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Lysophosphatidylcholine negatively reverses the effects of human umbilical cord-derived mesenchymal stem cells on high glucose-induced cell dysfunction

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Summary. Background. Increasing attention has been attracted to the application of human umbilical cord-derived mesenchymal stem cells (HUCMSCs) in the cell therapy of various diabetic complications, including diabetic retinopathy (DR). Lysophosphatidylcholine (LPC) has been reported to induce cell apoptosis and an inflammatory response. The present study aimed to investigate the mechanism of HUCMSCs in high glucose (HG)-treated retinal microvascular endothelial cells (RMECs) and the effect of LPC on this mechanism.

Methods. To mimic DR *in vitro*, RMECs were treated with HG. Flow cytometry analysis was used to identify HUCMSCs and the expression of their surface markers. The apoptosis of RMECs was also accessed using flow cytometry analysis. A CCK-8 assay was performed to measure the viability of RMECs. ELISA was used to detect the concentration of inflammatory cytokines (TNF-α, IL-6, and IL-1β) in RMECs. The protein expression of tight junction proteins in RMECs was examined using western blot analysis.

Results. HUCMSCs were identified to present positive markers (CD105, CD73, and CD90) and loss of negative markers (CD45, CD34, and HLA-DR). In RMECs, HG significantly induced a decrease in cell viability and an increase in cell apoptosis and tight junction proteins. Moreover, HG treatment promoted the production of inflammatory cytokines (TNF-α, IL-6, and IL-1β) and facilitated oxidative stress. However, these dysregulated cellular behaviors were alleviated by the treatment of the culture medium of HUCMSCs. Furthermore, LPC treatment reversed the effect of HUCMSCs on HG-induced RMEC injury and impaired the blood-retinal barrier. Moreover, the effect of HUCMSCs on the inflammatory response and oxidative stress of RMEC was also neutralized by LPC treatment.

Conclusion. LPC reverses the effects of HUCMSCs on HG-induced RMEC dysfunction, impaired blood-retinal barrier, inflammation, and oxidative stress.

Key words: HUCMSCs, LPC, High glucose, RMEC

Introduction

Diabetic retinopathy (DR) is one of the most common and serious complications of diabetes, leading to sight loss among the working-age population (Mohamed et al., 2007). It was estimated that approximately one-third of the 246 million diabetic patients show signs of DR, and one-third of them face the risks of severe retinopathy (Saaddine et al., 2008; Cheung et al., 2010). Apart from the risk of vision loss, DR also contributes to vascular damage and induces many systemic vascular complications with high mortality (Cheung and Wong, 2008). DR is a retinal disorder characterized by the leakage and obstruction of microvessels in the retina, resulting from diabetes mellitus (Cheung et al., 2010; Tan and Wong, 2023). The risk factors of DR include hyperglycemia, hypertension, and dyslipidemia, as revealed by numerous epidemiologic studies and clinical trials (Klein, 2007). According to the Wisconsin Epidemiologic Study of Diabetic Retinopathy, the overall 25-year incidence rate of any retinopathy is around 97%, and the rate of progression of retinopathy is 83% (Klein et al., 2008). Anti-angiogenic agents, surgical resection, laser photocoagulation, and glycemic control have been extensively employed in the management of DR (Kollias and Ulbig, 2010); however, patients still have poor outcomes (Chaudhary et al., 2021). Therefore, it is imperative to explore promising methods for the treatment of DR patients.

Human umbilical cord-derived mesenchymal stem cell (HUCMSC) refers to a type of multipotent stem cell

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present in the umbilical cord tissue of newborns, with the ability to differentiate into various types of tissue cells (Liu et al., 2016). Mesenchymal stem cells (MSCs) are indicated to inhibit immune responses. Due to these specific properties, HUCMSCs have been used in cellular therapy for diabetes mellitus and its complications. For example, HUCMSCs improve cardiac function by inhibiting myocardial fibrosis and autophagy in rat models of diabetic cardiomyopathy (Zhang et al., 2022; Liu et al., 2024b). HUCMSCs improve renal function and attenuate kidney fibrosis and the inflammatory response by restoration of autophagy in diabetic nephropathy mice (He et al., 2024). Moreover, previous studies have also revealed that HUCMSCs can be used in DR therapy. In detail, HUCMSC-derived exosomes induce the upregulation of miR-30c-5p, which suppresses oxidative stress, inflammation, and apoptosis of HG-treated RMECs by targeting MAPK/ERK axis repression in DR (Fu et al., 2022; He et al., 2022; Liu et al., 2022). It is noteworthy that stem cells have been extensively utilized in the treatment of diabetes and its associated complications, particularly DR (Kramerov and Ljubimov, 2016; Gaddam et al., 2019). However, the underlying mechanism of HUCMSC therapy of DR has not been thoroughly explored, which urges further investigation.

Lysophosphatidylcholine (LPC) is a lipid biomolecule produced by Lp-PLA 2 and plays an important role in various diseases (Law et al., 2019). LPC has been documented to facilitate the migration of lymphocytes and macrophages, promote the secretion of proinflammatory factors, and enhance cell apoptosis (Liu et al., 2020). The plasma level of LPC is increased in many diseases, such as cardiovascular diseases, renal failure, and diabetes (Itabe et al., 1994; Rabini et al., 1994; Sasagawa et al., 1998). Moreover, LPC leads to dysfunction of endothelial cells and prolonged endothelial activation (Kugiyama et al., 1990; Li et al., 2018). However, the role of LPC on HUCMSC-conditioned medium (CM)-mediated effects on RMECs remains unknown.

In this study, we proposed a hypothesis that LPC reversed the effect of HUCMSC-CM-mediated on HG-induced cell injury, blood-retinal barrier injury, inflammation, and oxidative stress in RMECs. This study may provide novel clues for DR cell therapy.

Materials and methods

Cell culture

The HUCMSCs were obtained from Guangzhou Saliai Stemcell (Guangzhou, China), seeded into 10 cm dishes, and grown to 80~90% confluence in α-MEM containing 10% fetal bovine serum (FBS; Invitrogen, Carlsbad, CA, USA) and 1% penicillin-streptomycin solution (Invitrogen). After removing the supernatant, endothelial cell medium was added to the dishes. After 48h, the replaced supernatant was harvested as the conditioned medium of HUCMSCs (HUCMSC-CM). RMECs (Ningbo Mingzhou Biotechnology, Zhejiang, China) were incubated in endothelial cell medium containing 1% penicillin/streptomycin (Invitrogen) and 10% FBS (Invitrogen) at 37°C in a humidified atmosphere with 5% CO_2. The conditioned medium of RMEC (RMEC-CM) was prepared similarly and was used as a parallel control for HUCMSC-CM.

Flow cytometry identification of HUCMSCs

Flow cytometry was used to identify HUCMSCs. A total of 5×10^8 cells per tube were collected. Hereafter, CD34, CD90, CD473, CD45, HLADR, and CD105 antibodies were incubated with cell lysates for 30 min at 4°C in the dark. The positive rate of these markers was determined by flow cytometry (BD Accuri C6, NY, USA).

Cell treatment

For glucose or mannitol treatment, different concentrations (5, 15, 25, or 35 mM) of glucose (Sigma-Aldrich, St. Louis, MO, USA) or mannitol (35 mM) were added to the endothelial cell medium. After 24h, RMECs were treated with HUCMSC-CM or RMEC-CM and LPC or normal saline for another 24h. The cell treatment timeline is shown in figure 2C.

CCK-8

RMECs were seeded into 96-well plates, and 10 μL of CCK-8 solution (Dojindo, Tokyo, Japan) was added and cultured for 4h at ambient temperature. The absorbance of RMECs was measured using a microplate reader (Reagen, Shenzhen, China) at 0, 12, 24, 36, and 48h at 450 nm.

Flow cytometry analysis

The apoptosis of RMECs was detected by flow cytometry analysis using an Annexin V-FITC Apoptosis Detection Kit (Sigma-Aldrich, St. Louis, MO, USA). RMECs, after the indicated treatment, were washed with PBS and then stained with 5 μL Annexin V-FITC and PI for 10 min at 28°C in the dark. Finally, the flow cytometer (FACSCanto II, BD Biosciences, San Jose, CA, USA) was used to access the cell apoptosis rate.

Enzyme-linked immunosorbent assay (ELISA)

The supernatant of RMECs was collected as previously reported. The concentrations of TNF-α, IL-6, and IL-1β in the supernatant of RMECs were measured with an enzyme-linked immunosorbent assay kit (ELISA) (Sigma-Aldrich). A microplate reader was used to read the optical density value at 450 nm. Furthermore, the content of glutathione (GSH) and the activities of catalase (CAT) and superoxide dismutase (SOD) in

the supernatants of cells were examined with a glutathione detection kit (A006-2, Nanjing Jiancheng Bioengineering Institute, Nanjing, China), human catalase ELISA kit (ab277396, Abcam), and human superoxide dismutase 1 ELISA kit (ab119520, Abcam) according to the manufacturer's protocols.

Western blot

RMECs were harvested and lysed in radioimmunoprecipitation assay (RIPA) lysis buffer (Beyotime, Shanghai, China). Proteins were extracted using a protein extraction kit (Thermo Fisher Scientific). The protein concentration was determined using the BCA protein assay kit (Boster Biological Technology, Wuhan, China). Proteins were subjected to 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to PVDF membranes (Pall Company, New York, USA). The membrane blocked with 5% nonfat milk powder was then incubated at 4°C overnight with the primary antibodies. Subsequently, the membranes were washed and incubated with horseradish-peroxidase-conjugated secondary antibody at room temperature for 2h. The protein bands were detected using an enhanced chemiluminescence reagent (ECL) kit (Pierce, Rockford, IL, USA). The primary antibody information is shown in Table 1.

Table 1. Primary antibodies information.

Antibody	Dilution	Number	Source
ZO-1	1: 1000	ab276131	Abcam
Occludin	1: 1000	ab216327	Abcam
Claudin-5	1: 1000	ab131259	Abcam
GAPDH	1: 1000	ab8245	Abcam

Statistical analysis

All data in this study are exhibited as the mean ± standard deviation. The statistical analyses were performed using SPSS 17.0 (IBM, Armonk, NY, USA). The two-group comparison was analyzed by Student's t-test, and one-way analysis of variance was used to evaluate differences among multiple groups. A value of p less than 0.05 was regarded as statistically significance.

Results

Identification of HUCMSCs

Flow cytometry analysis was used to identify HUCMSCs. As shown in figure 1A, HUCMSCs were positive for CD73, CD105, and CD90 but negative for CD45, CD34, and HLA-DR, which confirms their identity as HUCMSCs.

HG treatment inhibited RMEC viability

CCK-8 assay revealed that the viability of RMECs was gradually decreased by increasing doses (15, 25, or

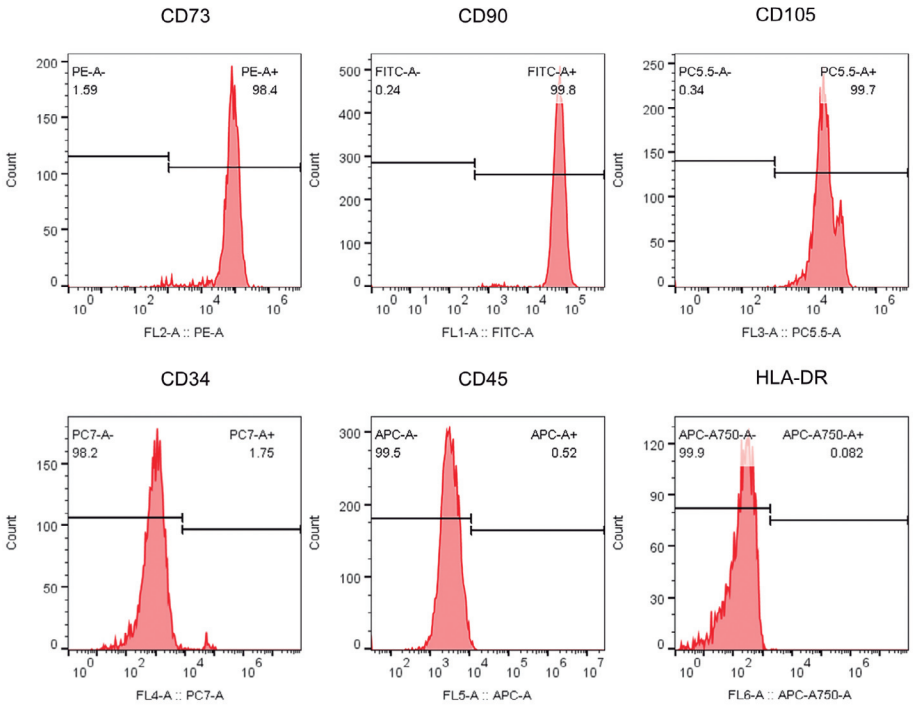

Fig. 1. Identification of HUCMSCs. Flow cytometry analysis was used to detect the expression of CD73, CD105, CD90, CD45, CD34, and HLA-DR in HUCMSCs.

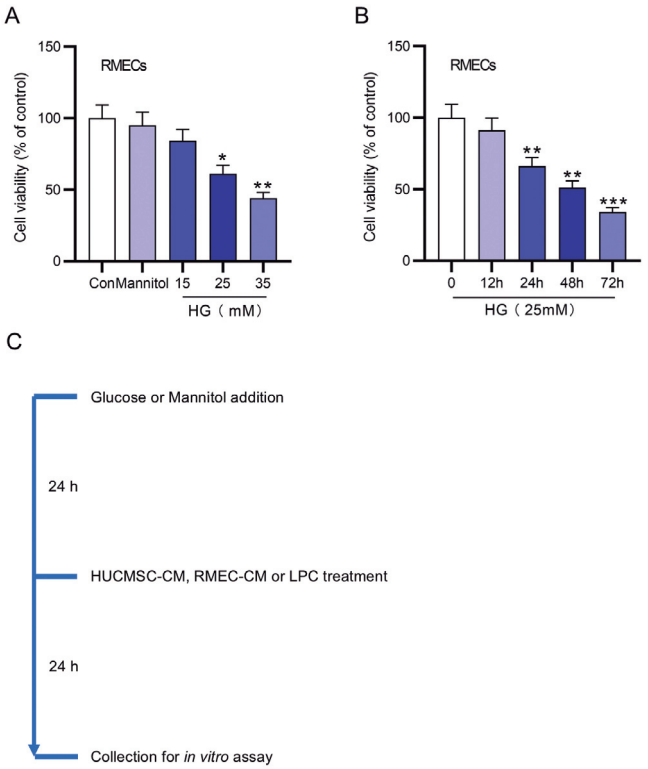

Fig. 2. HG treatment inhibited RMEC viability. **A, B.** A CCK-8 assay was used to measure the viability of RMEC. **B, C.** The timeline of cell treatment. *p<0.05, **p<0.01, ***p<0.001.

35 mM) of glucose treatment for 24h (Fig. 2A). To avoid excessive cell apoptosis, 25 mM of glucose treatment was used for subsequent experiments. Moreover, glucose treatment inhibited cell viability in a time-dependent manner (Fig. 2B). Collectively, HG treatment inhibited RMEC viability in a dose and time-dependent manner.

HUCMSC-CM alleviated HG-induced RMEC damage

To evaluate the effect of HUCMSCs on RMECs, RMECs were treated with HUCMSC-CM or RMEC-CM. According to figure 3A, the decrease in RMEC viability induced by HG treatment was neutralized by HUCMSC-CM administration. Moreover, HG induced an increase in the apoptosis of RMECs, and HUCMSC-CM significantly reduced the RMEC apoptosis caused by HG treatment (Fig. 3B,C). Furthermore, the downregulation of tight junction proteins (ZO-1, Occludin, and Claudin-5) induced by HG treatment was increased by HUCMSC-CM administration (Fig. 3D-E). All in all, HUCMSC-CM alleviated HG-induced RMEC damage.

HUCMSC-CM attenuated HG-induced inflammation and oxidative stress of RMEC

The concentration of inflammatory cytokines (TNF-α, IL-6, IL-8) in HG-induced RMECs was detected using ELISA. The result showed that HG caused an elevation in the content of TNF-α, IL-6, and IL-1β in

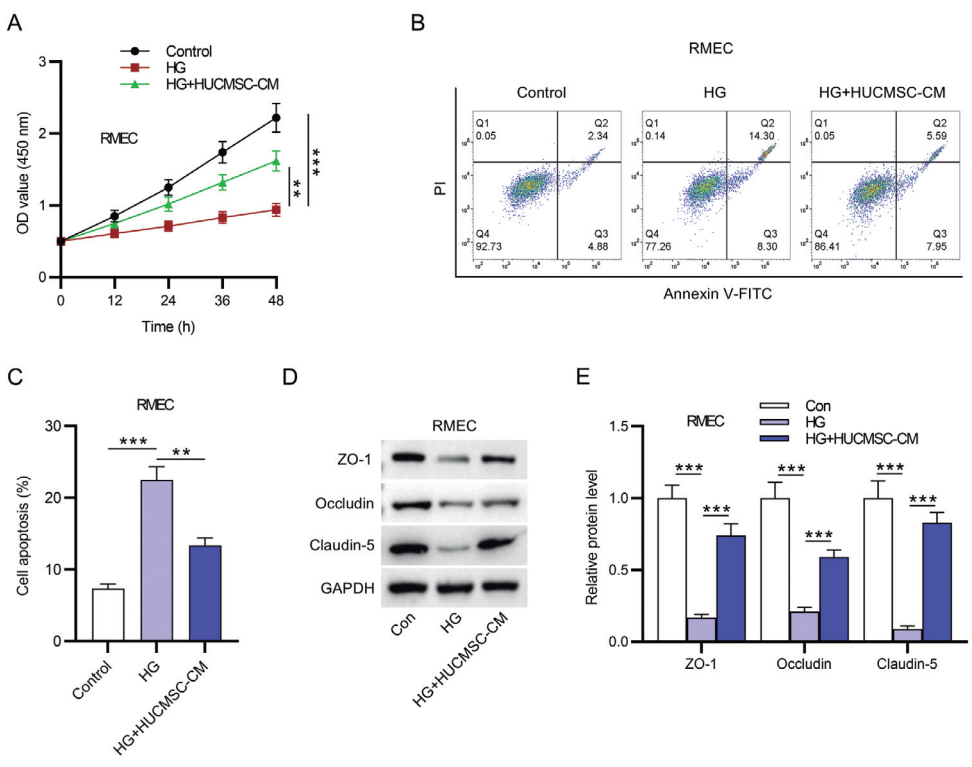

Fig. 3. HUCMSC-CM alleviated HG-induced RMEC damage. **A.** A CCK-8 assay was used to measure the viability of HG-induced RMEC. **B, C.** Flow cytometry analysis was used to assess the apoptosis of RMECs. **D, E.** The measurement of protein levels of tight junction proteins. **p<0.01, ***p<0.001.

RMECs, which was demonstrated to be reversed by HUCMSC-CM treatment (Fig. 4A). Besides, CAT, SOD, and GSH contents were reduced in response to HG treatment, and HUCMSC-CM administration reversed these results (Fig. 4B). Moreover, the downregulation of the SOD2 protein was observed in the HG group compared with the control group, and HUCMSC-CM treatment enhanced this protein level (Fig. 4C). To summarize, HUCMSC-CM attenuated HG-induced inflammation and oxidative stress of RMEC.

LPC neutralized the effect of HUCMSC-CM on RMEC viability and apoptosis

Based on the CCK-8 assay, the increased cell viability of HG-induced RMECs in the HUCMSC-CM + NS (normal saline) group was inhibited by LPC treatment (Fig. 5A). In addition, flow cytometry revealed that HUCMSC-CM ameliorated the HG-induced RMEC apoptosis, while LPC treatment counteracted this effect and increased the cell apoptosis rate (Fig. 5B,C). Moreover, the HUCMSC-CM mediated the upregulation of tight junction proteins (ZO-1, Occludin, and Claudin-5) in HG-induced RMECs was then reduced by LPC treatment (Fig. 5D-E). To conclude, LPC neutralized the effect of HUCMSC-CM on RMEC viability and apoptosis.

LPC reversed the effect of HUCMSC-CM on the inflammatory response and oxidative stress of RMECs

ELISA showed that the concentration of inflammatory cytokines (TNF-α, IL-6, IL-1β) was decreased by HUCMSC-CM treatment in HG-induced

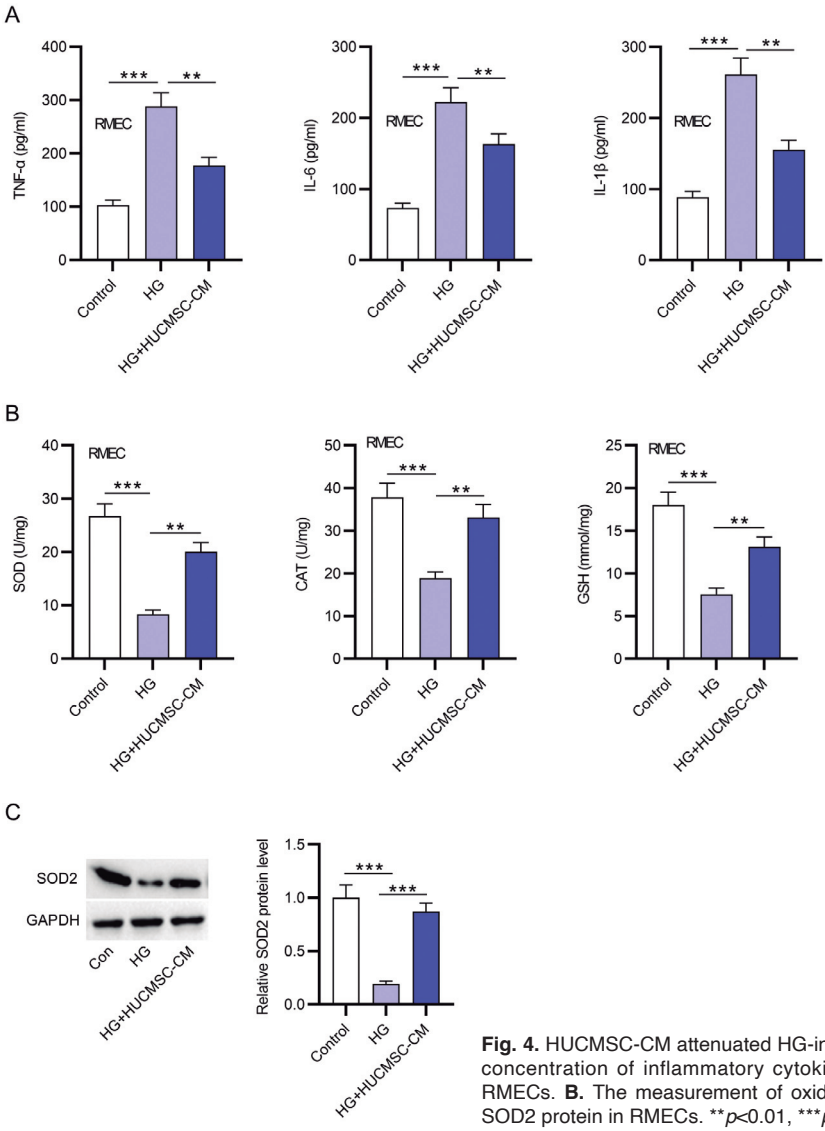

Fig. 4. HUCMSC-CM attenuated HG-induced inflammation and oxidative stress of RMEC. **A.** The concentration of inflammatory cytokines (TNF-α, IL-6, IL-8) was measured using ELISA in RMECs. **B.** The measurement of oxidative stress markers in RMECs. **C.** The measurement of SOD2 protein in RMECs. **p<0.01, ***p<0.001.

RMECs, and LPC treatment elevated the levels of inflammation factors in HG-induced RMECs (Fig. 6A). The CAT, SOD, and GSH contents were elevated in the HUCMSC-CM + NS group, while in the HUCMSC-CM + LPC group, the expression of these antioxidant enzymes was decreased by LPC treatment (Fig. 6B). Furthermore, the upregulated SOD2 protein in the HUCMSC-CM group was decreased by LPC administration (Fig. 6C). All in all, LPC negatively reversed the effect of HUCMSCs on the inflammatory response and oxidative stress of RMECs.

Discussion

Their self-renewal and differentiation properties have enabled the clinical trials of stem cell therapy for decades. It is estimated that every year, over 50,000 bone marrow transplants are conducted globally (Kimbrel and Lanza, 2020). HUCMSCs have been reported to provide a promising approach for DR treatment (Gaddam et al., 2019; Li et al., 2021a,b), while the underlying mechanisms are not fully investigated. In the present study, we identified HUCMSCs by detecting

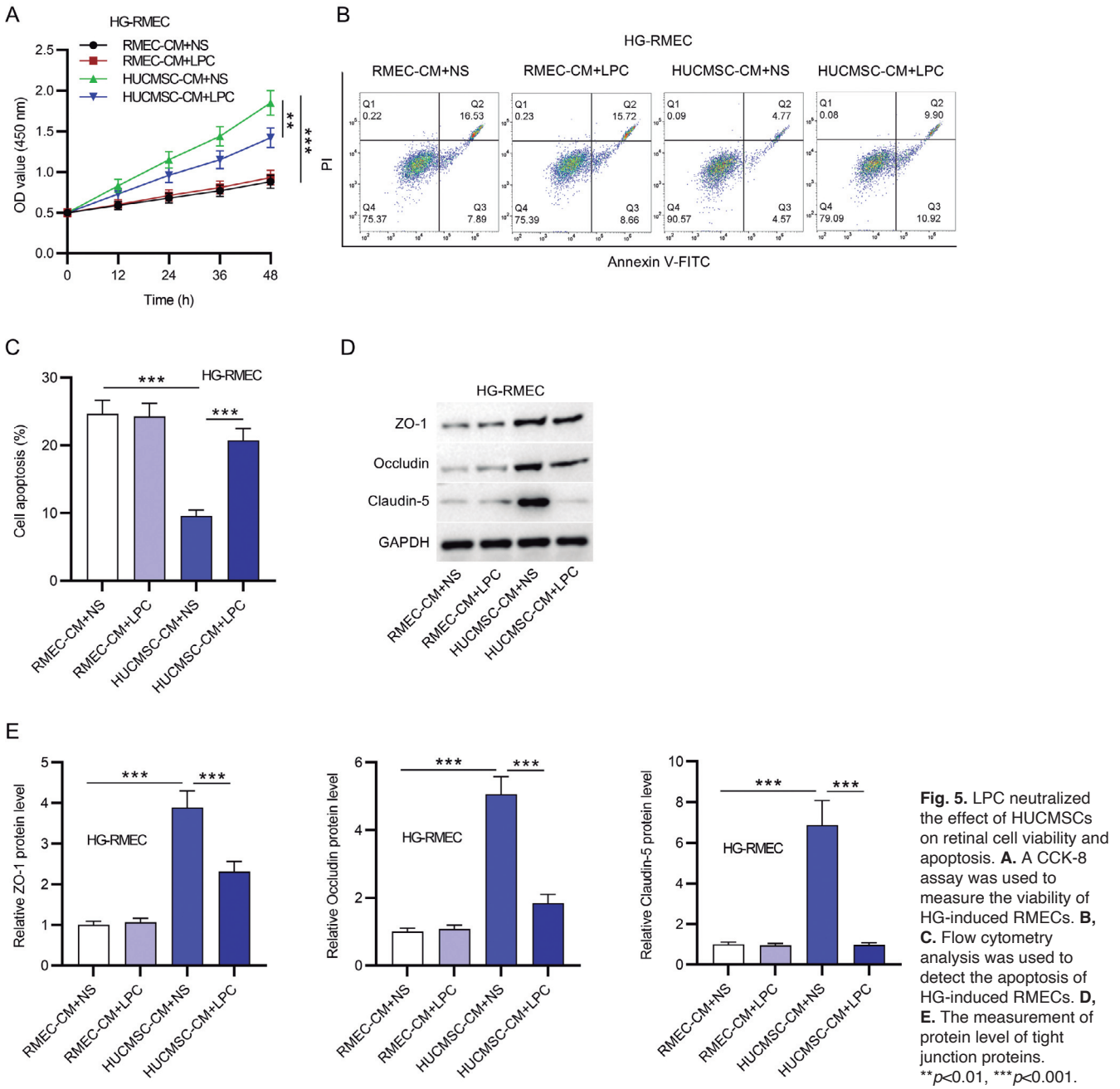

Fig. 5. LPC neutralized the effect of HUCMSCs on retinal cell viability and apoptosis. **A.** A CCK-8 assay was used to measure the viability of HG-induced RMECs. **B, C.** Flow cytometry analysis was used to detect the apoptosis of HG-induced RMECs. **D, E.** The measurement of protein level of tight junction proteins. $^{**}p<0.01$, $^{***}p<0.001$.

the expression of surface markers, which were positive for CD73, CD105, and CD90 but negative for CD45, CD34, and HLA-DR. Emerging literature proposes that HUCMSCs affect the cell viability and apoptosis of endothelial cells (Liu et al., 2022; Deng et al., 2023). In detail, HUCMSCs protect the vascular endothelium from diabetic damage via a paracrine mechanism and inactivating the MAPK/ERK signaling pathway (Liu et al., 2022). Similarly in our study, the viability of RMECs was significantly decreased in the HG group, while co-incubation with HUCMSC-CM reversed this decrease. On the contrary, the apoptosis of RMECs was elevated after the HG treatment and showed a reduction after co-incubation with HUCMSC-CM. These results demonstrated a protective effect on RMEC injury.

According to previous studies, the inner blood-retina barrier (iBRB) plays a crucial role in maintaining the homeostasis of the inner retina by regulating the exchange of nutrients, ions, and other molecules between the blood and neural tissue (Felinski and Antonetti, 2005; Someya et al., 2022). This barrier is formed by specialized endothelial cells that line the microvasculature of the inner retina, which are interconnected by tight junction proteins (ZO-1, Occludin, and Claudin-5), creating a physical barrier that restricts paracellular diffusion and prevents harmful substances from entering the retinal tissue (Yan et al., 2022; O'Leary and Campbell, 2023). Coincidently, HUCMSC-CM was reported to improve blood brain barrier leakage and enhance vascular remodeling in stroke rats (Zhao et al., 2015; Liu et al., 2024a). In our study, HG treatment decreased tight junction proteins while HUCMSC-CM administration counteracted these trends, implying a protective effect of HUCMSC-CM on the BRB.

As a neurovascular diabetic complication, DR showed elevated levels of pro-inflammatory factors (TNF-α, IL-6, IL-1β) and antioxidant enzymes (SOD, CAT, and GSH) in RMECs (Dehdashtian et al., 2018; Gui et al., 2020; Andrés-Blasco et al., 2023). Our study indicated that HG treatment induced the RMEC inflammatory response and facilitated oxidative stress

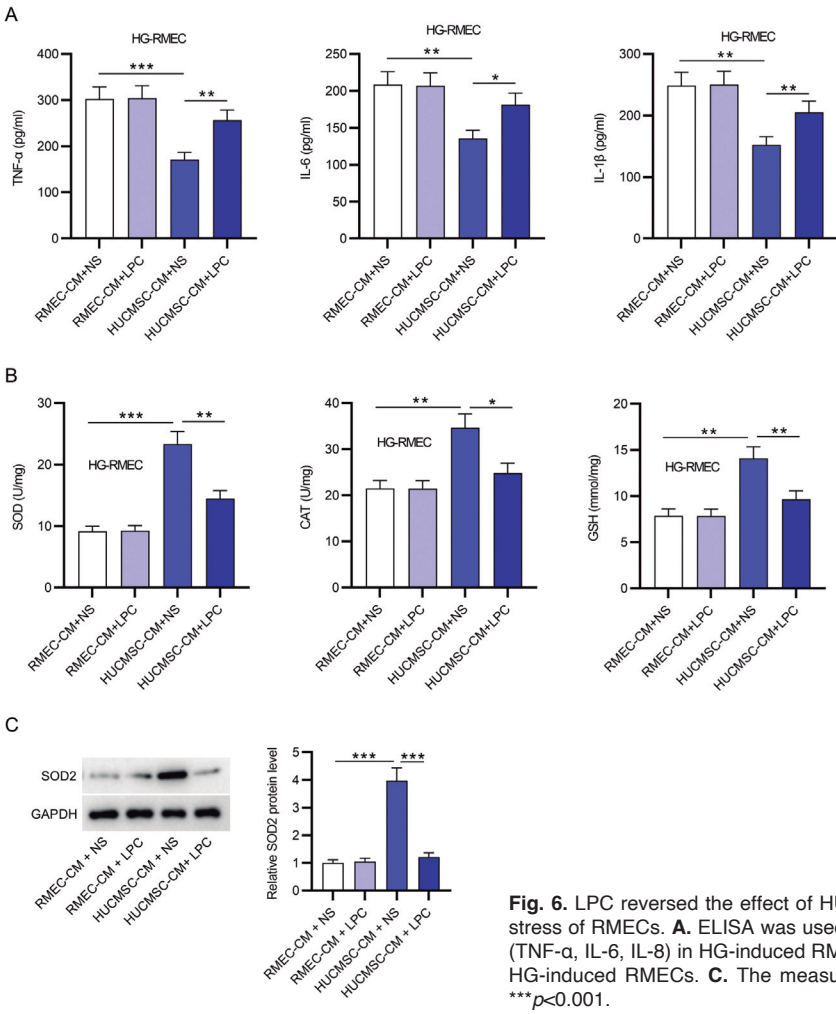

Fig. 6. LPC reversed the effect of HUCMSC-CM on the inflammatory response and oxidative stress of RMECs. **A.** ELISA was used to measure the concentration of inflammatory cytokines (TNF-α, IL-6, IL-8) in HG-induced RMECs. **B.** The measurement of oxidative stress markers in HG-induced RMECs. **C.** The measurement of SOD2 protein in RMECs. *p<0.05, **p<0.01, ***p<0.001.

injury and that these results were neutralized by HUCMSC-CM treatment. To be specific, the decreased concentration of inflammatory cytokines and increased antioxidant enzymes were observed in RMECs treated with HUCMSC-CM.

LPCs, a class of lipid biomolecule, are critically involved in the pathogenesis of various diseases, such as cardiovascular diseases (Stegemann et al., 2014), brain diseases (Koizumi et al., 2010), and diabetes (Petersen et al., 2017). Particularly, LPC was reported to neutralize the effect of BMSC (bone marrow-derived mesenchymal stem cell) on inflammation and oxidative stress in DR (Zhao and He, 2021). To be specific, LPC mitigated the protective effects of BMSCs on the inflammatory response and oxidative stress injury in retinal endothelial cells via the TLR4/NF-κB signaling pathway (Zhao and He, 2021). In the present study, we found that LPC treatment reversed the effect of HUCMSC-CM on the viability and apoptosis of HG-treated RMECs. Moreover, the effect of HUCMSC-CM on tight junction proteins in HG-induced RMECs was counteracted by LPC treatment. Furthermore, the concentrations of inflammation factors were significantly increased while antioxidant enzymes were reduced in the HUCMSC-CM + LPC group, which indicated that LPC negatively reversed the inhibitory impact of HUCMSC-CM on inflammation and oxidative stress in RMECs.

In conclusion, HUCMSC-CM was demonstrated to promote cell viability and inhibit cell apoptosis, tight junction proteins, inflammation factors, and antioxidant enzymes in HG-induced RMECs. LPC treatment reversed the protective effects of HUCMSC-CM on RMECs, which provides clues for the underlying mechanism of stem cell therapy in DR.

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Competing interests. The authors declare that they have no competing interests.

Consent to participate. Not applicable.

Consent for publication. Not applicable.

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Authors' Contributions. Xian Liu conceived and designed the experiments. Xian Liu, Yong Chai, Han Liu, Yao Zhao, ChunYi Liu, Rui Luo, and Qiang Gan carried out the experiments. Xian Liu, Yong Chai, Han Liu, Yao Zhao, ChunYi Liu, Rui Luo, and Qiang Gan analyzed the data. Xian Liu and Yong Chai drafted the manuscript. All authors agreed to be accountable for all aspects of the work. All authors have read and approved the final manuscript.

Data Availability Statement. The datasets used or analyzed during the current study are available from the corresponding author upon reasonable request.

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