To investigate the mechanism by which miR-126-Exos inhibit inflammation, we treated HREC cells with exosomes derived

from miR-NC-transfected hUCMSCs or miR-126-transfected hUCMSCs. After 6 hours, we treated cells with high glucose (25 nM) for 24 hours. Consistent with our in vivo results, the protein levels of HMGB1, NLRP3 inflammasome, and NF- κ B/P65 greatly decreased in the miR-126-Exo group at 24 hours compared to the miR-NC-Exo group (Figs. 6A–D). RT-PCR measurements demonstrated that miR-126-Exo treatment did not affect the expression of HMGB1 mRNA (Fig. 6E); the level of miR-126 significantly increased in high glucose-stimulated HREC cells in the miR-126-Exo treated group (Fig. 6F). ELISA analysis showed that the HRECs secreted low levels of IL-1 β (Fig. 6G), IL-18 (Fig. 6H), and caspase-1 (Fig. 6I) after miR-126-Exo administration compared to the HRECs in the other treatment groups. Our results indicated that miR-126 suppressed high glucose-induced inflammation through downregulating the expression of HMGB1 protein.

DISCUSSION

In this study, we found that MSC-Exo administration can inhibit inflammation induced by hyperglycemia. We showed that miR-126 in the exosomes plays an important role in regulating the inflammatory response. Our results indicate that miR-126-Exos

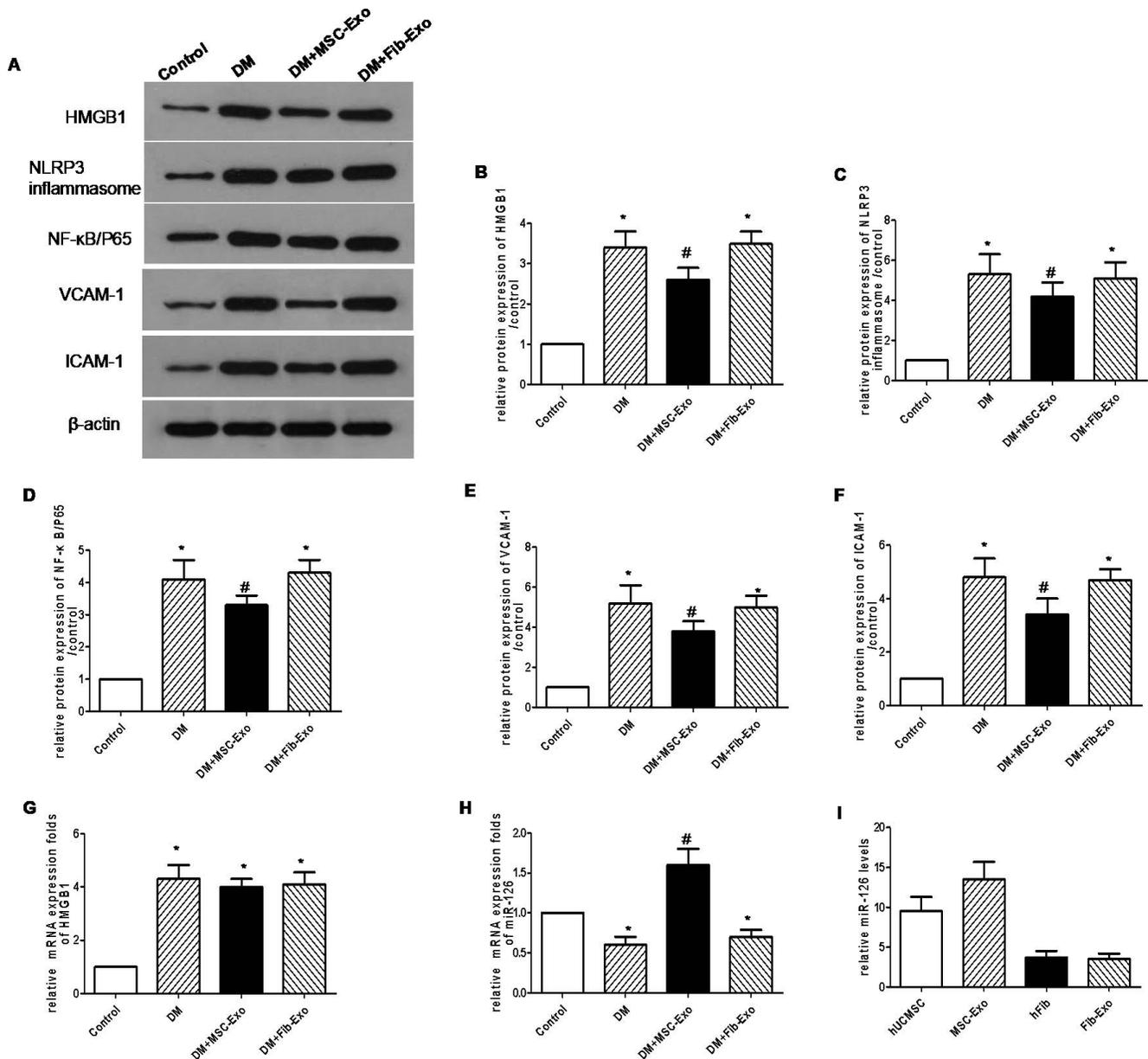


FIGURE 3. MSC-Exos inhibited HMGB1 signaling pathway activation in diabetic rats. (A–F) Western blot assay for expression of HMGB1 and its downstream target proteins the NLRP3 inflammasome and NF- κ B/P65, as well as expression of VCAM-1 and ICAM-1 in the retinas of rats from different groups. $n = 6$ for each treatment. Relative protein levels were measured by ImageJ software. (G) Quantitative analysis of relative HMGB1 mRNA expression in the retinas of rats from different groups. $n = 6$ for each treatment. (H) Quantitative analysis of relative miR-126 expression in the retinas of rats from different groups. $n = 6$ for each treatment. (I) miR-126 levels in hUCMSCs, hUCMSC-exosomes, hFib, and hFib-exosomes were detected by quantitative RT-PCR. $n = 5$ for each treatment. * $P < 0.05$ versus control group; # $P < 0.05$ versus DM group.

more effectively inhibit the HMGB1 signaling pathway and reduce inflammation in diabetic rats. Importantly, xenogeneic MSC-Exos from humans were also effective in alleviating the inflammatory reaction in rats.

Currently, MSC therapy is gradually being adapted into clinical applications for various diseases, and the mechanism of its therapeutic effect is mainly due to paracrine activity. MSCs are reported to secrete exosomes, which are enriched in the extracellular condition.²⁶ Exosomes have been recognized to be an important medium for cellular communication. They can regulate a variety of pathophysiological processes by delivering mRNAs, miRNAs, and various proteins to receptor cells.²⁷ In retinal ischemia injury, exosomes have

been found to reduce the area of retinal infarction and the inflammatory response.²⁸ In laser-induced retinal injury, exosomes could decrease the expression of MCP-1, improve cell proliferation, and inhibit retinal cell apoptosis in vivo and in vitro.²⁹ Bai et al.³⁰ found that local administration of MSC-Exos effectively inhibited experimental autoimmune uveoretinitis in rats by decreasing the migration of inflammatory cells and indicated a potential new therapy for the treatment of uveitis by MSC-Exos. In general, MSC-Exos are increasingly regarded as an attractive candidate to repair tissue damage and regulate the inflammatory response. The role of MSC-Exos in retinal inflammation induced by high glucose is still unknown.

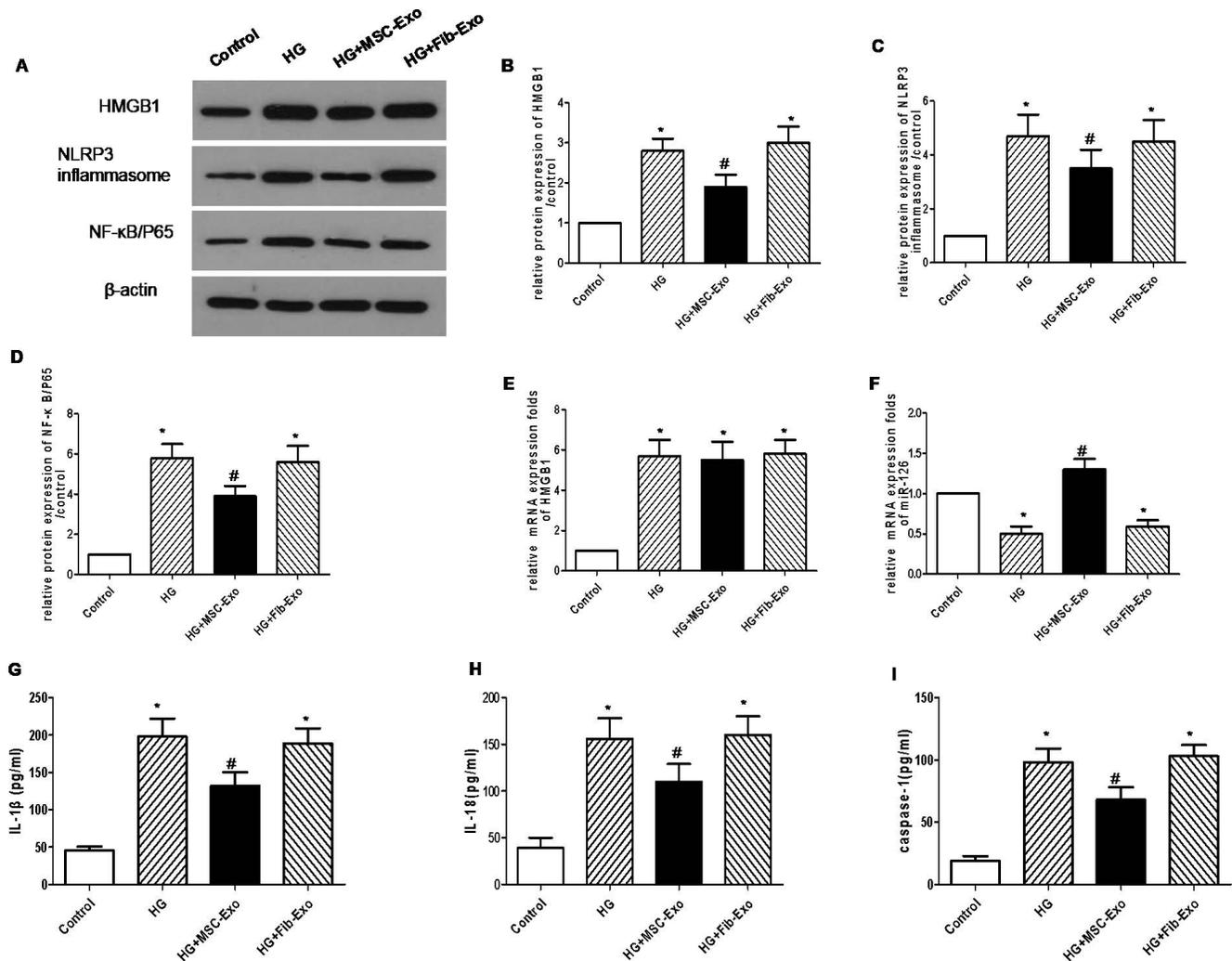


FIGURE 4. MSC-Exos suppressed high glucose-induced inflammation in human retinal endothelial cells. (A–D) Western blot assay for HMGB1 expression and expression of its downstream target proteins the NLRP3 inflammasome and NF-κB/P65 in HREC cells from different groups. $n = 6$ for each treatment. Relative protein levels were measured by ImageJ software. (E–F) Quantitative analysis of relative HMGB1 levels and miR-126 levels in the HREC cells from different groups, $n = 6$ for each treatment. (G–I) ELISA analysis for IL-1β, IL-18, and caspase-1 levels in the HREC cells from different groups. $n = 5$ for each treatment. * $P < 0.05$ versus control group; # $P < 0.05$ versus HG group.

Many proinflammatory factors are found to be increased in vitreous of patients with DR and in the retinas of diabetic rats.³¹ Consistent with previous studies, we found an increase in IL-1β, IL-18, and caspase-1 in diabetic rats. The activation of the NLRP3 inflammasome produces a caspase-1 precursor, which results in the maturation of IL-1β and IL-18. NLRP3-activated proinflammatory cytokines have been shown to play an important role in DR formation. We found that MSC-Exos downregulated the expression of HMGB1, NLRP3 inflammasome, and NF-κB/P65 proteins, which could inhibit the production of several inflammatory factors such as IL-1β, IL-18, and caspase-1. Reducing the level of miR-126 induced endothelial injury and contributed to the occurrence and development of diabetic vascular inflammation.²⁵ In agreement with these findings, we found that DR cases had greatly decreased retinal miR-126 expression when compared with controls. We confirmed that MSC-Exo administration had an important beneficial effect on severe high glucose-induced inflammation. Furthermore, we showed that MSC-Exos over-expressing miR-126 more successfully inhibited the HMGB1 signaling pathway and ameliorated the inflammatory response

in diabetic rats, suggesting that miR-126-Exos may be important regulators of the inflammatory response and vascular integrity in retinal endothelial cells.

MiR-126 has a variety of target genes, such as HMGB1.³² HMGB1 can promote the occurrence of inflammation and the pathogenesis of autoimmune diseases.³³ Since the level of HMGB1 is increased in various inflammatory and autoimmune diseases, HMGB1 could be used as a special biomarker or a target for novel therapies.³⁴ HMGB1 is generated actively in HRECs and is released passively after tissue injury or pathogenic change in the retina.³⁵ Our study showed that the expression levels of HMGB1 in both high glucose cultured HRECs in vitro and in the retina of diabetic rats were significantly increased. Moreover, levels of HMGB1 protein were significantly attenuated by miR-126-Exo treatment of HRECs subjected to high glucose. HMGB1 is reported to bind to and signal through receptors on the surface of the cell, including the NLRP3 inflammasome, TLR4, and TLR2.³⁶ The downstream effects are very complicated and include the activation of NF-κB signaling. This induces the general initiation and regulation of the inflammatory response as well

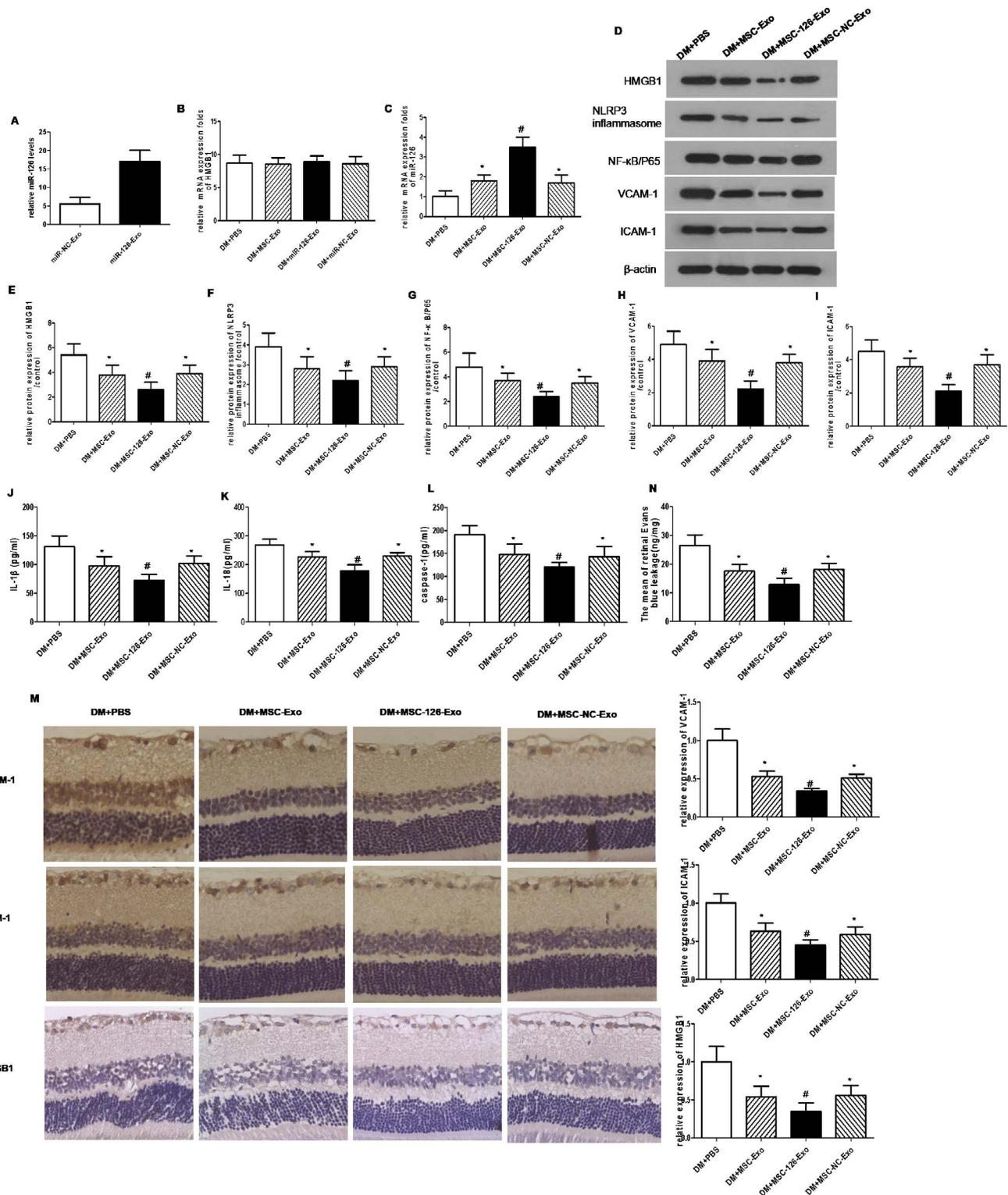


FIGURE 5. MiR-126 inhibited HMGB1 expression and suppressed the inflammatory reaction. **(A)** Quantitative analysis for relative miR-126 expression in hUCMSCs transfected with miR-126. **(B, C)** Quantitative analysis for relative HMGB1 levels and miR-126 levels in the retinas of diabetic rats after intravitreal injection of hUCMSC-exosomes, miR-126-exosomes, miR-NC-exosomes or PBS. *n* = 6 for each treatment. **(D–I)** Western blot assay for HMGB1 expression and expression of its downstream target proteins the NLRP3 inflammasome and NF-κB/P65 as well as expression of VCAM-1 and ICAM-1 in the retinas of rats from different groups. *n* = 6 for each treatment. **(J–L)** ELISA analysis for IL-1β, IL-18, and caspase-1 levels in the retinas of rats from different groups. *n* = 5 for each treatment. **(M)** Representative positive ICAM-1, VCAM-1, and HMGB1 staining in the retinas of rats from different groups were examined by immunohistochemistry. **(N)** Mean retinal EB dye leakage in the retinal blood vessels. **P* < 0.05 versus DM+PBS group; #*P* < 0.05 versus DM+MSC-Exo group.

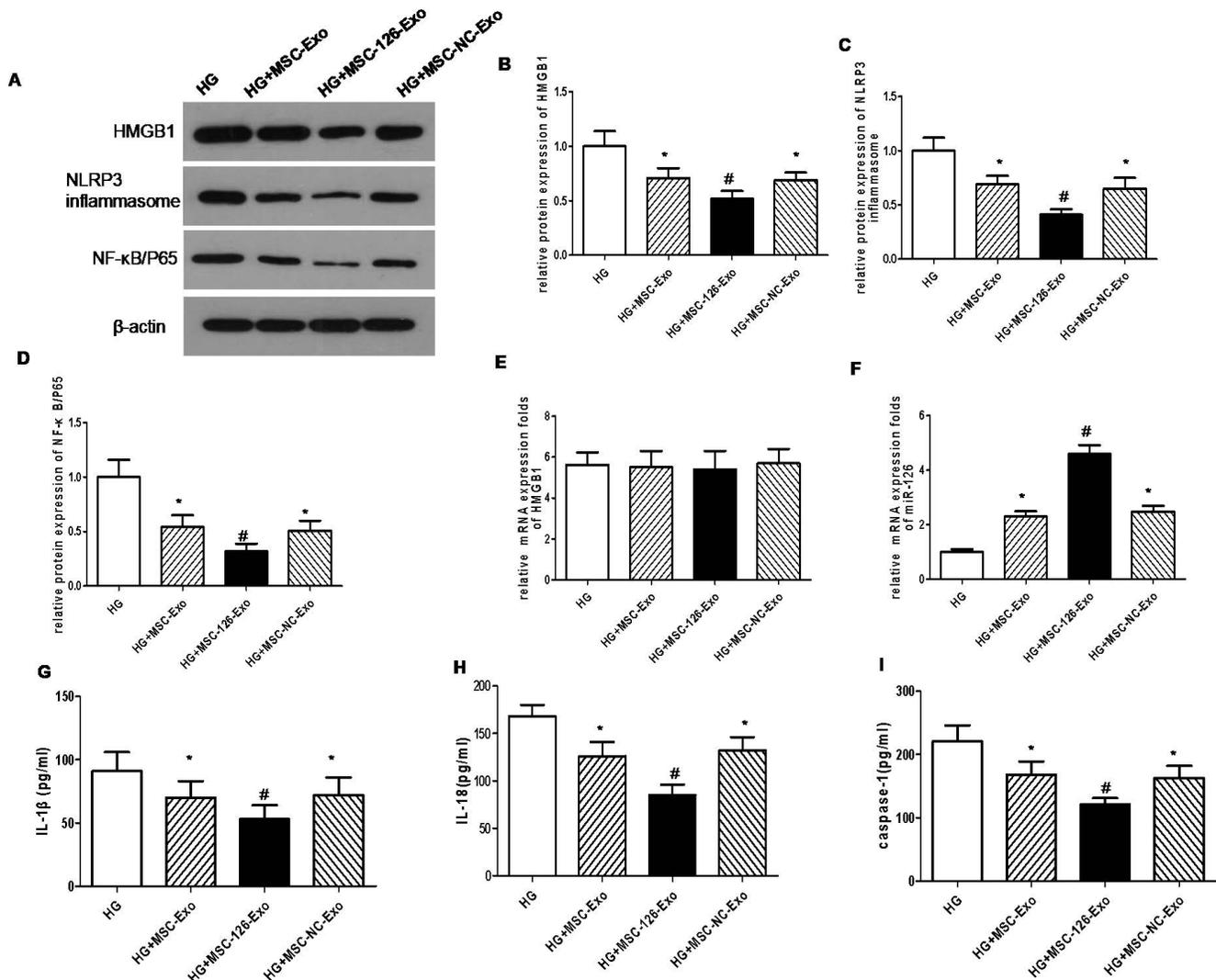


FIGURE 6. MSC-Exo-derived miR-126 restricted high glucose-induced inflammation by targeting HMGB1. (A–D) Western blot assay for HMGB1 expression and expression of its downstream target proteins the NLRP3 inflammasome and NF-κB/p65 in HREC cells from different groups. $n = 6$ for each treatment. Relative protein levels were measured by ImageJ software. (E–F) Quantitative analysis of relative HMGB1 levels and miR-126 levels in the HREC cells from different groups, $n = 6$ for each treatment. (G–I) ELISA analysis for IL-1 β , IL-18, and caspase-1 levels in HREC cells from different groups. $n = 5$ for each treatment. * $P < 0.05$ versus HG group; # $P < 0.05$ versus HG+MSC-Exo group.

as the production of proinflammatory cytokines. Our study showed that MSC-Exos reduce hyperglycemia-induced retinal inflammation by downregulating the HMGB1 signaling pathway, which may decrease NLRP3 inflammasome and p65 levels to regulate the expression of inflammatory factors.

In conclusion, we found that miR-126 contributed to the downregulation of HMGB1 and of its downstream molecules involved in inflammation. MiR-126-Exos decreased HMGB1 expression and inhibited activation of NF-κB/p65, which is a crucial mediator in modulating the expression of inflammatory factors and is likely the mechanism behind the amelioration of the high glucose-induced inflammatory response. Further studies of the mechanism of miR-126-Exos would be of great significance for the development of new prevention measures and treatment targets for DR.

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