TBHQ Regulates the Nrf2/HO-1 Pathway to Enhance Stem Cell Treatment of Diabetic Retinopathy

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Research Article

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Abstract

Objective: To investigate the therapeutic effect of human umbilical cord mesenchymal stem cells (hUCMSCs) on diabetic retinopathy (DR) in diabetic rats, and to study the mechanism of hUCMSCs in treating diabetic retinopathy by tert-butylhydroquinone (tBHQ) regulation of the Nrf2/HO-1 pathway.

Methods: The diabetic rat model was induced by intraperitoneal injection of streptozotocin (STZ). The experimental animals were divided into six groups: Normal, diabetes mellitus (DM), hUCMSCs, tBHQ, combined tBHQ-hUCMSCs, and all-trans-retinoid acid (ATRA)-hUCMSCs combined group. Visual function experiments and histological analyses were performed eight weeks post intravitreal injection. Biochemical and molecular analyses were used to assess the hUCMSCs composition and its biological effects.

Results: Improvements in systemic oxidative stress and inflammation were found in the tBHQ group. Although hUCMSCs had no significant effect on oxidative stress, retinal structure was improved, visual defects reduced and expression of local retinal inflammatory factors were inhibited following its application. The effect of combined therapy was better than that of single therapy. Inhibition of the Nrf2/HO-1 pathway can promote the expression of systemic inflammatory factors and inhibit the therapeutic effect of hUCMSCs in the retina.

Conclusions: Intravitreal administration of hUCMSCs triggers an effective cytoprotective microenvironment in the retina of diabetic mice. Alone, however, it may not significantly improve the systemic inflammatory response of diabetes. In combination with tBHQ it may promote Nrf2expression, systemic antioxidant stress and therapeutic effects of hUCMSCs.

Background

According to a report released by the International Diabetes Association in 2019, the number of people suffering from diabetes mellitus (DM) globally is about 463 million, and it is estimated that this number will rise to 578 million by 2040[1]. A common ocular complication of DM is diabetic retinopathy (DR). More than 60% of DM patients with disease duration of more than 20 years will have DR changes[2]. According to a global epidemiological survey, it is estimated that 36 million people are blind and 216 million are visually impaired. DR has become the second most prevalent cause of visual impairment and blindness in the world, so attention should be paid to its treatment[3].

Elevated blood glucose causes local retinal ischemia and hypoxia, destruction of retinal pigment epithelial cells, capillary endothelial cells, pericytes, thinning of capillary walls and local compensatory hyperplasia[4]. Due to metabolic needs, some hemangiomas can be slightly dilated to increase blood flow and adapt to local ischemia and hypoxia. If ischemia and hypoxia persist or increase, excessive telangiectasia will cause destruction of the blood-retinal barrier, aggravation of leakage, retinal hard exudates and edema, most of these changes occurring in the outer plexiform layer[5]. Hard exudates are

formed by leakage of plasma and local lipids, becoming localized waxy plaques in the outer plexiform layer when the lipid is gradually absorbed[6]. Ischemia and hypoxia continue to destroy blood vessels, resulting in local secretion and regulation disorder, hypoxia inducing factor-1 α (HIF-1 α), and increased vascular endothelial growth factor (VEGF)[7] secretion inducing angiogenesis. When neovascularization occurs, the disease has entered the proliferative phase. Traction from the new fibrovascular membrane causes the capillaries to rupture, leading to vitreous hemorrhage and perhaps retinal detachment.

It has been found that the mechanisms of mesenchymal stem cells (MSCs) involved in tissue repair may be migration and homing to the injured site promoting tissue repair[8], reducing inflammation and regulating immunity[9], inhibiting the mitochondrial apoptosis pathway[10], and secreting angiogenic factors, neurotrophic factors and other cytokines[11]. Human umbilical cord MSCs (hUCMSCs) are rich in sources, high primitive, expand easily, and have low immunogenicity. In addition, some studies have found that hUCMSCs are more active in secreting cytokines than other MSCs[12], which makes hUCMSCs more attractive.

However, the success rate of MSC transplantation has always been a key factor limiting its clinical application. At present, the majority view is that the high level of oxidative stress in vivo leading to apoptosis after transplantation is the main factor affecting the therapeutic effect of MSCs.

Normally, reactive oxygen species (ROS) maintain homeostasis by participating in the normal metabolic process of the body and clearing cellular metabolites[13]. ROS are mainly produced by the mitochondrial respiratory chain. The abnormal glucose metabolism caused by long-term hyperglycemia in DM patients will stimulate the mitochondrial electron transport chain to produce ROS, excess ROS attack mitochondrial DNA (mtDNA), oxidative damage of which will lead to mitochondrial protein damage in the respiratory chain and release more ROS, forming a feedback loop[14]. Moreover, this phenomenon was not immediately alleviated by the correction of blood glucose level, and in vitro experiments have confirmed that even after withdrawal from hyperglycemia, the mitochondria of retinal pericytes continue to produce excess ROS, until the latter is cleared to a controllable level[15]. Excessive ROS can also cause mitochondrial fission, energy depletion[16], and leakage from damaged mitochondria, leading to damage to other organelles and cell necrosis[17], thereby promoting the expression of inflammatory cytokines. Thus it can be seen that ROS is not only a pathological product of ischemia and hypoxia, but also an important link in strengthening oxidative stress injury and tissue necrosis[18, 19].

Nuclear factor erythroid-2-related factor 2 (Nrf2) is central to cellular antioxidation and reduction and plays a key role in cellular oxidative stress responses [20]. In the physiological state, the Neh2 domain of Nrf2 is coupled with the DGR region of Keap1, and Nrf2 is in a state of low activity. Under oxidative stress, ROS change the Keap1 domain, phosphorylate Nrf2, uncouple Nrf2 from Keap1, and Nrf2 transfers from the cytoplasm to the nucleus. The phosphorylated Nrf2 binds to the Maf protein through the domain Neh1 to form a heterodimer, which regulates the activity of downstream target genes such as phase II detoxifying enzymes in vivo. Heme-oxygenase-1 (HO-1) is an important phase II detoxification enzyme,

together with other cellular endogenous substances. Under the action of biliverdin reductase, heme is reduced to biliverdin, with Fe^{2+} and CO as intracellular redox regulators participating in the regulation of cellular oxidative stress and inflammation.

Since hUCMSCs would encounter an inflammatory milieu in the diabetic eye, we increased expression of the Nrf2/HO-1 pathway to determine whether tBHQ is beneficial to the success rate of hUCMSCs transplantation.

Methods

Animals

Male Sprague Dawley (SD) rats were purchased from the Guangdong animal experimental center, Hospital of Guangzhou University of Traditional Chinese Medicine. Rats were housed in pathogen-free microisolator cages with access to food and water ad libitum with a 12:12 hour light–dark cycle and constant temperature and humidity.

Grouping and drug administration

Table 1. Drug administration

Group	Detail
Normal	intraperitoneal injection of citrate buffer
DM	intraperitoneal injection of streptozotocin (STZ)
hUCMSCs	intraperitoneal injection of STZ + intravitreal injection of hUCMSCs
tBHQ	intraperitoneal injection of STZ + Oral tBHQ
tBHQ-hUCMSCs	intraperitoneal injection of STZ + Oral tBHQ + intravitreal injection of hUCMSCs
ATRA-hUCMSCs	intraperitoneal injection of STZ + Oral ATRA + intravitreal injection of hUCMSCs

All of the eight-week-old male rats were fixed and received an intraperitoneal injection of 60mg/kg STZ (Sigma, Code: S0130) immediately after dissolving it in 0.1M citrate buffer pH 4.5, or citrate buffer only (Normal rats). One week later, 1mL/100g peanut oil was administered to the Normal and DM groups orally once per day. Ten weeks later, in the hUCMSCs, tBHQ-hUCMSCs and ATRA-hUCMSCs groups, hUCMSCs were injected into the vitreous. tBHQ and ATRA were dissolved in peanut oil at concentrations of 15mg/mL and 0.1mg/mL respectively. One week after modeling, 15mg/100g of tBHQ per day was administered orally to the tBHQ and tBHQ-hUCMSCs groups, and 0.1mg/100g of ATRA per day was administered orally to the ATRA-hUCMSCs group.

Blood glucose quantification

Blood samples were collected from the tail vein of non-fasted alert rats, and glucose levels were determined with the glucometer system Accu-Chek Performa from Roche Diagnostic (Mannheim, Germany).

Glycated hemoglobin and plasma insulin quantification

After the rats were anesthetized, blood was collected from the abdominal aorta. The percentage of HbA1c was measured using the DCA2000 Analyzer (Bayer). The insulin concentrations were assessed using a mouse insulin ultrasensitive ELISA kit (Mercodia, Code: 10-1250-10).

Ex vivo expansion and characterization of hUCMSCs

The hUCMSCs were purchased from Vcanbio Cell & Gene Engineer Corp (Tianjin, China). The seed cells of P2 generation were resuscitated in a water bath at 37℃. The cells were carefully blown and resuscitated with complete culture medium. The formula of the medium was as follows: 89% DMEM-F12 (Gibco, Code: 11330), 10% FBS (Biosharp, Code: 04-001-1ACS), 1% penicillin and streptomycin (Beyotime Biotechnology, Code: C0222). The solution was centrifuged, the supernatant discarded, and the medium was re-suspended, blown and well shaken, then syringed into an aseptic culture bottle, which was labeled and placed in a 5% CO_2, saturated humidity incubator at a constant 37 ℃ temperature to maintain the culture. When the cell density reached about 80%, the culture medium was discarded, the cells rinsed twice with normal saline, TrypLE (Gibco, Code: 125603-029) digestive juice added to digest the cells for two minutes in the cell incubator. After this period 10mL saline (Shijiazhuang No.4 Pharmoceutical, Code: H13023201) was added to end digestion and the solution was again centrifuged. After discarding the supernatant, the medium was returned to the centrifuge tube, and the cells were gently blown for uniform distribution in the culture medium. The cell suspension was syringed into the culture flask and placed into the cell incubator to maintain culture.

Once the cells were cultured to P5 generation, they were characterized. The digested cells fell away and were diluted to a density of 1×10^5/μL, incubated with FITC-CD34 (Abcam, Code: ab131589), FITC-CD45 (Abcam, Code: ab27287) and FITC-CD90 (Abcam, Code: ab11155) for 1 hour and washed again with buffered saline. The residual first antibody was removed, FITC-IgG (Abcam, Code: ab6854) secondary antibody diluent was added, and the solution was incubated at room temperature without light for one hour, then washed again with buffered saline to prevent unbound secondary antibodies from affecting the experimental results. The cells were transferred for detection of immunofluorescent markers by flow cytometry.

Intravitreal administration of hUCMSCs

Rats were anesthetized using intraperitoneal injection of 3% pentobarbital sodium. Pupils were dilated with tropicamide acetate (Dirui, Code: 20103127) before surgery, and 5μL hUCMSCs cell suspension was administered using a microsyringe after topical anesthesia. The conjunctival sac was rinsed with normal saline, then the syringe needle was inserted normal to the scleral surface at the 1-3 o'clock position at

the limbus. The syringe was pressed slowly to inject the cell diluent into the vitreous cavity, kept in place for a few seconds, then was slowly withdrawn and the ocular surface at this location was gently pressed using a cotton swab for several seconds to prevent the liquid from escaping.

HE staining

Rats were euthanized by cervical dislocation and their eyes were enucleated and fixed in 4% paraformaldehyde (Biosharp, Code: BL539A). The eyes were immersed in 50%, 70%, 85% and 95% ethanol for 1 hour each, completely immersed in anhydrous ethanol Ⅰ, Ⅱ and Ⅲ for 1 hour for dehydration, then in xylene Ⅰ and Ⅱ for 30 minutes. After heating to dissolve the paraffin the transparent eyeball was soaked in the paraffin for 1 hour. Serial sections were made along the axis parallel to the optic disc and cornea, about 4pm. The slices were dyed in the following order: Xylene Ⅰ, then Ⅱ, 10 minutes each; a 1:1 mixture of xylene and anhydrous ethanol 5 minutes; 100%, 95%, 85%, 75% ethanol, 5 minutes each; distilled water, 2 minutes; hematoxylin staining, 8 minutes; distilled water, 2 minutes; 1% hydrochloric acid alcohol, 0.5 minute; tap water, 1 minute; 5% ammonia, 1 minute; eosin, 2 minutes; distilled water, 2 minutes; 75%, 85%, 95%, 100% ethanol, 5 minutes each; Xylene Ⅰ and Ⅱ, 5 minutes each. A neutral resin sealing film was applied, and the sections were observed and photographed under a light microscope.

Quantification of retinal ganglion cells (RGCs)

The number of RGCs in the ganglion cell layer was quantified using a PM-10AD microscope (Olympus, Japan). The labeled cells from temporal lobe to serrated nostril were counted on five consecutive sections. The samples were blindly analyzed by two independent observers. The data are expressed as the number of RGCs per 100 μm retinal length.

Quantification of blood inflammatory factors

The serum was diluted five times with sample dilution. According to the ELISA kit instructions (Mbbiology, Yancheng, China; IL-6 Code: MM-0190R2; TNF-α Code: MM-0180R2; iNOS Code: MM-0454M2; IFN-γ, Code: MM-0198R2), the optical density (OD) value was detected by enzyme labeling instrument at 450nm wavelength. The concentrations of IL-6, IL-10, TNF- α, iNOS and IFN- γ were calculated.

Quantification of antioxidant capacity

Quantity of SOD in rats' blood was determined using the xanthine oxidase method, MDA using the thiobarbituric acid method[21] and GSH-Px using chemical colorimetry[22].

Western Blot Analysis

Target proteins were detected via western blotting. VEGF, Nrf2, HO-1, NF-κBp65 and cyclooxygenase-2 (COX-2) were targeted. β-actin was used as a reference protein. Each protein was repeated at least three times. The bands were analyzed by densitometry using the image analysis system (Image J, National Institute of Health, Bethesda, USA). 30~60 μg of proteins were transferred to a polyvinylidene fluoride

membrane (BIO-RAD, Code: 162-0177) via immunoblotting after the electrophoresis using the Bio-Rad Mini-Protean Tetra electrophoresis “wettransfer” system (Bio-Rad, USA). Anti-VEGF (1:2000, Abcam, Code: ab47154), anti-Nrf2 (1:1000, Abcam, Code: ab89443), anti-HO-1 (1:1000, Abcam, Code: ab13248), anti-NF-κBp65 (1:2000, Cell Signaling Technology, Code: 3033S), anti-COX-2 (1:2000, Abcam, Code: ab188184) and anti-β-actin (1:5000, Abcam, Code: ab8226) were used as primary antibodies. Blotting was performed at least three times to confirm the reproducibility of the results. Bands were analyzed densitometrically using an image analysis system.

Quantitative real-time polymerase chain reaction (qRT-PCR)

Retinal RNA was extracted using a One Step PrimeScript™ RT-qPCR Mix RNA extraction kit (Takara, Code: RR600S). The purity and concentration of RNA were determined using a spectrophotometer (Nano Photometer NP80, IMPLEN, Germany). ReverTra Ace qPCR RT Master Mix with gDNA remover kit (TOYOBO, 96600) was used for reverse transcription and synthesis of RNA. The mRNA expression of IL-1β, TNF-α, Nrf2, HO-1 were quantitatively detected using a SYBR Green Realtime PCR Master Mix kit (TOYOBO, 0722-841400). The mRNA expression was normalized to β-actin. The sequence of forward and reverse primers of RNA are shown in Table 2. Each group consisted of three samples, each in triplicate. Relative gene expression between groups was calculated using the $2^{-\triangle\triangle CT}$ method.

Table 2 qRT-PCR Forward/Reverse(F/R) primers sequences

Primers	Sequences 5'-3'
β-actin	5'-CCTCTATGCCAACACAGTGC-3'
	5'-CATCGTACTCCTGCTTGCTG-3'
Nrf2	5'-TCCCATTTGTAGATGACCATGAG-3'
	5'-CCATGTCCTGCTCTATGCTG-3'
HO-1	5'-ACAGAGGAACACAAAGACCAG-3'
	5'-GTGTCTGGGATGAGCTAGTG-3'
IL-1β	5'-TCCAGGATGAGGACATGAGCAC-3'
	5'-GAACGTCACACACCAGCAGGTTA-3'
TNF-α	5'-CAGGCGGTGCCTATGTCTCA-3'
	5'-GGCTACAGGCTTGTCACTCGAA-3'

Statistical analyses

SPSS version 20.0 was used for statistical analysis. A Shapiro-Wilk test was used to check normality, mean ± standard deviation (SD) was used to describe normally distributed measurement data, and parametric tests were used for statistical analysis. A one-way ANOVA test was used to compare the

means among the three groups and Levene Statistic was used to test the homogeneity of variance. If the variance was uniform, Bonferroni correction was used for pairwise comparison between groups. If the variance was uneven, Dunnett T3 test was used. P values < 0.05 were considered statistically significant.

Results

Diabetes induction

To induce DM, SD male rats in the DM group received an intraperitoneal injection of 60mg/kg STZ. As reported previously[23], intraperitoneal injection of STZ can induce apoptosis of pancreatic cells, and hyperglycemia is maintained without insulin supplementation, which can lead to a variety of diabetic complications, including DR. Three days after administration of STZ, the DM group was established, the blood glucose of each individual was more than 16.6 mmol/L, and remained at a high level at the follow-up test (Fig. 1b). Weight in the Normal group increased with time, and was significantly reduced in the DM group ($p<0.01$; Fig. 1a). Eighteen weeks after DM induction, reduction in plasma insulin level was correlated with a severe elevated glycated hemoglobin level (Fig. 1c and d).

Ex vivo expansion and characterization of hUCMSCs

Under the microscope, the cells adhered well after resuscitation. Three to 4 days after hUCMSCs passage, the cells grew to about 80% fusion degree in the culture flask. The growth state of the cells was good, the size was uniform, and the cells showed a long fusiform swirl-shaped adherent growth state (Fig. 1a). Hematopoietic stem cell surface markers CD34 and CD45 were at low levels of expression on cultured hUCMSCs (positive rate $\leq$ 5%) (Fig. 1b and c), while mesenchymal stem cell surface marker CD90 was expressed at a high level (positive rate $\geq$ 95%) (Fig. 1d).

Combination therapy of hUCMSCs and tBHQ prevents RGCs loss

The inner limiting membrane of the retina in the Normal group was smooth and complete. The RGCs were neat and regular, with good shape, no obvious abnormalities, and a single layer arrangement. The inner nuclear layer was also neatly arranged, and the outer nuclear layer nuclei were darkly colored, neatly and tightly arranged. The boundaries between the layers were clear. Eighteen weeks after modeling, the RGC layer showed clear vacuolization, the inner and outer nuclear layers were disordered and the thickness of the retina had reduced. The structure of the retina in the tBHQ and hUCMSCs groups was significantly better, and further significant improvement in structure, including vacuolation of the RGC layer, was found in the combined tBHQ-hUCMSCs group. In the ATRA-hUCMSCs group, however, the retinal thickness was reduced and the RGC layer was vacuolated (Fig. 3a).

Loss of RGCs is an indicator of severity of DR and is one of the early lesions in this condition[24]. As expected, we found that RGCs in the retina of diabetic rats decreased significantly after 18 weeks of modeling. However, the administration of hUCMSCs and tBHQ can reduce the damage to retinal RGCs caused by diabetes, and combination therapy of hUCMSCs and tBHQ offered greater protection of rat

retinal RGCs. Despite these effects, after 18 weeks of modeling, the blood glucose levels of these three groups remained at a high level. The addition of Nrf2 inhibitor ATRA can weaken the protective effect of hUCMSCs (Fig. 3b).

tBHQ improves the ability of hUCMSCs to resist oxidative stress and reduces inflammation

The high oxidative stress level and inflammatory response of diabetes will continue to aggravate the damage to the retina and be related to the early death of retinal neurons[25, 26]. As expected, tBHQ can play a role in anti-oxidative stress and anti-inflammation by activating the Nrf2/HO-1 pathway[27]. The results showed inhibited antioxidant capacity in diabetic rats.

However, the blood levels of SOD (Fig. 4e) and GSH-Px (Fig. 4g) in the tBHQ and tBHQ-hUCMSCs groups added with Nrf2/HO-1 agonists increased. It presents an opposite trend on MDA (Fig. 4f). After administration of ATRA, the antioxidant capacity was inhibited in a sustained manner. Systemic inflammation may be significantly aggravated by diabetes, and TBHQ can significantly inhibit the systemic inflammatory response. Most inflammatory factors increased with the administration of ATRA (Fig. 4a, b and d). Interestingly, after oral administration of ATRA, blood iNOS content was higher than that in the DM group (Fig. 4c), but the difference was not statistically significant ($P > 0.05$). This may suggest that the pro-inflammatory effect of ATRA is independent of the iNOS pathway. Vitreous injection of hUCMSCs had no significant effect on oxidative stress and inflammation.

Combination therapy of hUCMSCs and tBHQ reduces the retinal oxidative and inflammatory damage

Diabetic retinopathy can significantly increase the level of oxidative stress and inflammation in the eye[28, 29]. In addition, it will stimulate the secretion of VEGF and cause angiogenesis[30]. We found that the levels of retinal inflammatory factors NF–κ B p65 and COX-2 were significantly increased in 18-week-old diabetic rats. In addition, the expression of VEGF, Nrf2 and HO-1 was enhanced. Vitreous injection of hUCMSCs can inhibit the expression of inflammatory factors. Although the expression of Nrf2 and HO-1 also increased slightly, the difference was not statistically significant ($P > 0.05$). On the other hand, the combination of hUCMSCs and tBHQ, compared with either one of these treatments, enhanced antioxidation and the inhibition of inflammation. After inhibiting the Nrf2/HO-1 pathway, the therapeutic effect of hUCMSCs was weakened. Compared with the hUCMSCs group, the expression of NF- κ B p65, COX-2 and VEGF was increased, while the expression of Nrf2 and HO-1 was inhibited.

The expression of mRNA is similar to that of protein. The expression of both IL-1β and TNF- α mRNA were significantly inhibited by hUCMSCs, while neither Nrf2 nor HO-1 were affected. tBHQ can not only increase the expression of Nrf2 and HO-1, but also enhance the ability of antioxidation and inhibit the inflammatory reaction in DR. The combined treatment of the two is helpful to reduce the oxidative and inflammatory damage of the retina. If the Nrf2/HO-1 pathway is inhibited, the therapeutic effect of hUCMSCs may be weakened and the oxidative and inflammatory damage of the retina may be aggravated.

Discussion

Diabetic retinopathy is one of the leading causes of impaired vision, causing blindness in millions of sufferers[3]. Mesenchymal stem cells are a potentially feasible treatment method which may protect the retina by migration and repairing, inhibiting angiogenesis and by anti-inflammatory and antioxidant effects. In this sense, due to the molecular and cellular mechanisms associated with the reported MSCs therapeutic effects on pathological events that occur at the initial stages of DR, this approach has been applied to the treatment of DR and many other diseases[31].

In this experiment, we used an animal model of DM induced by a single high dose of STZ[32]. This model is characterized by high modeling rate and low mortality. STZ can rapidly kill islet β cells, resulting in hypoinsulinemia and hyperglycemia. Long-term hyperglycemia is linked to the various complications of diabetes. The eye is one of the main organs affected by DM, and more than 60% of DM patients with disease duration of more than 20 years will have DR changes[2]. For these reasons, this model is widely used to study diabetes and its complications, including DR. A previous report using this model found that STZ significantly reduced blood insulin levels and increased oxidation and inflammation levels throughout the body including the eyes. In addition, the retinal structure of DM rats was seriously damaged, and the number of RGCs was reduced by about one-third[33].

The evidence for the application of MSCs in the treatment of DR originated from an STZ-induced DM rat model[34] with intravenous administration of adipose-derived mesenchymal stem cells (AMSCs). The results showed AMSCs in the retinas of rats in the treatment group and it was suggested that AMSCs can differentiate into retinal photosensitive and glial-like cells. In addition, intravenous injection of AMSCs can reduce blood glucose levels and improve integrity of the blood-retinal barrier (BRB) in diabetic rats. Other researchers have transplanted AMSCs into the eyes of rats via the vitreous cavity[35, 36]. Studies have shown that AMSCs can reside on damaged RPE cells and participate in retinal repair[35, 36]. However, not all researchers are in agreement. Previous studies have analyzed the migration of MSCs to the retina and the potential of these cells to differentiate into RGCs. Unfortunately, most cells cannot pass the BRB and differentiate into RGCs. They play a therapeutic role but remain in the vitreous cavity[37, 38]. However, MSCs pretreated by alpha-aminoadipic acid may improve the structural integration of vitreous transplanted cells[38]. In addition, some researchers have found that transplantation of MSCs into the subretinal space is a more effective route than the vitreous chamber[36, 39]. In addition, some clinical trials have used suprachoroidal application[40] and tenon delivery[41]. With the advancement of purification technology, extracellular vesicles (Evs) of MSCs are slowly being introduced[31].

In addition to migrating to damaged areas to participate in tissue repair, MSCs can treat diabetes in a variety of other ways. In vitro, RGCs were exposed to a specified concentration of H_2O_2 to establish an oxidative damage model. In a group treated with MSCs the intracellular oxidation factor MDA was down-regulated, and the intracellular antioxidant factor SOD was up-regulated demonstrating that MSCs can protect RGCs in vitro[42]. In an in vivo experiment involving cats, MSCs were injected into the vitreous

cavity of an eye with optic nerve damage. The study found that although the survival rate of RGCs in the treatment group decreased with time, compared with a control group injected with buffered saline, the decrease in the MSCs treatment group was moderately significant[43]. This suggests that MSCs may play a therapeutic role by reducing the injury to the optic nerve. Other researchers also agreed with this conclusion[44, 45] providing evidence that the nerve protection characteristic of MSCs is related to the modulation of glial cells[46]. However, Holan et al speculated that this protective effect may be achieved by promoting the secretion of neuroprotective factors and inhibiting inflammation[45].

Previous studies have also shown that MSCs increase retinal vascular density and reduce the number of acellular capillaries[47]. It is well known that VEGF is an effective factor to promote vascular growth. It not only enhances vascular permeability, but also stimulates endothelial cell proliferation and migration, which promotes retinal angiogenesis[48]. Many types of anti-VEGF drug are available to clinicians, and offer an important treatment option for DR[49, 50]. One of the main concerns about MSCs is the promotion of angiogenesis,[51, 52] which may aggravate retinal damage. However, MSCs have the positive effect of significantly inhibiting retinal neovascularization and reshaping capillary networks during DR treatment[53], consistent with the present findings. We speculate that the special microenvironment of the retina is responsible for this phenomenon, with MSCs reduced the ineffective perfusion of blood vessels. This peculiarity needs to be further studied. Maintaining the integrity of the BRB may also be one of the important therapeutic effects of MSCs. BRB leakage was clearly apparent in STZ-induced DM rats 4 months after modelling. The treatment group showed less vascular leakage and BRB damage in diabetes. The results of pathological sections also showed more ordered retinal structure in the treatment group[54].

The protective effect of MSCs on blood vessels and nerves has been found to depend on the inhibition of oxidation and inflammation, which play fundamental roles in the pathogenesis of DR[55]. The retina is rich in unsaturated fatty acids and has a high demand for oxygen and energy, so it is particularly vulnerable to oxidative stress. In the present study, transplantation of hUCMSCs into the vitreous cavity resulted in significant inhibition of the retinal inflammatory response significant decrease in inflammatory factors such as IL-1β and NF- κB p65. This has also been found in other studies[56, 57]. In general, high immunosuppression is a key characteristic of MSCs. Previous studies have shown that T and B cell proliferation and NK cell activity may be inhibited by MSCs, leading to the down-regulation of inflammatory factors[58]. In addition, many studies have shown that MSCs regulate the expression of a variety of molecules related to immunosuppression, including COX-2, TNF- α and IFN- γ[59, 60]. In an STZ-induced DM rat model, Evs from AMSCs effectively down-regulate the expression of IL-1 β, IL-6 and TNF-α, and alleviate inflammation and retinal injury[55].

Our study also found that hUCMSCs may moderately down-regulate retinal oxidation, although this regulatory effect is much weaker than that of tBHQ, consistent with research by Eggenhofer et al[61]. It is possible that high levels of oxidation and inflammation in the transplant environment reduces the survival rate of MSCs, but some researchers have proposed the alternative view that intravenous

hUCMSCs can effectively inhibit oxidation of the retina[62, 63]. We speculate that the lack of agreement may reflect different methodology, including hUCMSCs dosage and the transplantation mode and timing. Our study also found that vitreous injection of hUCMSCs had no significant effect on systemic oxidation and inflammatory factor expression. This suggests that local administration of hUCMSCs does not influence systemic blood glucose and inflammatory response, and that its biological function is performed locally.

In addition, we found that long-term oral administration of the Nrf2/HO-1 pathway activator tBHQ can reduce retinal injury and improve retinal structure, consistent with previous studies[64, 65]. Unfortunately, low bioavailability and BRB pass rate limit application, so large dosage continuous treatment would be needed to maintain the effective concentration of drugs in the retina, and may bring adverse side effects. Although we observed no significant adverse reactions in the tBHQ group in the present study, such reactions may arise with different treatment protocols.

Another outstanding issue is the fate and immune function of MSCs after transfer to the inflammatory environment of the diseased retina. Garrido et al[66] found that when MSCs are transplanted to an injured site, their survival and implantation rates are low, mainly due to the harsh microenvironment characterized by inflammation and oxidative stress. With enhancement of oxidative stress in vivo, ROS can directly inhibit the osteogenic differentiation of MSCs. In addition, the accumulated ROS further activates NF- κB and TNF-α pathways, forming a pre-feedback loop, inhibiting the differentiation function and activity of MSCs[67]. Increased expression of Nrf2 raises the levels of SOD and GSH-Px, inhibiting oxidative stress and reducing expression of aging-related proteins. However, inhibition of Nrf2 function can also weakens MSCs activation[68]. In addition, a number of studies have shown that modulation of Nrf2 has a positive therapeutic effect on MSCs in the treatment of a variety of oxidative stress, inflammation and other aspects of systemic function[69, 70]. It is unclear, however, whether MSCs have anti-inflammatory and antioxidant effects, or whether tBHQ inhibits oxidation and is conducive to the survival and activation of MSCs. Our findings suggest the latter.

We also found that the combination of hUCMSCs and tBHQ had a synergistic effect, with better systemic and retinal response to treatment than hUCMSCs treatment alone, regardless of the level of oxidation or inflammation. Compared with the tBHQ group, the tBHQ-hUCMSCs group had less retinal damage, but blood levels of inflammation and oxidation did not decrease significantly. No adverse events occurred during the experiment. Therefore, we speculate that tBHQ can activate Nrf2/HO-1 pathway, inhibit oxidation and inflammation, promoting hUCMSCs to perform a therapeutic role in the local retina and reduce retinal injury.

Conclusion

Using a STZ induced DM model, we demonstrated that one single intravitreal injection of hUCMSCs therapeutically benefit the retina by suppressing oxidation and inflammation. However, it seems to have no effect on systemic blood sugar and inflammation. The combination of hUCMSCs and tBHQ can down-

regulate the levels of systemic oxidative and inflammatory factors. Molecular and histological studies further confirm the beneficial effects of this treatment in vivo. This may be a potentially feasible treatment strategy and future studies are needed to identify optimal location, time and dose of injection. Preclinical studies will focus on the safety and efficacy of combination therapy.

Abbreviations

hUCMSCs: human umbilical cord mesenchymal stem cells; Nrf2: nuclear factor erythroid-2-related factor 2; HO-1: heme oxygenase-1; tBHQ: tert-butylhydroquinone

Declarations

Acknowledgements

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Authors' contributions

Ze-Peng XU is the first author on this paper. Ze-Peng XU and Ni TIAN designed and carried out the research, analyzed the data, and drafted the manuscript. Ze-Peng XU, Song-Tiao LI, Kun-Meng LI, Xiao-Yu WANG and Xiao-Jie LI performed the research. Yan DONG□Xiao-Yi YU, Rui-Ying ZHONG, Qiu-Hong LIU, Xiao-Chuan WANG, Yan YANG and Ying-Zi LUO provided technical support. Mei-Xia AN helped to review the data and the manuscript.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Ethics approval and consent to participate

All animal procedures reported in this study follow the "Regulations on the Administration of Laboratory Animals" promulgated by the Chinese Science and Technology Commission.

Consent for publication

Not applicable.

Competing interests

There is no conflict of interest to declare.

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Figures

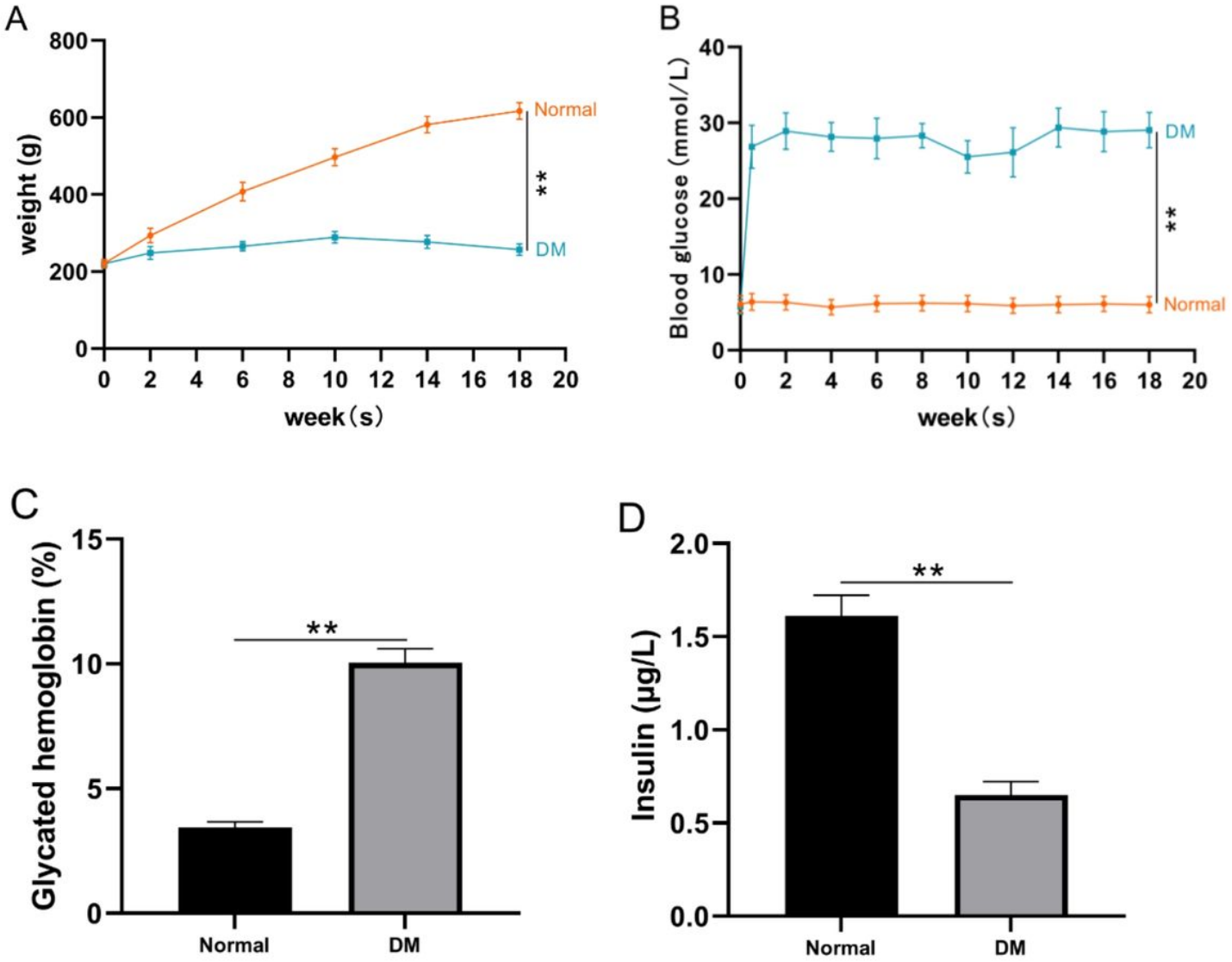

Figure 1

Characterization of diabetes mellitus stage between the Normal and DM groups without treatment. a Weight was determined once every two weeks. b Blood glucose level was determined once every two weeks in venous blood samples obtained from alert non-fasted animals. c Glycated hemoglobin and d insulinemia levels were determined 18 weeks after the model was established. **p < 0.01 vs. Normal group. Error bars indicate SEM.

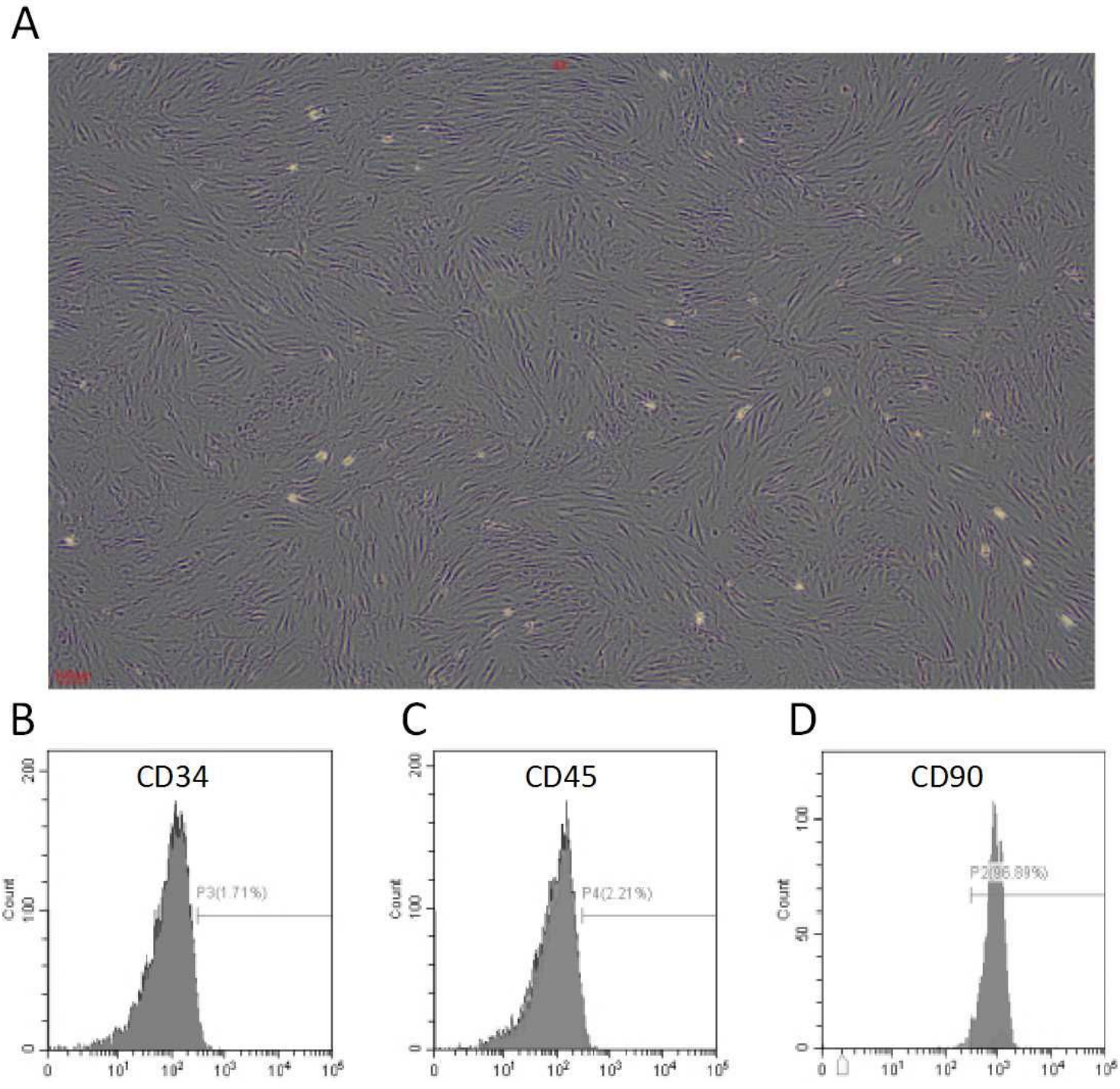

Figure 2

Characterization of hUCMSCs. After the resuscitated hUCMSCs were passaged according to the above method, a the P5 generation after passage was photographed under the light microscope. According to

the expression of hUCMSCs marker CD90 and the non-expression of hematopoietic marker CD30 and CD45, the immunophenotype of the cells was analyzed. FITC-labeled b CD34, c CD45 and d CD90 antibodies were added to identify the phenotype of the cells.

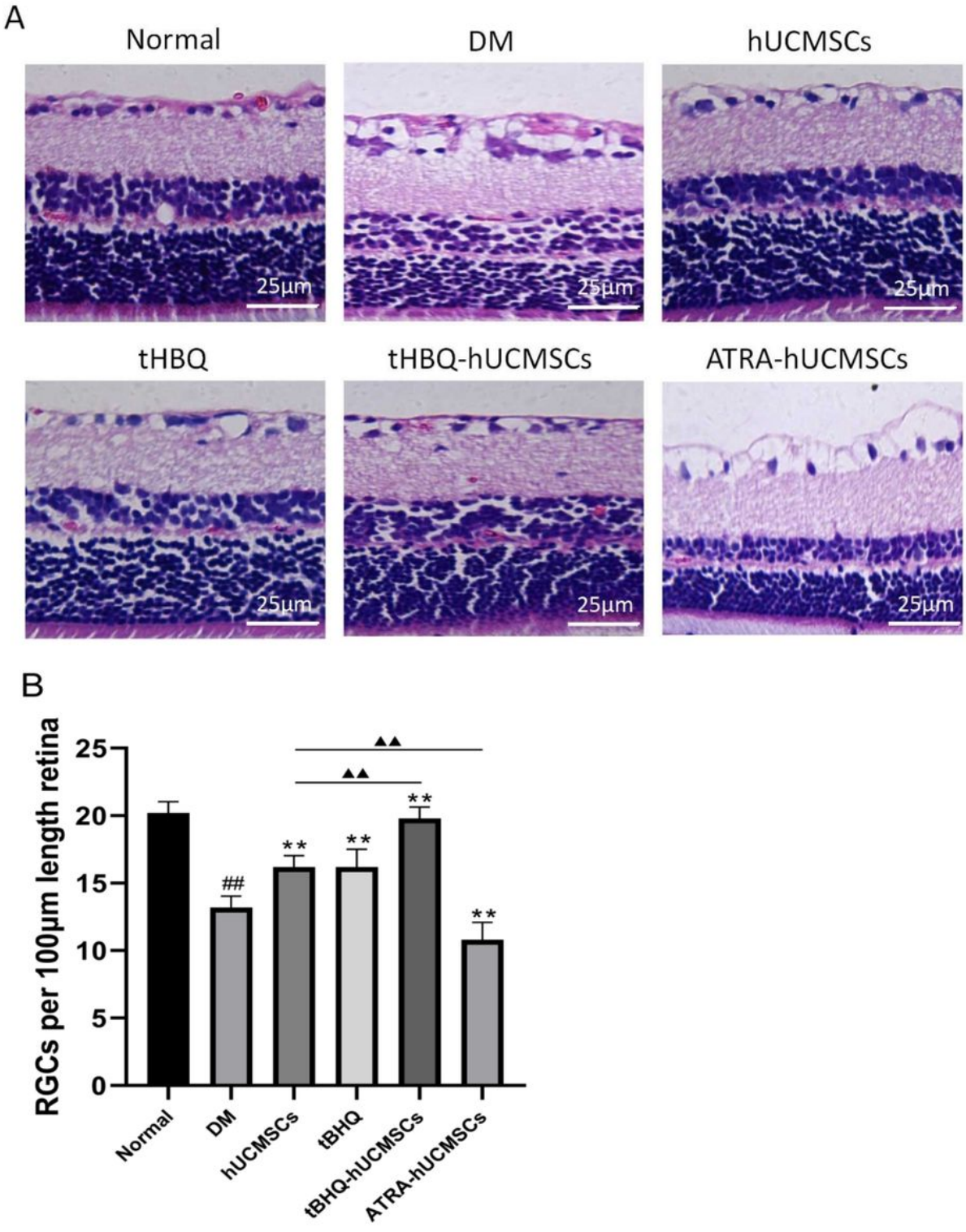

Figure 3

Combination therapy of hUCMSCs and tBHQ prevents RGC loss. One week after STZ administration, 15mg/100g tBHQ was administered to tBHQ and tBHQ-hUCMSCs groups respectively. The ATRA group

was treated with 0.1mg/100g ATRA. 5×105 hUCMSCs was injected into the vitreous cavity 10 weeks after STZ administration. Serial sections were made along the axis parallel to the optic disc and cornea, about 4pm. a After 18 weeks of modeling, the samples were observed by focusing on the RGC layer. b RGCs were quantified and are presented as the mean ± SEM from n = 5 animals/group normalized to SD rats. ##p < 0.01 vs. Normal group, **p < 0.01 vs. DM group, ▲▲p < 0.01 vs. hUCMSCs group. Error bars = SEM.

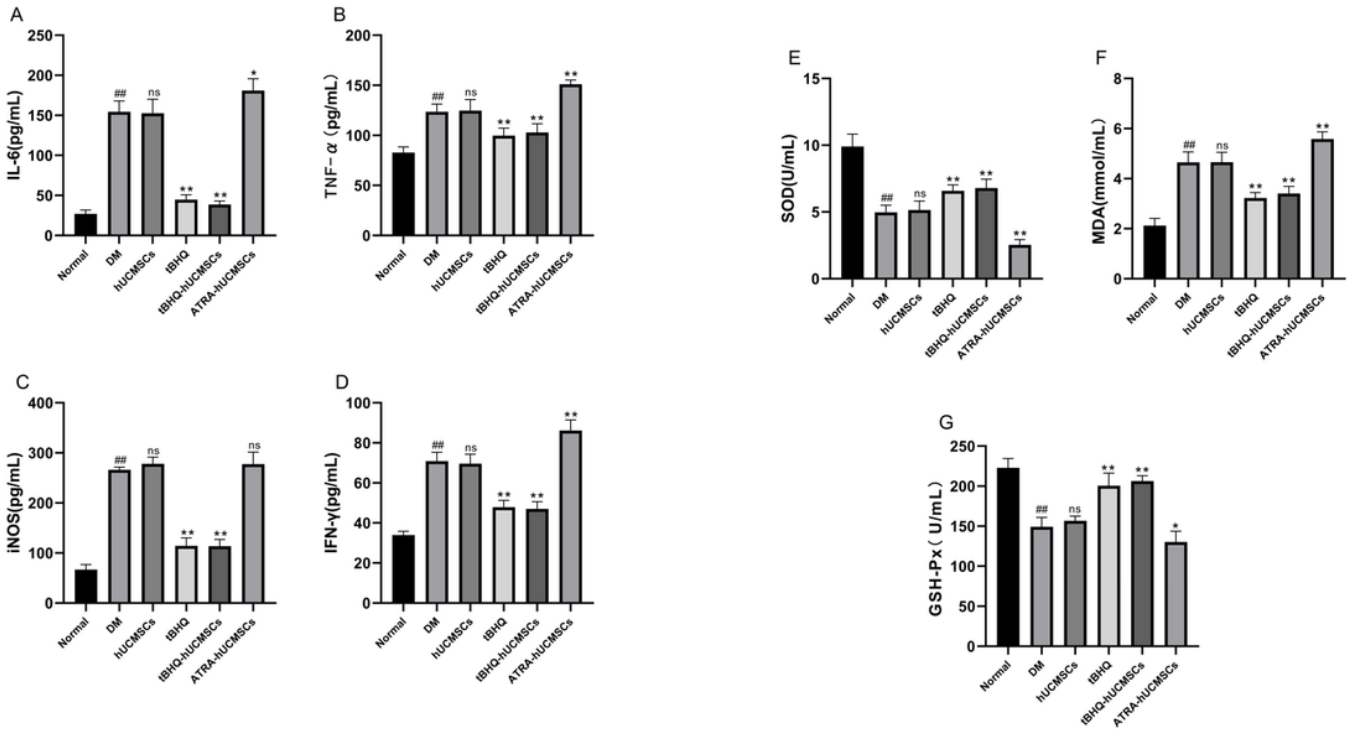

Figure 4

Quantification of blood inflammatory factors and antioxidant capacity. After 8 weeks of hUCMSCs administration, the rats were anesthetized and blood was collected from the abdominal aorta. The inflammatory factors and antioxidant capacity in the blood are compared. Data are represented as the mean ± SEM from n = 5 animals/group normalized to SD rats. ##p < 0.01 vs. Normal group, *p < 0.05 vs. DM group, **p < 0.01 vs. DM group, ns = not significant (p>0.05).

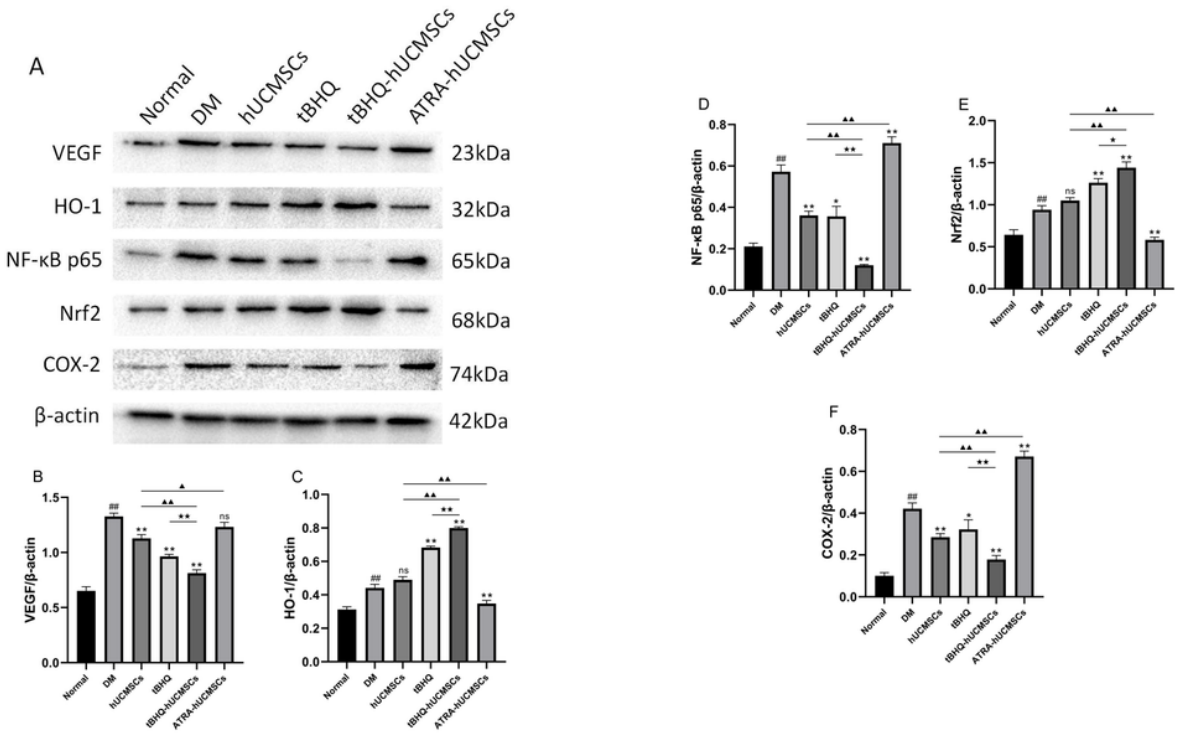

Figure 5

Differences in retinal protein expression among different groups. After 8 weeks of hUCMSCs administration, the rats were sacrificed, the eyes were removed and the retinas were detached. a The intensity of the western blot bands was quantified by densitometric analysis. β-actin was used as a loading control. Data represent the mean ± SEM from n = 3 animals/group normalized to SD rats. ##p < 0.01 vs. Normal group, *p < 0.05 vs. DM group, **p < 0.01 vs. DM group, ▲p < 0.05 vs. hUCMSCs group, ▲▲p < 0.01 vs. hUCMSCs group, □p < 0.05 vs. tBHQ group, □□p < 0.01 vs. tBHQ group

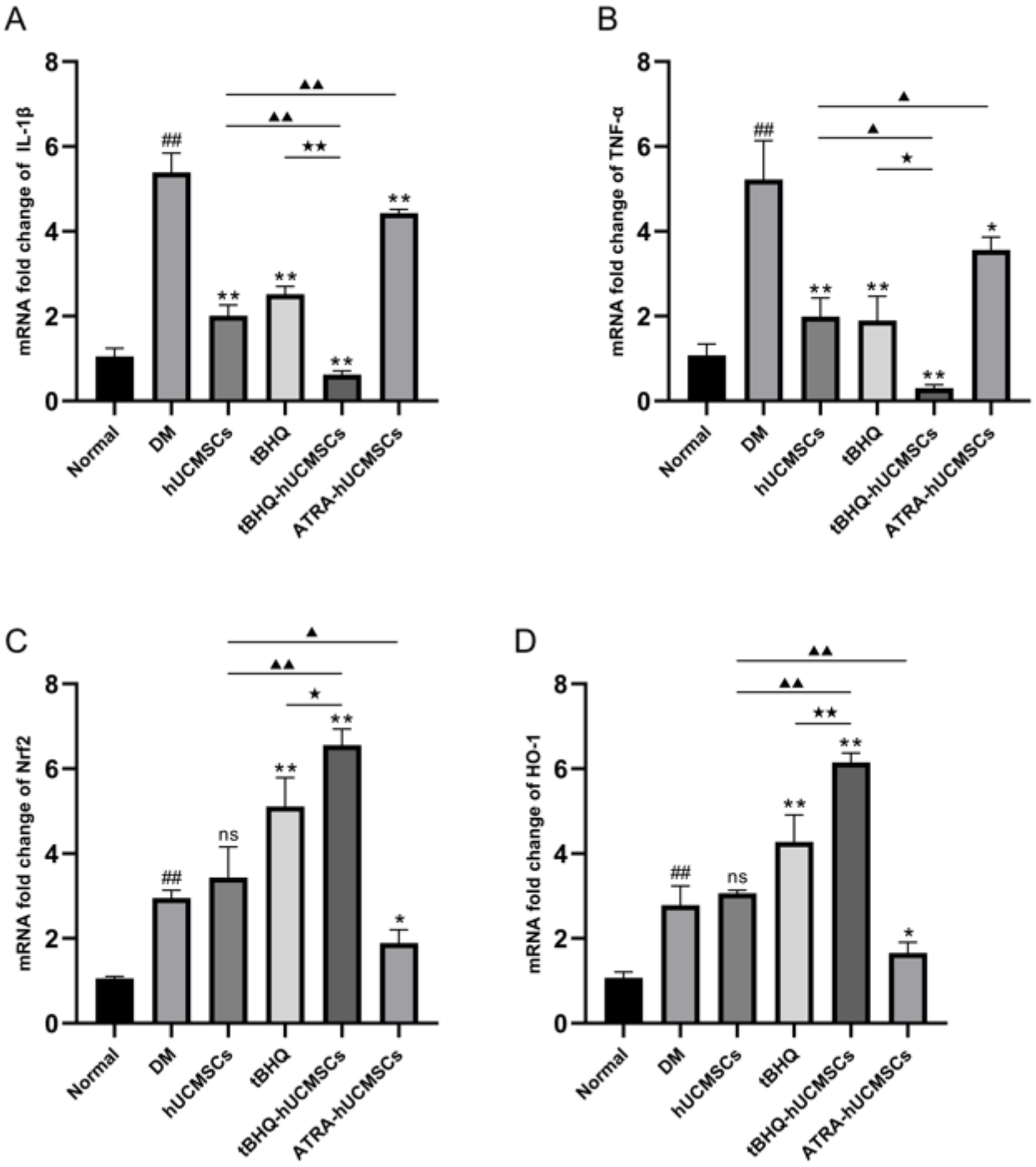

Figure 6

Quantification of mRNA of a IL-1β, b TNF-α, c Nrf2 and d HO-1expression among different groups. β-actin was used as a loading control. Data represent the mean ± SEM from n = 3 animals/group normalized to SD rats. ##p < 0.01 vs. Normal group, *p < 0.05 vs. DM group, **p < 0.01 vs. DM group, ▲p < 0.05 vs. hUCMSCs group, ▲▲p < 0.01 vs. hUCMSCs group, ★p < 0.05 vs. tBHQ group, ★★p < 0.01 vs. tBHQ group