• Basic Research •

Human umbilical cord mesenchymal stem cells derived-exosomes on VEGF-A in hypoxic-induced mice retinal astrocytes and mice model of retinopathy of prematurity

Xiao-Tian Zhang[1,2], Bo-Wen Zhao[1,2], Yuan-Long Zhang[1,2], Song Chen[1,2]

[1]Tianjin Eye Hospital, Tianjin 300020, China
[2]Tianjin Key Lab of Ophthalmology and Visual Science, Tianjin 300020, China
Co-first authors: Xiao-Tian Zhang, Bo-Wen Zhao, and Yuan-Long Zhang
Correspondence to: Song Chen. Tianjin Eye Hospital, Tianjin Key Laboratory of Ophthalmology and Visual Science, Tianjin 300020, China. chensong9999@126.com
Received: 2023-12-17 Accepted: 2024-04-01

Abstract

● **AIM:** To observe the effect of human umbilical cord mesenchymal stem cells (hUCMSCs) secretions on the relevant factors in mouse retinal astrocytes, and to investigate the effect of hUCMSCs on the expression of vascular endothelial growth factor-A (VEGF-A) and to observe the therapeutic effect on the mouse model of retinopathy of prematurity (ROP).

● **METHODS:** Cultured hUCMSCs and extracted exosomes from them and then retinal astrocytes were divided into control group and hypoxia group. MTT assay, flow cytometry, reverse transcription-polymerase chain reaction (RT-PCR) and Western blot were used to detect related indicators. Possible mechanisms by which hUCMSCs exosomes affect VEGF-A expression in hypoxia-induced mouse retinal astrocytes were explored. At last, the efficacy of exosomes of UCMSCs in a mouse ROP model was explored. Graphpad6 was used to comprehensively process data information.

● **RESULTS:** The secretion was successfully extracted from the culture supernatant of hUCMSCs by gradient ultracentrifugation. Reactive oxygen species (ROS) and hypoxia inducible factor-1α (HIF-1α) of mice retinal astrocytes under different hypoxia time and the expression level of VEGF-A protein and VEGF-A mRNA increased, and the ROP cell model was established after 6h of hypoxia. The secretions of medium and high concentrations of hUCMSCs can reduce ROS and HIF-1α, the expression levels of VEGF-A protein and VEGF-A mRNA are statistically significant and concentration dependent. Compared with the ROP cell model group, the expression of phosphatidylinositol 3-kinase (PI3K)/protein kinase B (AKT)/mammalian target of rapamycin (mTOR) signal pathway related factors in the hUCMSCs exocrine group is significantly decreased. The intravitreal injection of the secretions of medium and high concentrations of hUCMSCs can reduce VEGF-A and HIF-1α in ROP model tissues. HE staining shows that the number of retinal neovascularization in ROP mice decreases with the increase of the dose of hUCMSCs secretion.

● **CONCLUSION:** In a hypoxia induced mouse retinal astrocyte model, hUCMSCs exosomes are found to effectively reduce the expression of HIF-1α and VEGF-A, which are positively correlated with the concentration of hUCMSCs exosomes. HUCMSCs exosomes can effectively reduce the number of retinal neovascularization and the expression of HIF-1α and VEGF-A proteins in ROP mice, and are positively correlated with drug dosage. Besides, they can reduce the related factors on the PI3K/AKT/mTOR signaling pathway.

● **KEYWORDS:** human umbilical cord mesenchymal stem cells; retinal astrocytes; retinopathy of prematurity; vascular endothelial growth factor; hypoxia inducible factor

DOI:10.18240/ijo.2024.07.07

Citation: Zhang XT, Zhao BW, Zhang YL, Chen S. Human umbilical cord mesenchymal stem cells derived-exosomes on VEGF-A in hypoxic-induced mice retinal astrocytes and mice model of retinopathy of prematurity. *Int J Ophthalmol* 2024;17(7):1238-1247

INTRODUCTION

The exact mechanism of retinopathy of prematurity (ROP) remains unclear, but this condition is common in preterm, low-birth-weight infants and is primarily characterized by retinal vascular proliferation. Relevant factors include insulin-like growth factor (IGF), placental growth factor (PIGF), platelet-derived growth factors (PDGFs), fibroblast growth factors (FGFs), and vascular endothelial growth factor (VEGF)[1-5]. Intravitreal VEGF levels are significantly elevated in ROP infants.

Astrocytes originate from ectodermal neuronal cells extending from the optic nerve head and morph into a network of stellate cells in the inner surface layer of the retina. In the development of ROP, astrocytes are a significant source of VEGF along the central pathway, and it has been demonstrated that under hypoxic conditions, retinal astrocytes increase the secretion of VEGF-A, and their upstream mRNA expression is similarly upregulated[6-10].

Currently, numerous researchers are focusing on the application of human umbilical cord mesenchymal stem cells (hUCMSCs) in ophthalmologic patients[11-12]. Ha *et al*[13] discovered anti-VEGF-A effects of hUCMSC exosomes in wet age-related macular degeneration (AMD) cells and animal models. As a result, hUCMSC exosomes are likely to have an anti-VEGF effector in ROP models.

The current treatment methods for ROP include cryotherapy, laser photocoagulation, anti-VEGF therapy and adjunct therapy. The gold standard for ROP treatment in the Asian region is laser photocoagulation delivered through laser indirect ophthalmoscopy[14]. However, both cryotherapy and laser photocoagulation have not addressed the potential mechanisms of retinal vascular proliferation in ROP, and the potential disadvantages of anti-VEGF therapy include the frequency and duration of follow-up, detection and management of recurrences, persistent peripheral avascularity, long-term effect on visual acuity and fields, systemic adverse effects particularly involving neurodevelopmental delay.

To improve existing therapies, we innovatively applies extracellular vesicles derived from mesenchymal stem cells to the ROP model, exploring their role in downregulating VEGF *in vitro* and in ROP animal models, as well as their possible mechanisms, providing new ideas for the treatment of ROP.

MATERIALS AND METHODS

Ethical Approval The study was conducted in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research (Approval No.YSY-DWLL-2022262).

Materials hUCMSCs came from Tianjin Eye Hospital Research Institute). C57BL/6J mice came from Yishengyuan Gene Technology Co., Ltd. (Laboratory Animal Use License No.SYKZ (Jin) 2021-0003); Exosome Extraction and Purification Kit came from Shanghai Yumeibo Biotechnology Co., Ltd; Reverse Transcription Kit came from Yijin Biology; anti-cluster of differentiation 9 (CD9) antibody, anti-integrin beta 3 antibody, anti-HSP70 antibody, anti-hypoxia inducible factor-1α (HIF-1α) antibody, anti-VEGF-A antibody, anti-phospho-protein kinase B (p-AKT) antibody, anti-phosphatidylinositol 3-kinase (PI3K) antibody, anti-phospho-mammalian target of rapamycin (p-mTOR) antibody, anti-m TOR antibody, anti-beta actin antibody, anti-AKT antibody, anti-phospho-phosphatidylinositol 3-kinase (p-PI3K) antibody were from Abcam; VEGF-A antibody came from proteintech; ranibizumab came from Lucentis

Methods

Culture of hUCMSCs and extraction of exosomes hUCMSCs were frozen in tubes and added to culture medium for culture and passaging. The supernatant of the cells was collected and centrifuged, and the exosomes were collected. Western blot was used to detect the expression levels of CD9, CD61 and HSP70 proteins in the exosomes. The size of pelleted structures was determined with dynamic light scattering (DLS) using a Zeta View®, a nanoparticle tracking analyzer for hydrodynamic particle size[15].

Effect of hUCMSCs exosomes on VEGF-A expression in hypoxia-induced mouse retinal astrocytes Retinal astrocytes were taken from C57BL/6J mice of 2d and cultured. The cells were divided into control group and hypoxia group. Control group: incubated in a conventional cell culture incubator; hypoxia group: 400 μmol/L cobalt chloride was added, and the cells were divided into 2, 6, 12, and 24h groups according to the hypoxia time. Methylthiazolyldiphenyl-tetrazolium bromide (MTT) assay was used to detect the proliferation rate of the cells in each group. Flow cytometry was used to detect the reactive oxygen species (ROS) of the cells in each group. The mRNA expression was detected by reverse transcription-polymerase chain reaction (RT-PCR). Western blot was used to detect the expression of HIF-1α and VEGF-A proteins in the cells in each group.

The cells were divided into control group, hypoxia group, hypoxia+low concentration (100 μg/mL) exosome group, hypoxia+medium concentration (200 μg/mL) exosome group, and hypoxia+high concentration (300 μg/mL) exosome group. MTT assay was used to detect the activity of the cells in each group at 24h. Western blot was used to detect the expression of HIF-1α and VEGF-A in different groups, RT-PCR detected the expression of VEGF-A mRNA in cells of different groups, and the kit detected the expression of ROS.

Exploration of possible mechanisms by which hUCMSCs exosomes affect VEGF-A expression in hypoxia-induced mouse retinal astrocytes Primary mouse retinal glial cells were cultured in the following groups. Control group: cultured in normal environment; hypoxia group: cultured in 400 μmol/L cobalt chloride; hypoxia+medium concentration exosome group: cultured in hypoxia for 12h, 200 μg/mL exosome was added; hypoxia+IGF-1 group: cultured in hypoxia for 12h, 200 ng/mL IGF-1; hypoxia+LY-294002 (protein kinase inhibitors that block phosphotidylinositol-3-kinase) group: hypoxia culture for 12h, add 10 μmol/L LY-294002; hypoxia+medium concentration exosome+IGF-1 group: hypoxia culture for 12h, add 200 μg/mL exosome

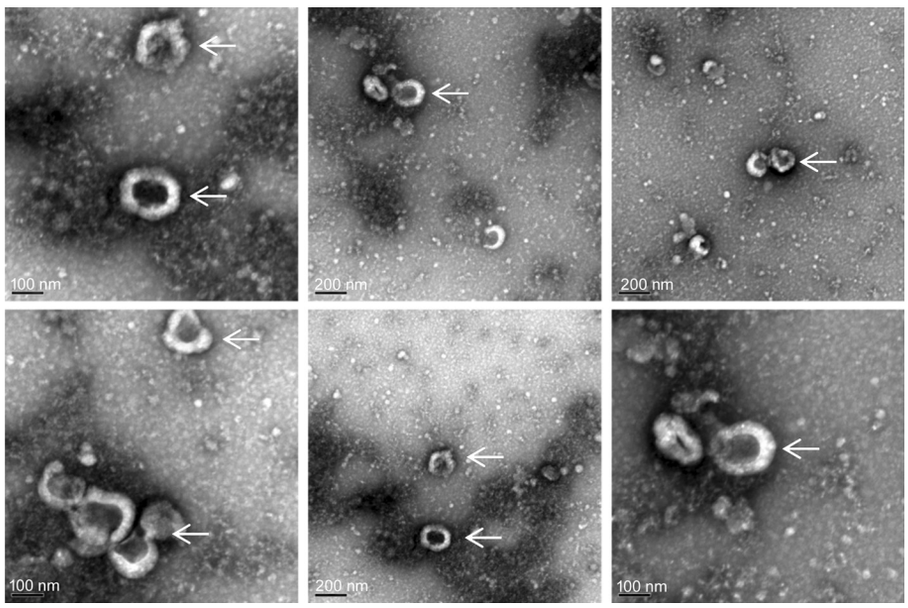

Figure 1 Exosomes of hUCMSCs observed under electron microscope Arrow: Exosomes. hUCMSCs: Human umbilical cord mesenchymal stem cells.

and 200 ng/mL IGF-1; hypoxia+medium concentration exosome+LY-294002 group: hypoxia culture for 12h, 200 μg/mL exosomes and 10 μmol/L LY-294002 were added.

After 12h of culture in each group, MTT was performed to detect cell activity, and Western blot was performed to detect the expression levels of p-AKT/AKT, p-PI3K/PI3K, p-mTOR/mTOR proteins in the cells of each group.

Efficacy of exosomes of UCMSCs in a mouse model of ROP C57BL/6J mice of 7–12d old were grouped: Group A (normal group): 5 mice cultured in normal environment; Group B (model group): 5 mice cultured in 75% high oxygen environment for 5d and then cultured in normal environment for 7d; Group C (low-dose hUCMSCs exosome group): the left eyes of 5 mice were injected with 2 μL 50 μg/mL hUCMSCs exosome on the 5th day and cultured in a normal environment for 7d; Group D (medium-dose huCMSCs exosome group): the left eyes of 5 mice were injected with 2 μL 100 μg/mL hUCMSCs exosome on the 5th day and cultured in a normal environment for 7d; Group E (high-dose hUCMSCs exosome group): the left eye of 5 mice was injected with 2 μL of 150 μg/mL hUCMSCs exosome on the 5th day and cultured in a normal environment for 7d; Group F (saline group): the left eye of 5 mice was injected with 2 μL of sterile saline on the 5th day and cultured in a normal environment for 7d; Group G (ranibizumab group): the left eye of 5 mice was injected with 2 μL of ranibizumab on the 5th day and cultured in a normal environment for 7d.

Hematoxylin-eosin (HE) staining was performed to observe the retinal structure, Western blot was performed to detect the expression of HIF-1α and VEGF-A proteins in each group, and RT-PCR was used to perform assays regarding the expression of HIF-1α mRNA and VEGF-A mRNA.

Statistical Analysis Graphpad6 was used to comprehensively process data information. All values are shown as the means ± standard deviation (SD). Student's *t* test analysis was performed to analyze all data. $P<0.05$ was considered significant difference.

RESULTS

Culture of hUCMSCs and Extraction of Exosomes The hUCMSCs grew well. HUCMSCs exosomes could be observed under transmission electron microscopy (Figure 1). Nanoparticle tracking analysis assay revealed that the diameter of exosomes was 138.8±49.1 nm (Figure 2). Western blot assay showed that the expression of CD9, CD61 and HSP70 was upregulated in the mesenchymal stem cell (MSC) group ($P<0.05$; Figure 3).

Effects of hUCMSCs Exosomes on VEGF-A Expression in Hypoxia-Induced Mouse Retinal Astrocytes MTT test showed that cell damage increased with increasing hypoxia time (Figure 4), of which the difference was significant ($P<0.05$) at 12h and 24h of hypoxia (Table 1). The results of Western blot test, RT-PCR test, and flow cytometry showed that HIF-1α, VEGF-A protein expression, VEGF-A mRNA and ROS expression gradually increased with hypoxia time ($P<0.05$; Figures 5–7, Table 2). The 6h hypoxia group's cellular activity did not undergo a significant decrease, and the related protein expression and mRNA expression levels were increased, so the 6h hypoxia group was selected as the model group.

The results of MTT assay, flow cytometry, RT-PCR assay, and Western blot assay showed that the cellular activity was decreased in hypoxia group, and the protein expression of

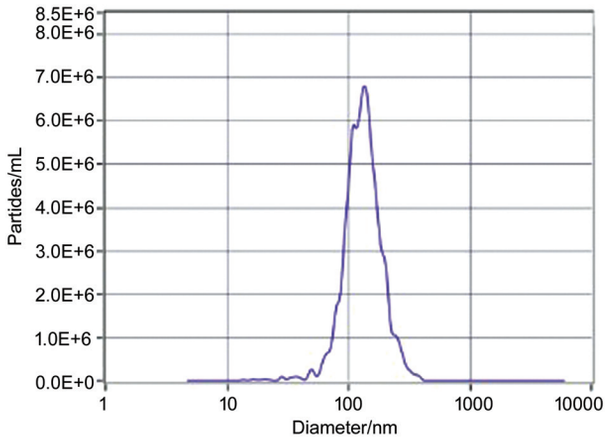

Figure 2 Nanoparticle tracking analysis (NTA) detection of exosome diameter.

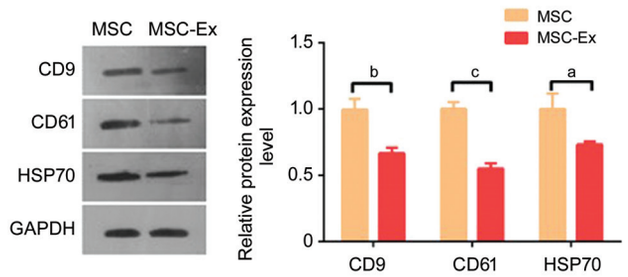

Figure 3 Western blot detection of CD9, CD61, HSP70 in exosomes [a]*P*<0.05; [b]*P*<0.01; [c]*P*<0.001. MSC: Mesenchymal stem cells; MSC-Ex: Mesenchymal stem cells exsomes; CD9: Cluster of differentiation 9; CD61: Cluster of differentiation 61; HSP70: Heat shock 70 kDa protein; GAPDH: Glyceraldehyde-3-phosphate dehydrogenase.

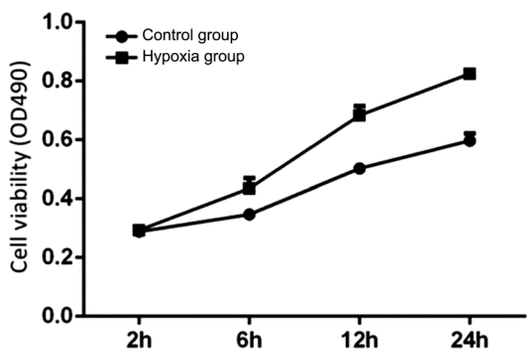

Figure 4 Assay of cell activity in each group.

ROS, VEGF-A mRNA, HIF-1α, and VEGF-A was increased (*P*<0.05), but the decrease in cellular activity was mitigated with the increase of exosomal concentration of hUCMSCs, and the protein expression of ROS, VEGF-A mRNA, HIF-1α, and VEGF-A protein expression were gradually decreased, in which the difference between the middle and high exosome concentration groups was statistically significant (*P*<0.05; Table 3, Figures 8–11). Starting from exosomes 200 μg/mL, the level of cellular oxidative stress decreased with the increase of exosomes concentration, and the expression of related proteins and mRNAs were also down-regulated with the increase of exosomes concentration, so exosomes 200 μg/mL was selected as the concentration of model group.

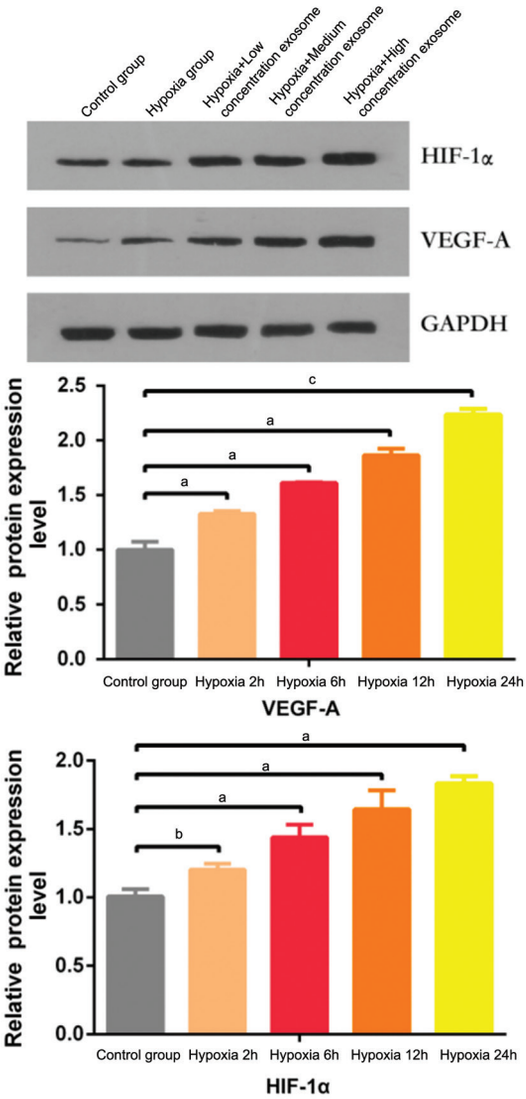

Figure 5 The expression levels of proteins of HIF-1α and VEGF-A were detected by Western blot [a]*P*<0.05; [b]*P*<0.01; [c]*P*<0.001. VEGF-A: Vascular endothelial growth factor-A; HIF-1α: Hypoxia inducible factor-1α; GAPDH: Glyceraldehyde-3-phosphate dehydrogenase.

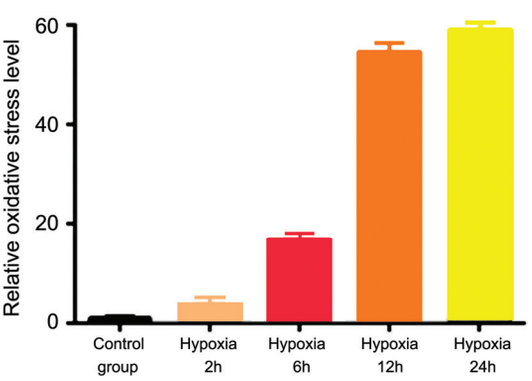

Figure 6 ROS expression level in cells in each group of flow cytometry ROS: Reactive oxygen species.

Exploration of Possible Mechanisms by Which hUCMSCs Exosomes Affect VEGF-A Expression in Hypoxia-Induced Mouse Retinal Astrocytes MTT assay and Western blot assay showed that the hypoxia group showed decreased

Table 1 OD values of MTT test for each groups of cells

Groups	2h	6h	12h	24h
Control group	0.289±0.012	0.347±0.026	0.503±0.032	0.597±0.038
Hypoxia group	0.294±0.020	0.385±0.019	0.581±0.029	0.695±0.041
P	0.812	0.143	0.031[a]	0.004[a]

OD: Optical density; MTT: Methylthiazolyldiphenyl-tetrazolium bromide. [a]*P*<0.05.

Table 2 ROS expression levels in each group

Parameters	Control group	Hypoxia 2h	Hypoxia 6h	Hypoxia 12h	Hypoxia 24h
ROS	1.69±0.43	6.55±1.73	28.31±1.98	93.20±2.14	99.92±0.14
P		0.031[a]	0.007[a]	0.000[a]	0.000[a]

ROS: Reactive oxygen species. [a]*P*<0.05 *vs* control group.

Table 3 OD values of MTT test, ROS expression levels, HIF-1α concentration, and VEGF-A concentration in each group

Parameters	Control group	Hypoxia group	Hypoxia+Low concentration exosome	Hypoxia+Medium concentration exosome	Hypoxia+High concentration exosome
OD	0.47±0.09	0.82±0.12	0.82±0.18	0.69±0.21	0.56±0.17
t			1.892	4.870	5.195
P			0.892	0.013[a]	0.007[a]
ROS	2.91±0.68	93.60±2.36	93.03±2.06	33.43±4.37	7.67±1.46
t			2.781	4.598	7.891
P			0.782	0.013[a]	0.002[a]
HIF-1α concentration	0.99±0.04	1.65±0.02	1.63±0.04	1.46±0.05	1.22±0.02
t			1.980	3.679	4.294
P			0.082	0.031[a]	0.001[a]
VEGF-A concentration	1.01±0.14	2.09±0.04	2.03±0.05	1.77±0.06	1.61±0.05
t			1.362	2.867	4.562
P			0.769	0.021[a]	0.001[a]

OD: Optical density; MTT: Methylthiazolyldiphenyl-tetrazolium bromide; ROS: Reactive oxygen species; VEGF-A: Vascular endothelial growth factor-A. [a]*P*<0.05 *vs* hypoxia group.

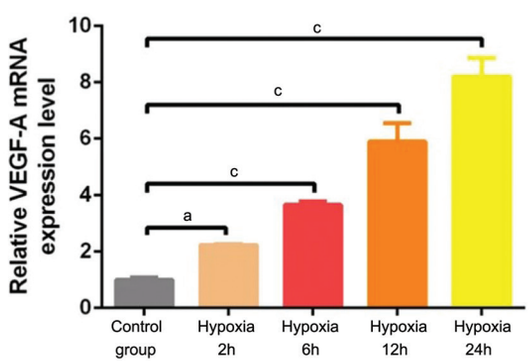

Figure 7 The expression levels of VEGF-A mRNA were detected by RT-PCR [a]*P*<0.05; [c]*P*<0.001. VEGF-A: Vascular endothelial growth factor-A; RT-PCR: Reverse transcription-polymerase chain reaction.

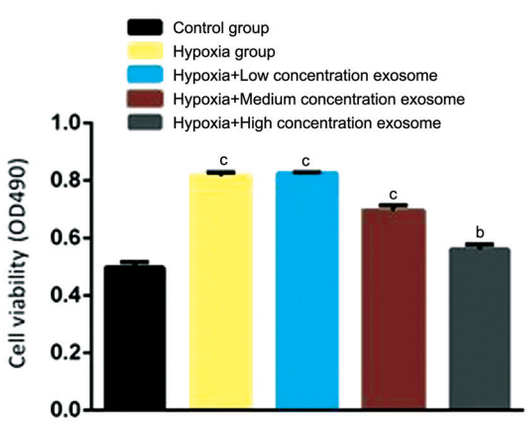

Figure 8 Cell activity assay in each group [b]*P*<0.01; [c]*P*<0.001.

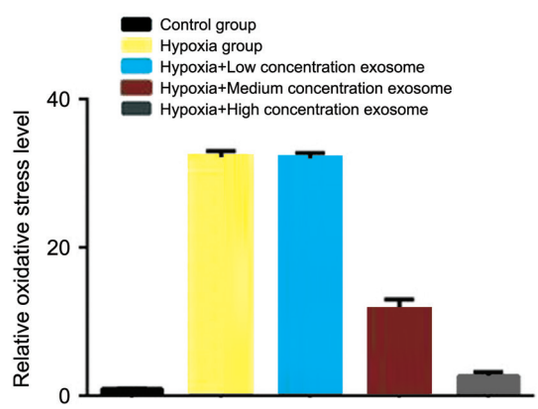

Figure 9 ROS expression level of cells in each group of flow cytometry ROS: Reactive oxygen species.

cell activity, up-regulation of p-AKT, p-PI3K, and p-mTOR expression compared with the control group (*P*<0.05), while the hypoxia group+medium-dose exosome group+LY-294002 group showed improved cell activity, down-regulation of p-AKT, p-PI3K, and p-mTOR expression compared with the hypoxia group (*P*<0.05), while the cellular activity of the hypoxia group+LY-294002 group improved, p-AKT, p-PI3K, p-mTOR expression was down-regulated compared with that of the hypoxia group+medium-dose exosome group, but the difference was not statistically significant (*P*>0.05), and the cellular activity of the group that added both medium-

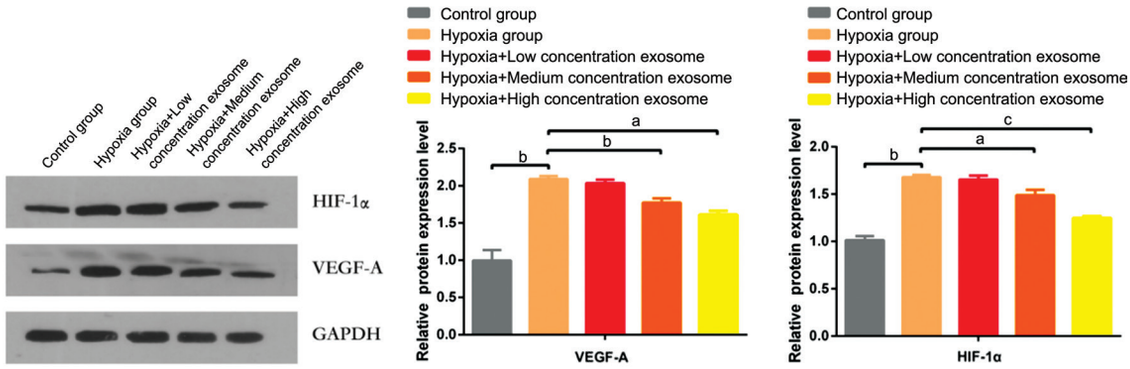

Figure 10 The expression levels of proteins of HIF-1α and VEGF-A were detected by Western blot [a]P<0.05; [b]P<0.01; [c]P<0.001. VEGF-A: Vascular endothelial growth factor-A; HIF-1α: Hypoxia inducible factor-1α; GAPDH: Glyceraldehyde-3-phosphate dehydrogenase.

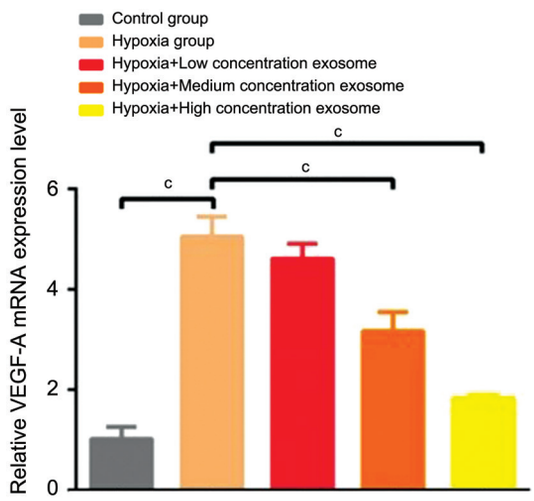

Figure 11 The levels of mRNA of HIF-1α and VEGF-A were detected by RT-PCR [c]P<0.001. VEGF-A: Vascular endothelial growth factor-A; HIF-1α: Hypoxia inducible factor-1α; RT-PCR: Reverse transcription-polymerase chain reaction.

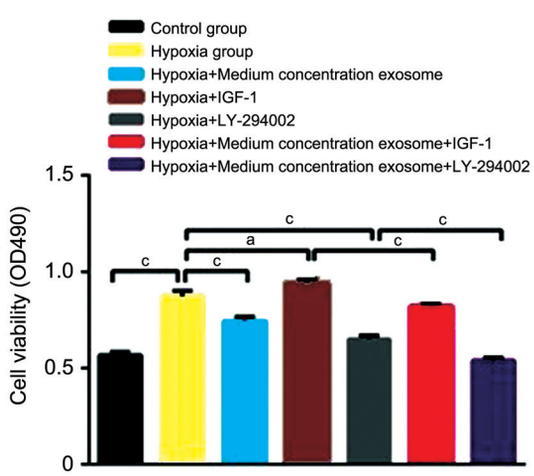

Figure 12 Cell viability assay in each group [a]P<0.05; [c]P<0.001. IGF-1: Insulin-like growth factor-1.

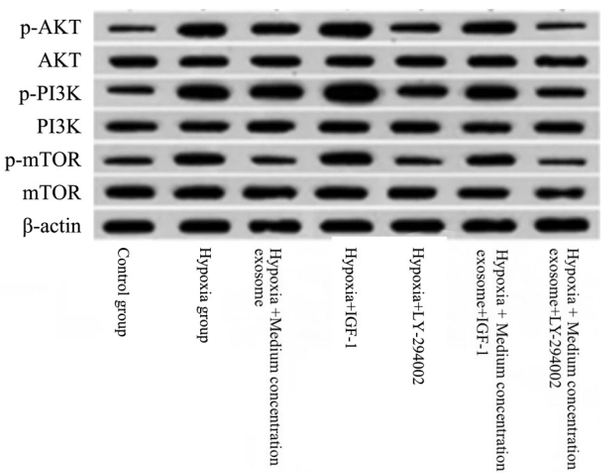

Figure 13 Western blot bands AKT: Protein kinase B; p-AKT: Phospho-AKT; PI3K: Phosphatidylinositol-3-kinase; p-PI3K: Phospho-PI3K; mTOR: Mammalian target of rapamycin; p-mTOR: Phospho-mTOR; IGF-1: Insulin-like growth factor-1.

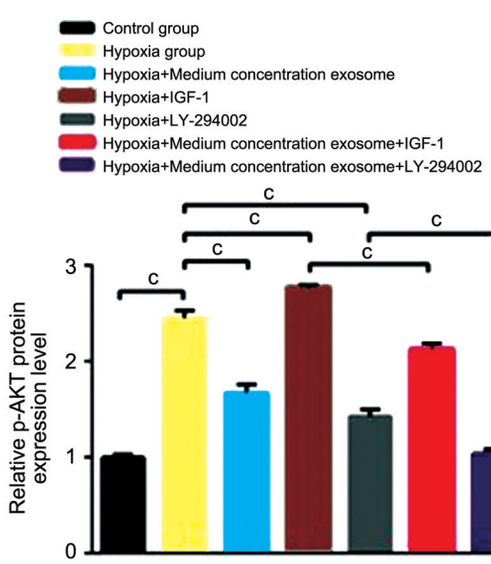

Figure 14 Western blot detection of p-AKT expression level in each group [c]P<0.001. p-AKT: Phospho-AKT; IGF-1: Insulin-like growth factor-1.

dose exosome and the inhibitor improved, p-AKT, p-PI3K, p-mTOR expression was down-regulated (P<0.05). The cellular model with the addition of IGF-1 activator had lower cellular activity, up-regulated p-AKT, p-PI3K, and p-mTOR expression (P<0.05) compared to the hypoxia model cells, while the addition of the medium-dose exosomes showed significant improvement in cellular activity, and significant

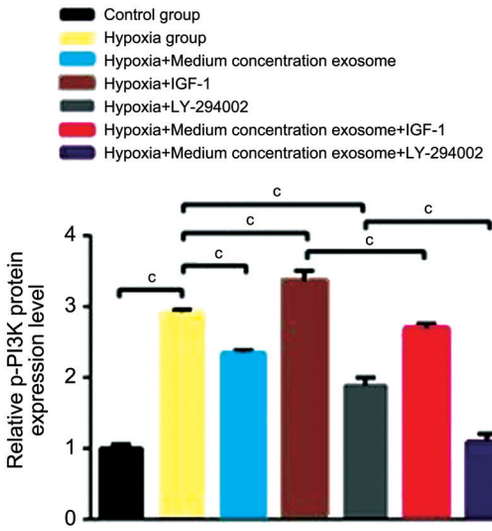

Figure 15 Western blot detection of p-PI3K expression level in each group [c]*P*<0.001. p-P13K: Phospho-PI3K; IGF-1: Insulin-like growth factor-1.

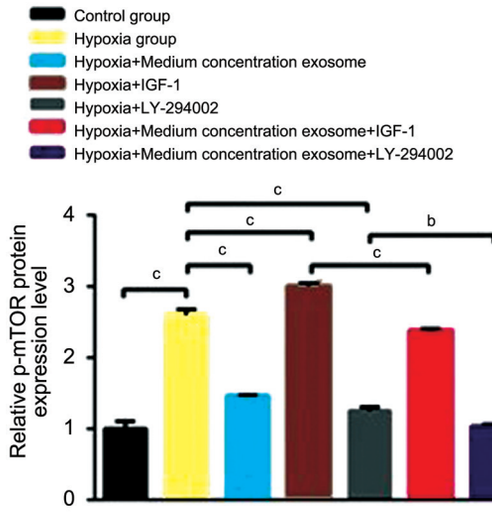

Figure 16 Western blot detection of p-mTOR expression levels in different treatment groups [b]*P*<0.01; [c]*P*<0.001. p-mTOR: Phospho-mTOR; IGF-1: Insulin-like growth factor-1.

down-regulation (*P*<0.05) of p-AKT, p-PI3K, and p-mTOR expression (Figures 12–16).

Efficacy of hUCMSCs Exosomes in a Mouse Model of ROP

All enrolled mice survived well, and no ocular inflammatory reaction was detected in any of the groups after drug injection (Figure 17).

The retina of mice in the HE-stained normal and saline groups was structurally intact with neat edges; a large number of endothelial cell nuclei breaking through the inner boundary membrane could be seen in the retina of mice in the model group; the structural characteristics of retinal disorders in mice in the dose-from-low-to-high-exosome group showed a tendency from poor to good, with a decrease in the number of endothelial cell nuclei breaking through the inner boundary membrane. Similarly, the number of mice breaking through the nucleus of the inner boundary membrane endothelial cells in the ranibizumab group was extremely low (Figures 18 and 19).

RT-PCR test and Western blot test showed that the expression of HIF-1α, VEGF-A mRNA and protein was up-regulated in the model group compared with the normal group (*P*<0.05), and the expression of related mRNA and protein was gradually decreased with the increase of exosomal injection of hUCMSCs, which was decreased in the medium- and high-dose exosome group and the razumab group compared with the model group (*P*<0.05). Although HIF-1α mRNA and protein were reduced in the high-dose exosome group compared with the razumab group, and VEGF-A mRNA and protein were reduced in the razumab group compared with the high-dose exosome group, the difference was not statistically significant (*P*>0.05; Figures 20 and 21).

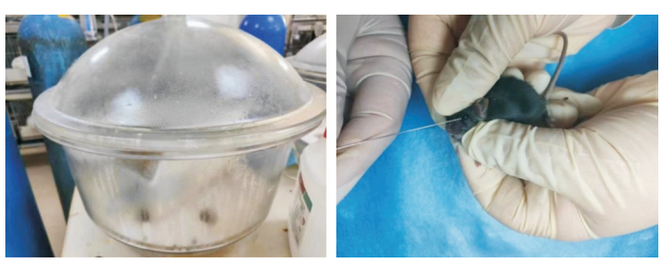

Figure 17 Oxygen chamber used in the experiment and drugs injected into the vitreous cavity of mice.

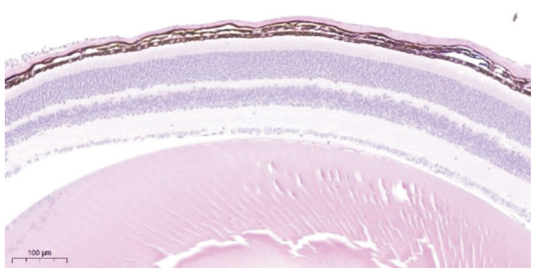

Figure 18 Hematoxylin-eosin staining of retinal sections of mice in the normal group.

DISCUSSION

Exosomes are extracellular vesicles approximately 100 nm in diameter surrounded by a lipid bilayer released in response to microenvironmental influences. They can deliver their membrane signals[16] or cellular contents (including nucleic acids, lipids, mitochondria, and proteins)[17-19] into the target cell, leading to physiological changes in the target cell. MSC exosomes have similar functions to MSCs, and compared with MSCs, MSC-derived exosomes (MSC-Exo) are stable, non-immunogenic, easy to transform, and can easily pass through crossing the biological barriers into target organs. A study showed that exosomes from MSCs could effectively improve hyperoxia-induced retinopathy, and the exosomes were well tolerated after intravitreal injection, providing a new idea for non-cellular treatment of ROP[20]. We successfully extracted exosomes from the culture supernatant of hUCMSCs and observed specific hUCMSC surface proteins CD9, CD61, and

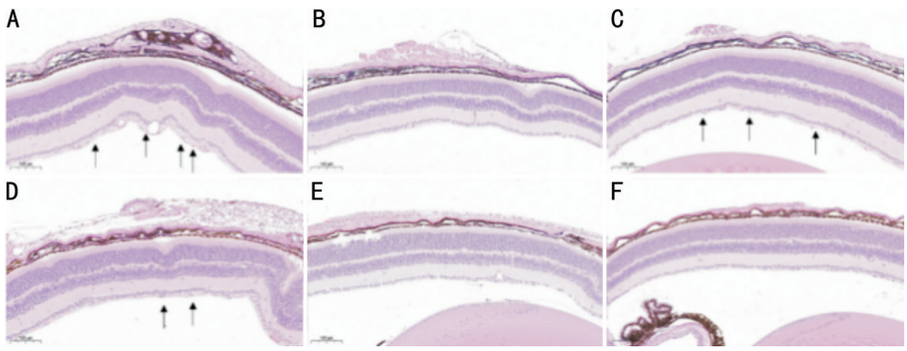

Figure 19 Hematoxylin-eosin staining of retinal sections of mice A: The model group; B: The saline group; C: The low-dose hUCMSCs exosome group; D: The medium-dose hUCMSCs exosome group; E: The high-dose hUCMSCs exosome group; F: The ranibizumab group. Arrow: Nuclei of endothelial cells breaking through the inner border membrane. hUCMSCs: Human umbilical cord mesenchymal stem cells.

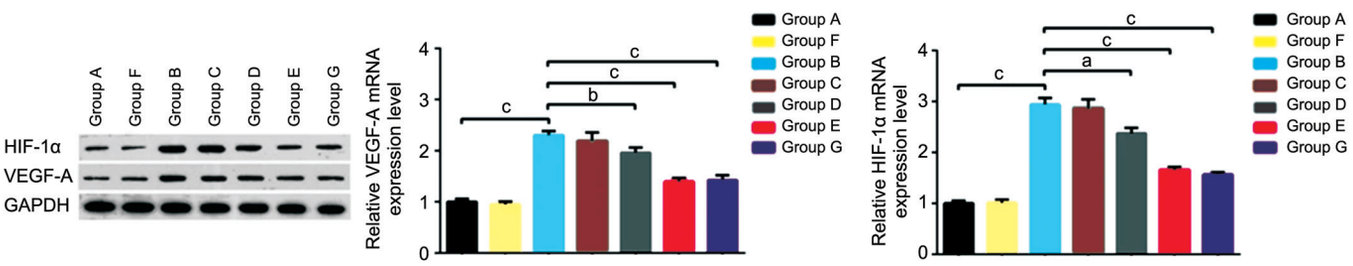

Figure 20 Western blot detection of HIF-1α, VEGF-A protein expression levels in different treatment groups [a]*P*<0.05; [b]*P*<0.01; [c]*P*<0.001. VEGF-A: Vascular endothelial growth factor-A; HIF-1α: Hypoxia inducible factor-1α; GAPDH: Glyceraldehyde-3-phosphate dehydrogenase. Group A: Normal group; Group B: Model group; Group C: Low-dose hUCMSCs exosome group; Group D: Medium-dose huCMSCs exosome group; Group E: High-dose hUCMSCs exosome group; Group F: Saline group; Group G: Ranibizumab group.

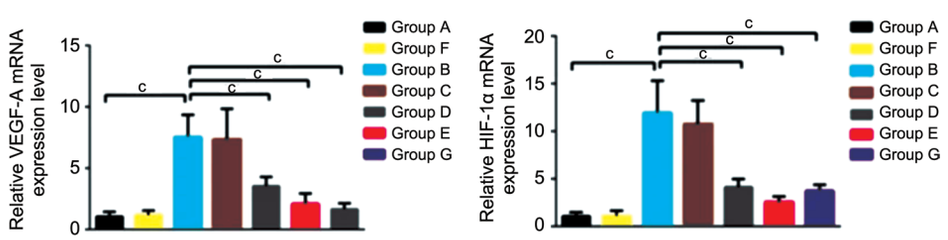

Figure 21 RT-PCR detection of mRNA expression levels in each group [c]*P*<0.001. VEGF-A: Vascular endothelial growth factor-A; RT-PCR: Reverse transcription-polymerase chain reaction. Group A: Normal group; Group B: Model group; Group C: Low-dose hUCMSCs exosome group; Group D: Medium-dose huCMSCs exosome group; Group E: High-dose hUCMSCs exosome group; Group F: Saline group; Group G: Ranibizumab group.

HSP70 by Western blot assay, which proved that the channel source was reliable and provided a solid foundation for subsequent experiments.

Preterm infants exhibit incomplete development of the retinal vasculature and a peripheral avascular zone[21]. In addition, an increase in oxygen bioavailability at birth exposes preterm infants to a relatively hyperoxic environment that, coupled with the infant's immature antioxidant system, leads to oxidative stress[22]. The pathophysiology of ROP is described by a two-phase hypothesis in which Phase I is characterized by compromised and delayed intraretinal vascularization, and Phase II is characterized by vision-threatening extraretinal or intravitreal neovascularization (IVNV)[23], pathologic IVNVs fail to supply blood to the nonperfused areas of the retina and even grow into the vitreous, ultimately causing retinal tugging, hemorrhage, and even detachment of the retina[24]. Studies in retinal vascular models have shown that astrocytes are a core pathway source of VEGF if hypoxia occurs during development. It has been demonstrated that under hypoxic conditions, the secretion of VEGF-A by retinal astrocytes is increased and its upstream mRNA expression is similarly upregulated[6-10]. The main molecular mechanism is the abnormal activation of the HIF pathway, which in turn regulates the expression of pro-angiogenic genes and their corresponding receptors, such as VEGF and its receptors VEGFR1/VEGFR2, stromal cell-derived factor-1 (SDF-1) and its receptor CXCR4, PDGF-B and its receptor platelet-derived growth factor-b receptor (PDGFRB), and angiopoietin-2 (ANGP2) and its receptor tyrosine kinase (TIE2)[25]. The interactions between these pro-angiogenic factors collectively lead to retinal neovascularization (RNV) development. In addition, oxidative stress and excessive accumulation of ROS are important mechanisms. In ROP, ROS are overproduced and Src/Ras/p38MAPK protein kinase and JNK protein kinase are

overactivated, which can oxidatively damage the transcription factors HIF-1, cMyb, Sp-1, EGR-1, cFos, cJun, AP-1, *etc.*, so that they are unable to bind with the target gene promoters, inhibit the expression of the target genes, and promote apoptosis, and at the same time activate the signaling pathway HIF, which ultimately led to the generation of IVNV[26]. Our experiments also verified this point, and we also found that the activity of retinal astrocytes in the 6h hypoxia group did not undergo a significant reduction, and the expression levels of ROS, HIF-1α, VEGF-A protein and VEGF-A mRNA were all increased, so we finally chose the 6h hypoxia group as the model group of ROP cells. Then different concentrations of hUCMSCs exosomes were applied to the biological cells of the standard model group at different times, and it was found that starting from hUCMSCs exosomes 200 μg/mL, the level of cellular oxidative stress was reduced, and the expression of related proteins and mRNAs were down-regulated at the same time. Therefore, 200 μg/mL (medium concentration) exosomes was finally selected as the experimental concentration. Then, by detecting the related factors on the PI3K/AKT/mTOR signaling pathway, it was found that hUCMSCs exosomes could inhibit the PI3K/AKT/mTOR signaling pathway as well as the pathway inhibitors, and reduce the expression of VEGF-A and HIF-1α proteins. This may be a possible mechanism by which hUCMSCs exosomes play a role in the ROP cell model.

Finally, we chose the mouse model of oxygen-induced retinopathy (OIR) to study the effects of drug administration *via* intravitreal administration on IVNV. Mouse OIR is the most commonly used model to mimic ROP pathology. Chen *et al*[27] successfully created a rat OIR model by exposing neonatal rats to alternating hyper/hypoxic environments. The success of this method demonstrated that intermittent hyperoxia/hypoxia exacerbated OIR, consistent with clinical findings[28-34]. HE staining showed a greater number of vascular endothelial cell nuclei breaching the inner border membrane present in the retina of the model group, which demonstrated the success of the modeling. In contrast, the protein expression and vascular endothelial cells of the control group injected with saline in the vitreous cavity did not differ from those of the normal group, which implies that the operation of vitreous cavity injection, *per se*, does not have an impact on the observables. In addition, we also quantitatively assessed the effect of intravitreal injection of hUCMSCs exosomes on VEGF-A, HIF-1α and corresponding mRNA expression in a mouse model of OIR. After injection of medium and high doses of hUCMSCs exosomes into the vitreous cavity, the expression of VEGF-A, HIF-1α and corresponding mRNAs were down-regulated in a concentration-dependent manner, and the corresponding number of vascular endothelial cell nuclei breaching the inner border membrane in the retina was reduced, which suggests that medium and high doses of hUCMSCs exosomes improve the retina of the mouse model of OIR through VEGF-A, HIF-1α RNV and achieved therapeutic effect; given that the results of comparing the medium and high doses of hUCMSCs exosomes with ranibizumab were not statistically significant, it can be concluded that the medium and high doses of hUCMSCs exosomes have the same effect as ranibizumab in reducing the RNV of the retina and lowering the VEGF-A and HIF-1α.

HUCMSCs exosomes have multiple roles, and there are also multiple ways to affect the generation of IVNV. In the combination of medium-dose hUCMSCs exosomes and pathway inhibitors, the down-regulation of the PI3K/AKT/mTOR signaling pathway was better than that of using the pathway inhibitors alone, which suggests that the PI3K/AKT/mTOR signaling pathway is one of the possible signaling pathways. Hence, further research is needed to explore whether there is any other mechanism. In addition, the potential toxicity of exosomes should not be ignored, due to the limited experimental grouping, failed to carry out higher concentrations of hUCMSCs exosomes test to observe its toxic side effects. And we should also add electrophysiological examination to improve the experimental results.

To summarize, our experiments took a combined *in vivo* and *in vitro* approach before concluding that down-regulation of hypoxia-induced expression of VEGF-A and HIF-1α in retinal astrocytes, and consequently reduction of the generation of IVNV, which creates more chances and possibilities for the treatment of ROP, it is still have a long way to go and need to keep experimenting until it is determined that it is safe to be applied in the clinic.

ACKNOWLEDGEMENTS

Foundation: Supported by Tianjin Key Medical Discipline Specialty Construction Project (No.TJXZDXK-016A); Science Foundation of Tianjin Eye Hospital (No.YKZD1901).

Conflicts of Interest: Zhang XT, None; **Zhao BW,** None; **Zhang YL,** None; **Chen S,** None.

REFERENCES

1 Dogra MR, Katoch D, Dogra M. An update on retinopathy of prematurity (ROP). *Indian J Pediatr* 2017;84(12):930-936.

2 Dammann O, Hartnett ME, Stahl A. Retinopathy of prematurity. *Develop Med Child Neuro* 2023;65(5):625-631.

3 Fu YY, Lei CY, Ran QB, Huang X, Chen YY, Wang M, Zhang MX. Insulin-like growth factor-1 and retinopathy of prematurity: a systemic review and meta-analysis. *Surv Ophthalmol* 2023;68(6): 1153-1165.

4 Wilkinson-Berka JL, Babic S, De Gooyer T, *et al*. Inhibition of platelet-derived growth factor promotes pericyte loss and angiogenesis in ischemic retinopathy. *Am J Pathol* 2004;164(4):1263-1273.

5 Dumbrăveanu L, Cușnir V, Bobescu D. A review of neovascular glaucoma. Etiopathogenesis and treatment. *Rom J Ophthalmol* 2021;65(4):315-329.

6 O'Sullivan ML, Puñal VM, Kerstein PC, Brzezinski JA 4th, Glaser T, Wright KM, Kay JN. Astrocytes follow ganglion cell axons to establish an angiogenic template during retinal development. *Glia* 2017;65(10):1697-1716.

7 Perelli RM, O'Sullivan ML, Zarnick S, Kay JN. Environmental oxygen regulates astrocyte proliferation to guide angiogenesis during retinal development. *Development* 2021;148(9):dev199418.

8 Mueller SK, Nocera AL, Bleier BS. Exosome function in aerodigestive mucosa. *Nanomedicine* 2018;14(2):269-277.

9 Yu B, Zhang XM, Li XR. Exosomes derived from mesenchymal stem cells. *Int J Mol Sci* 2014;15(3):4142-4157.

10 Nakano A, Kondo R, Kaneko Y, *et al*. Changes in components of the neurovascular unit in the retina in a rat model of retinopathy of prematurity. *Cell Tissue Res* 2020;379(3):473-486.

11 Wang Y, Long W, Cao Y, Li JY, You LH, Fan YR. Mesenchymal stem cell-derived secretomes for therapeutic potential of premature infant diseases. *Biosci Rep* 2020;40(5):BSR20200241.

12 Yaghoubi Y, Movassaghpour A, Zamani M, Talebi M, Mehdizadeh A, Yousefi M. Human umbilical cord mesenchymal stem cells derived-exosomes in diseases treatment. *Life Sci* 2019;233:116733.

13 Ha D, Yang NN, Nadithe V. Exosomes as therapeutic drug carriers and delivery vehicles across biological membranes: current perspectives and future challenges. *Acta Pharm Sin B* 2016;6(4):287-296.

14 Sen P, Wu WC, Chandra P, Vinekar A, Manchegowda PT, Bhende P. Retinopathy of prematurity treatment: Asian perspectives. *Eye* (*Lond*) 2020;34(4):632-642.

15 Wang YJ, Zhang L, Li YJ, *et al*. Exosomes/microvesicles from induced pluripotent stem cells deliver cardioprotective miRNAs and prevent cardiomyocyte apoptosis in the ischemic myocardium. *Int J Cardiol* 2015;192:61-69.

16 Gurung S, Perocheau D, Touramanidou L, Baruteau J. The exosome journey: from biogenesis to uptake and intracellular signalling. *Cell Commun Signal* 2021;19(1):47.

17 Kalluri R, LeBleu VS. The biology, function, and biomedical applications of exosomes. *Science* 2020;367(6478):eaau6977.

18 Edgar JR. Q&A: what are exosomes, exactly? *BMC Biol* 2016;14:46.

19 Xia XH, Wang Y, Qin Y, Zhao S, Zheng JC. Exosome: a novel neurotransmission modulator or non-canonical neurotransmitter? *Ageing Res Rev* 2022;74:101558.

20 Moisseiev E, Anderson JD, Oltjen S, Goswami M, Zawadzki RJ, Nolta JA, Park SS. Protective effect of intravitreal administration of exosomes derived from mesenchymal stem cells on retinal ischemia. *Curr Eye Res* 2017;42(10):1358-1367.

21 Erdöl H, Hacioglu D, Kola M, Türk A, Aslan Y. Investigation of the effect of hemoglobin F and A levels on development of retinopathy of prematurity. *J Am Assoc Pediatr Ophthalmol Strabismus* 2017;21(2):136-140.

22 Fevereiro-Martins M, Marques-Neves C, Guimarães H, Bicho M. Retinopathy of prematurity: a review of pathophysiology and signaling pathways. *Surv Ophthalmol* 2023;68(2):175-210.

23 Ramshekar A, Bretz CA, Hartnett ME. RNA-seq provides insights into VEGF-induced signaling in human retinal microvascular endothelial cells: implications in retinopathy of prematurity. *Int J Mol Sci* 2022;23(13):7354.

24 Hessvik NP, Llorente A. Current knowledge on exosome biogenesis and release. *Cell Mol Life Sci* 2018;75(2):193-208.

25 Shah N, Ishii M, Brandon C, Ablonczy Z, Cai JW, Liu YT, Chou CJ, Rohrer B. Extracellular vesicle-mediated long-range communication in stressed retinal pigment epithelial cell monolayers. *Biochim Biophys Acta Mol Basis Dis* 2018;1864(8):2610-2622.

26 Knickelbein JE, Liu BY, Arakelyan A, *et al*. Modulation of immune responses by extracellular vesicles from retinal pigment epithelium. *Invest Ophthalmol Vis Sci* 2016;57(10):4101-4107.

27 Chen B, Li Q, Zhao BZ, Wang Y. Stem cell-derived extracellular vesicles as a novel potential therapeutic tool for tissue repair. *Stem Cells Transl Med* 2017;6(9):1753-1758.

28 Vähätupa M, Järvinen TAH, Uusitalo-Järvinen H. Exploration of oxygen-induced retinopathy model to discover new therapeutic drug targets in retinopathies. *Front Pharmacol* 2020;11:873.

29 Lu F, Liu Y, Guo YL, Gao YX, Piao YG, Tan S, Tang YZ. Metabolomic changes of blood plasma associated with two phases of rat OIR. *Exp Eye Res* 2020;190:107855.

30 Fouda AY, Xu ZM, Suwanpradid J, *et al*. Targeting proliferative retinopathy: Arginase 1 limits vitreoretinal neovascularization and promotes angiogenic repair. *Cell Death Dis* 2022;13(8):745.

31 Zhao K, Jiang YP, Zhang J, Shi J, Zheng PX, Yang CX, Chen YH. Celastrol inhibits pathologic neovascularization in oxygen-induced retinopathy by targeting the miR-17-5p/HIF-1α/VEGF pathway. *Cell Cycle* 2022;21(19):2091-2108.

32 Ramshekar A, Hartnett ME. Vascular endothelial growth factor signaling in models of oxygen-induced retinopathy: insights into mechanisms of pathology in retinopathy of prematurity. *Front Pediatr* 2021;9:796143.

33 Yang JH, Gao F, Zhang YK, Liu Y, Zhang D. Buyang Huanwu Decoction (BYHWD) enhances angiogenic effect of mesenchymal stem cell by upregulating VEGF expression after focal cerebral ischemia. *J Mol Neurosci* 2015;56(4):898-906.

34 Guo XJ, Tian XS, Ruan Z, *et al*. Dysregulation of neurotrophic and inflammatory systems accompanied by decreased CREB signaling in ischemic rat retina. *Exp Eye Res* 2014;125:156-163.