·Basic Research·

Effect of endothelial progenitor cells derived from human umbilical cord blood on oxygen-induced retinopathy in mice by intravitreal transplantation

Dan Wang[1], Bo Zhang[2], Hui Shi[1], Wei Yang[1], Ming-Chao Bi[1], Xiang-Fu Song[3], Chen Zhang[4], Jian-Hui Cheng[5], Ji-Long Hao[1], E Song[1,5]

[1]Department of Ophthalmology, First Hospital, Jilin University, Changchun 130021, Jilin Province, China
[2]Department of Neurosurgery, First Hospital, Jilin University, Changchun 130021, Jilin Province, China
[3]School of Public Health, Jilin University, Changchun 130021, Jilin Province, China
[4]School of Medicine, Tongji University, Shanghai 200092, China
[5]Lixiang Eye Hospital, Soochow University, Soochow 215021, Jiangsu Province, China
Co-first authors: Dan Wang and Bo Zhang
Correspondence to: E Song. Department of Ophthalmology, Lixiang Eye Hospital, Soochow University, Soochow 215021, Jiangsu Province, China. Department of Ophthalmology, First Hospital, Jilin University, Changchun 130021, Jilin Province, China. songe23@163.com. Ji-Long Hao. Department of Ophthalmology, First Hospital, Jilin University, Changchun 130021, Jilin Province, China. haojl@jlu.edu.cn
Received: 2016-04-12 Accepted: 2016-06-06

Abstract

• AIM: To investigate the effect of endothelial progenitor cells (EPCs) labeled by carboxy fluorescein diacetate succinimidyl ester (CFSE) on murine oxygen-induced retinopathy (OIR) by intravitreal transplantation.

• METHODS: After isolated from human umbilical cord blood mononuclear cells, EPCs were cultivated and then labeled with CFSE *in vitro*. C57BL/6J mice were placed to 75% hyperoxia chamber from P7 to P12 to establish OIR model. At P12, OIR mice were intravitreally injected with 1 μL suspension contained 2×10^5 EPCs (EPCs group) or isometric phosphate buffered saline (PBS group). The contralateral eye of each mice received no injection (OIR group). Evans blue angiography and frozen section were examined to track the labeled cells in OIR group at P15 and P19. Using retina paraffin sections and adenosinediphos phatase staining at P12 and P19, the effect of EPCs on OIR mice was evaluated quantitatively and qualitatively.

• RESULTS: The retinas from EPCs group with less non-perfusion area and fewer peripheral tufts were observed at P19, comparing with that from PBS or OIR group. The retinopathy in EPCs group receded earlier with less non-ganglion cells and neovascular nuclei, together with relatively regular distribution. The counts of the neovascular nuclei at P19 were reduced by 44% or 45%, compared with those of OIR group or PBS group respectively. Three days after EPCs injection, a large number of EPCs appeared in the vitreous cavity and adhered to the retinal surface. While at one week, the cells gathered between the internal plexiform layer and the inner limiting membrane, and some EPCs appeared in retinal vessels.

• CONCLUSION: EPCs transplantation can participate in the reparative procedure of the neovascularization in OIR.

• **KEYWORDS:** endothelial progenitor cells; retinal neovascularization; oxygen induced retinopathy; retinopathy of prematurity; cell transplantation; mouse
DOI:10.18240/ijo.2016.11.07

Wang D, Zhang B, Shi H, Yang W, Bi MC, Song XF, Zhang C, Cheng JH, Hao JL, Song E. Effect of endothelial progenitor cells derived from human umbilical cord blood on oxygen-induced retinopathy in mice by intravitreal transplantation. *Int J Ophthalmol* 2016;9(11):1578-1583

INTRODUCTION

Retinal neovascularization (RNV), a kind of pathological angiogenesis induced by retinal ischemia and hypoxia, could initiate a series of complications which are the reasons for visual function hazard. A variety of blindness, such as diabetic retinopathy, retinopathy of prematurity (ROP), retinal vein occlusions, are accompanied by a large number of RNV at the advanced period of the diseases [1]. Different from normal blood vessel, RNV is defective in endothelial function[2].

Endothelial progenitor cells (EPCs) are the precursor cells of vascular endothelial cells with high proliferative potential and endothelium characteristic. They are one of the important protective factors of vascular endothelial functions[3]. Currently, EPCs transplantation has been used in the treatment of myocardial infarction and other coronary heart diseases, stroke, diabetic microvasculopathies, and ischemic

retinopathies with preferable curative effects [4-5]. In the field of ROP, it was investigated that EPCs not only participated in retinal angiogenesis process in both physiological and pathological conditions, but also played an important role in the animal model[6-12]. Based on the above, we transplanted a sufficient amount of EPCs with effective functions into vitreous bodies of mice with retinal ischemia and hypoxia to observe the effect.

SUBJECTS AND METHODS

Endothelial Progenitor Cells Isolation and Culture EPCs were isolated from human umbilical cord blood using the method of our lab [13-14]. With the approval of Institute Ethics Committee and the informed consent signed by the guardians of the neonates, the samples (about 50 mL for each) of human umbilical cord blood were collected from healthy newborns with the gestational ages from 38 to 40wk. Human umbilical cord blood-derived mononuclear cells (MNCs) were gained from human umbilical cord blood by density gradient centrifugation using Percoll (1.077, GE Healthcare Co. Sweden).

Murine Oxygen Induced Retinopathy Model Oxygen induced retinopathy (OIR) model was set up in 48 C57BL/6J mice purchased from HFK BIOSCIENCE Co. Ltd., Beijing, China. According to the Ministry of Science and Technology Guide of People's Republic of China for the Care and Use of Laboratory Animals and Principles of Laboratory Animal Care (NIH publication No. 86-23, revised 1985), the mice in this research were treated with humane care.

According to the classic method for OIR [15], litters of postnatal day (P) 7 mice were placed to hyperoxia (mixed gas with 75% oxygen and 25% nitrogen) chamber for 5d from P7 to P12. The 12 mice of normal control group (NC group, 24 eyes) were always living in room air.

Endothelial Progenitor Cells Labeling and Intravitreal Injection Procedure On day 10, most cells showed the form of elongated cobblestones. EPCs were labeled with 5 μmol/L 5-(and-6)-carboxy fluorescein diacetate succinimidyl ester (CFSE, Molecular Probes Biotec. Co.). This concentration was proved to be the optimal for EPCs labeling in our lab[14]. EPCs were diluted into 2×10^5 cells/μL for transplantation.

At P12, OIR mice were anesthetized by intraperitoneal injection of chloral hydrate (3 μg/10 g body weight) and pupils were dilated with troicamide (0.5%). As described by Greenberg *et al* [16], injection with the volume of 1 μL was *via* a blunt 33-gauge needle attached to a 10-μL glass syringe (Hamilton Company, Reno, NV, USA). The 48 OIR animals (48 eyes) were intravitreally injected with CFSE labeled EPCs (EPCs group, 30 eyes) or with PBS (PBS group, 18 eyes). In every OIR animal, the contralateral eye served as a control and received no injection (OIR group, 48 eyes). No injection was performed on each eye of NC group (24 eyes).

Evans Blue Angiography At P15 or P19, three mice for each from EPCs group were deeply anesthetized as described above and then perfused through the left ventricle with normal saline containing 2% Evans Blue (Sigma) with the volume of 0.5 mL/10 g body weight. The eyes were enucleated immediately after cervical dislocation and placed in 4% paraformaldehyde for 0.5h at 4℃. Lenses were removed and peripheral retinas were dissected to obtain flat-mounted retinas with glycerol-gelatin on them. The flat-mounted retinas were viewed by confocal laser scanning microscope and photographed under 200× magnification.

Retina Paraffin Sections and Frozen Sections At P12 or P19, three mice from NC group and six mice from EPCs or PBS group were sacrificed with overdosed chloral hydrate. The eyes were enucleated and dipped in 4% paraformaldehyde for 24h at room temperature, and then embedded in paraffin. Serial sections (5 μm thick) were sagittally cut (through the cornea, parallel to the optic nerve) and stained with hematoxylin (Sigma) and eosin (Fisher). The neovascularization level was assessed using 400 × magnification light microscope.

At P15 or P19, three mice for each from EPCs group were executed. The eyes were flash-frozen in optimum cutting temperature. Eyes were cut into serial sections (20 μm thick), placed onto microscope slides and immersed in 10% formaldehyde solution for 5min. Hoechst (Lifetech) solution which was diluted by PBS at the rate of 1:200 was used for 5min to perform the cell-permeant nuclear counterstain. The positions of CFSE-labeled EPCs in eyes could be traced using confocal laser scanning microscope and photographed under 200× magnification.

Adenosinediphos Phatase Staining for Retinal Vascular Morphology At P12 or P19, three mice from each group were sacrificed and the eyes were removed and placed in the 10% neutral buffered formalin for 2h. Retinal tissues were immersed in the neutral buffered formalin and performed magnesium-activated adenosinediphos phatase (ADPase) staining which is an established method[17].

Statistical Analysis Data are presented as mean±standard deviation (SD). Comparing among the three groups was analyzed by ANOVA using SPSS 12.0. P value <0.05 was supposed to be statistically significant.

RESULTS

Hyperoxia Induced Vascular Occlusion and Neovascular Retinopathy In mice of NC group, at P12 the retinas were almost entirely vascularized (Figure 1A). There were 4 to 6 large vessels radiating from the optic disc to the peripheral retina with regular distributions. No obvious change was observed at P19 (Figure 1B). On the contrary, for OIR mice, oxygen exposure from P7 to P12 significantly inhibited the development of the retinal vascularization when detected at P12 (Figure 1C). At P19, vascular tortuosity, dilatation and

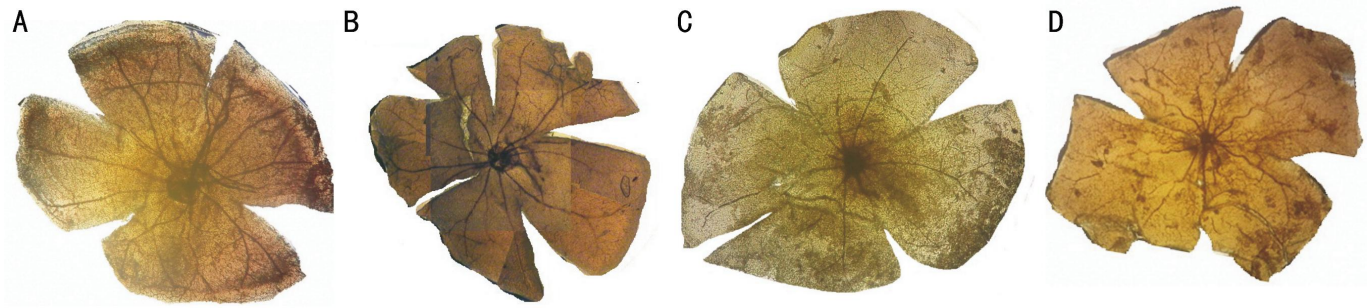

Figure 1 Hyperoxia-induced vascular occlusion observed by ADPase stain.

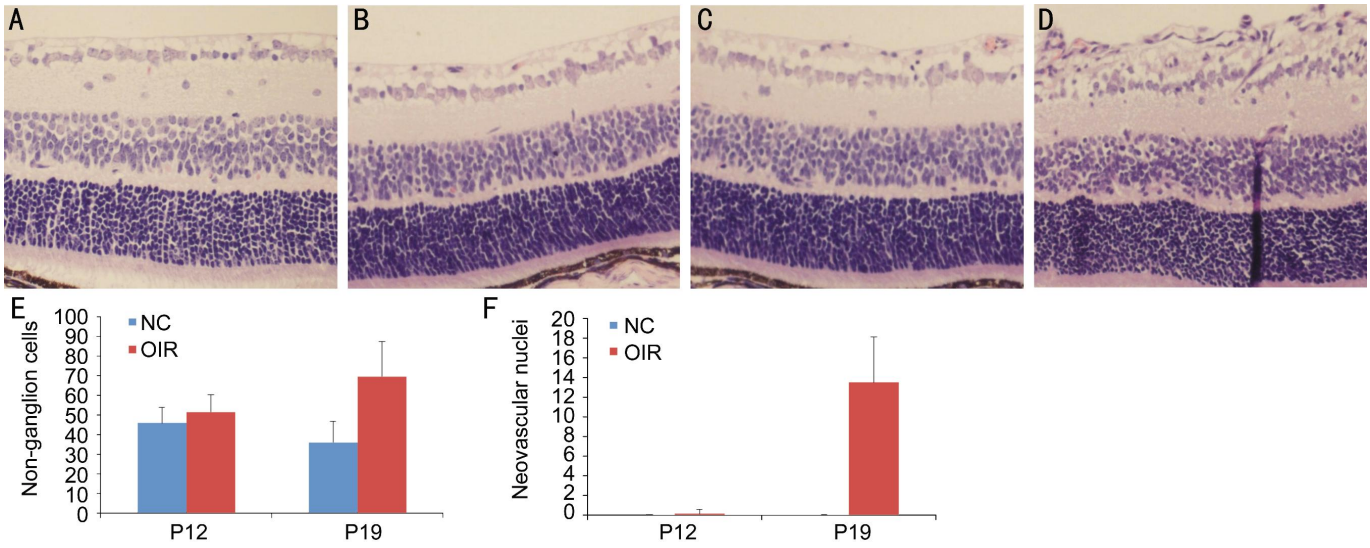

Figure 2 Hyperoxia induced neovascular retinopathy and quantitative analysis.

even occlusion with nonperfusion areas could be seen in the retinas from OIR group (Figure 1D). Some neovascular tufts showed up as well (Figure 1D).

Concerning changes of retinal cells, for retinas from NC group at P12 and P19, most cells between the internal plexiform layer and the inner limiting membrane were ganglion cells mostly with a single layer distribution, whose nuclei were round and big (Figure 2A, 2B). No nucleus which could break through the inner limiting membrane was observed in this group at P19 (Figure 2B). For the eyes from the OIR group, the cells between the internal plexiform layer and the inner limiting membrane presented similar to the retinas in NC group at P12 (Figure 2C). However, while at P19, the cells increased significantly, most of which were non-ganglion cells with various nuclei and also some neovascular nuclei (Figure 2D). The distributions were completely disordered and many neovascular nuclei broke through the inner limiting membrane. Compared with NC group, the counts of non-ganglion cells (Figure 2E, $P<0.01$; $n=6$) and neovascular nuclei (Figure 2F, $P<0.001$; $n=6$) in the eyes from the OIR group were significantly higher at P19.

Receding Oxygen Induced Retinopathy Earlier in Mice Injected Endothelial Progenitor Cells Intravitreally At P19, in comparison with the retinal performance in the eyes from the OIR group (Figure 3A), the eyes of PBS group presented the similar changes including vascular tortuosity, dilatation and occlusion with nonperfusion areas and neovascular tufts (Figure 3B). However, the retinas of the eyes of EPCs group displayed less nonperfusion area and fewer peripheral tufts at P19 (Figure 3C).

As for retinal cells between the internal plexiform layer and the inner limiting membrane, comparing with the retinas in the eyes from the OIR group at P19 (Figure 3D), the eyes of PBS group also displayed the increased number of non-ganglion cells with various nuclei and neovascular nuclei which had broken through the inner limiting membrane (Figure 3E). But for the eyes of EPCs group, the retinopathy receded earlier and could be observed at P19 with the representation of less non-ganglion cells and neovascular nuclei, together with relatively regular distribution (Figure 3F). In these eyes, the counts of the non-ganglion cells and neovascular nuclei at P19 were reduced by 10% and 44% for each, in comparison with the eyes from the OIR group (Figure 3G, for non-ganglion cells, $P=0.503$, $n=6$; while for neovascular nuclei, $P<0.05$, $n=6$), and reduced by 11% and 45% for each, in comparison with the eyes of PBS group (Figure 3G, for non-ganglion cells, $P=0.473$, $n=6$; while for neovascular nuclei, $P<0.05$, $n=6$).

Trace of the Carboxy Fluorescein Diacetate Succinimidyl Ester Labeled Endothelial Progenitor Cells After Intravitreal Transplantation in Mice At P12, OIR mice of the EPCs group were intravitreally injected with

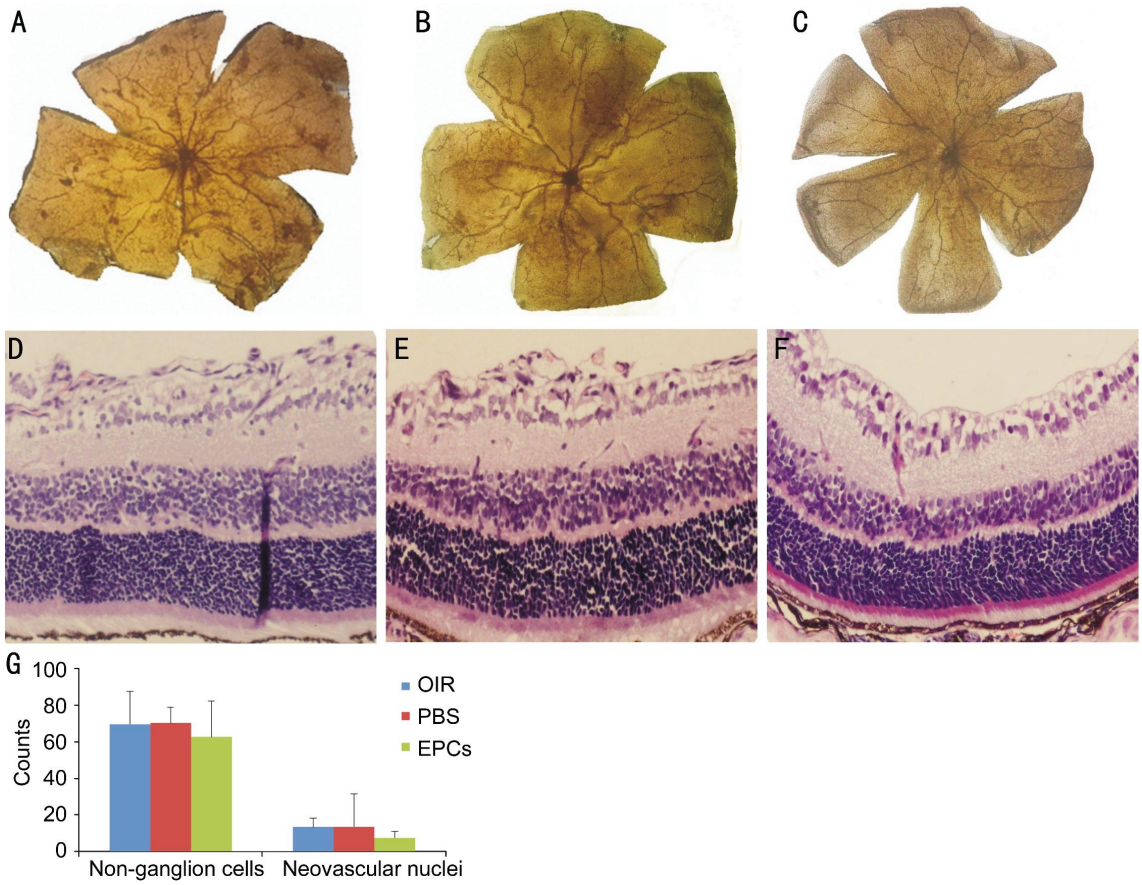

Figure 3 OIR could be receded earlier in mice injected EPCs intravitreally.

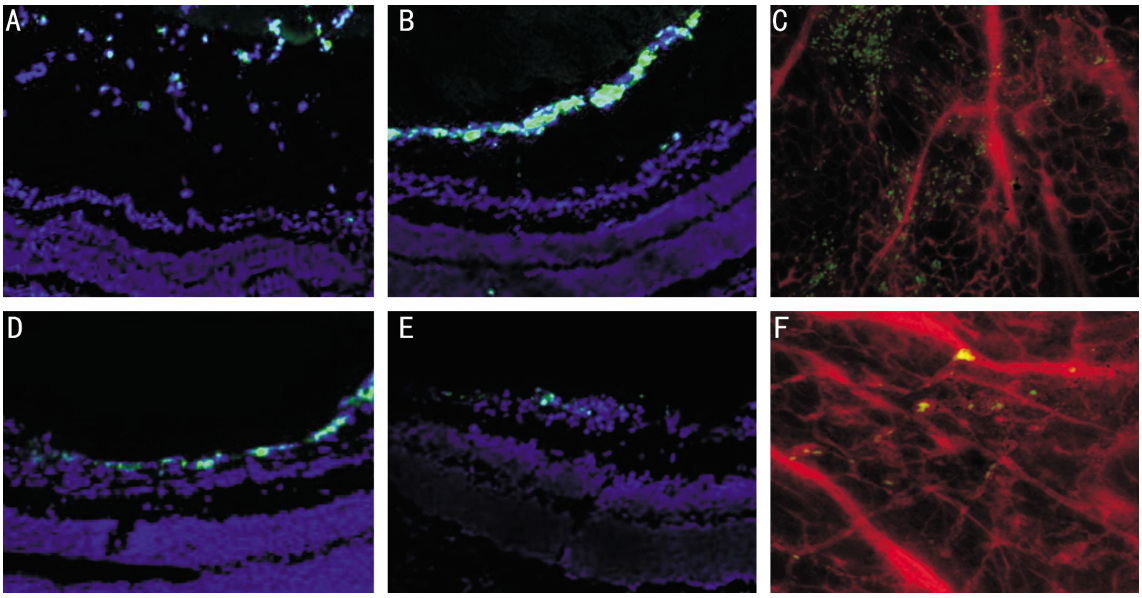

Figure 4 Track of the labeled EPCs after transplantation by Evans blue angiography and frozen section (200× magnification).

1 μL CFSE labeled EPCs suspension (2×10^5 cells/μL) using microsyringe. Three days since EPCs transplantation (P15), plenty of green EPCs showed up in the vitreous cavity (Figure 4A, 4B) and adhered to the retinal surface (Figure 4C). One week after EPCs transplantation (P19), the cells aggregated between the internal plexiform layer and the inner limiting membrane (Figure 4D, 4E) and some EPCs appeared in retinal vessels (Figure 4F). These EPCs could participate in the repairmen process of the pathological angiogenesis and attenuate OIR.

DISCUSSION

ROP could result in pathologic vascularization of retina, which frequently leads to nonreversible visual acuity decrease. Smith *et al*[15] established the OIR model could be easily set up and used to evaluate the qualitative and quantitative changes. To evaluate the extent of retinopathy of OIR, Liu *et al*[18] performed ADPase staining in retinal whole mounts, counted the numbers of neovascular nuclei, vessel lumens, and non-ganglion cells. According to their study, for OIR mice, the counts of non-ganglion cells and

neovascular nuclei increased significantly at P17 with disorganized distribution in more than one layer within or crossing the inner limiting membrane [18]. Compared with the normal control group, for OIR mice, non-ganglion cells increased by 1.50-fold higher at P14, peaked by 3.14-fold higher at P17, and regressed moderately by 1.55-fold higher at P21, and increased proliferation of neovascular nuclei was detected at P17[18].

In view of the classic researches about OIR, the greatest neovascular response was shown up from P17 to P21[15]. And according to the previous study of our lab [14], which was about the repair effect of EPCs intravitreal transplantation on the photocoagulation injury mice model, one week was long enough for the injected EPCs to congregate around the retinopathy. So we chose P12 as the injected time point and P19 as the observation time point. In OIR mice of our research, the inhibited development of the retinal vascularization was detected at P12, and vascular occlusion with nonperfusion areas and neovascular tufts could be seen at P19. Referring to the changes of retinal cells in OIR mice at P19, the non-ganglion cells with various nuclei and also some neovascular nuclei increased dramatically. The distributions were also disorganized. These findings were similar to the former study at P17[18].

At present, for the cell therapy of retinal diseases, there are different types of stem cells. For example, mononuclear cells were transplanted intravitreously for diabetic retinopathy[19], optic nerve atrophy[19-20], age-related macular degeneration[19], retinitis pigmentosa [21-23] and ischemic retinopathy [23] in human. So far, there have been various effective treatments for ROP which could hold back the progression of ROP [4, 24-25]. Nakagawa *et al* [6] demonstrated that the frequency of circulating EPCs in OIR mice decreased immediately at P12 (hyperoxia) , increased at P17 (hypoxic) compared with control and there was a dilatory mobilization and differentiation of the bone marrow (BM)-derived. EPCs resulting in an pathological vascular development by bone marrow-transplanting in mice. There was a completely supplement of EPCs into the pathologic vasculature.

In the present study, our results showed that the retinas of the eyes of EPCs group displayed less nonperfusion area and fewer peripheral tufts at P19 in comparison with that from the eyes of OIR or PBS group. As for retinal cells between the internal plexiform layer and the inner limiting membrane, in EPCs group, the retinopathy receded earlier and could be observed at P19 with the representation of less non-ganglion cells and neovascular nuclei, together with relatively regular distribution compared with OIR or PBS group. The counts of the non-ganglion cells and neovascular nuclei in EPCs group at P19 were reduced by around 10% ($P>0.05$) and 44% ($P<0.05$). There was significant difference about the changes of neovascular nuclei in EPCs group compared with OIR or PBS group, but not for the non-ganglion cells. The reason might be the neovascular nuclei returned to the space between the internal plexiform layer and the inner limiting membrane but still did not regress completely.

Caballero *et al* [7] once injected EPCs derived from pooled healthy human donors to the animal models of ischemic vascular damage, including chronic (diabetes) and acute (ischemia/reperfusion injury and OIR) models. The OIR animals were sacrificed 2d after injection with EPCs and the results illustrated robust, patent vessels with plenty of EPCs colocalizing with the vascular wall of primarily large vessels. Comparing with the study above, we observed the retinas with injected EPCs for longer time and obtained some more evidences for the positive therapic effect of EPCs intravitreal transplantation on OIR mice. Similarly to the previous study of our lab [14], at present we found that three days after EPCs transplantation, plenty of EPCs appeared in the vitreous cavity and adhered to the retinal surface. While one week after EPCs transplantation, the cells gathered between the internal plexiform layer and the inner limiting membrane, and some EPCs showed up in retinal vessels. These EPCs could accelerate the reparative procedure of the neovascularization of OIR.

In summary, using the OIR model with CFSE-labeled EPCs intravitreal transplantation, we demonstrated that at P19, the retinas from the eyes of EPCs group had less nonperfusion area, fewer peripheral tufts and neovascular nuclei with relatively regular distribution compared with that from the eyes of the OIR or PBS group. One week after EPCs transplantation, the cells aggregate between the internal plexiform layer and the inner limiting membrane in which the neovascular nuclei existed, and some EPCs could be seen in retinal vessels. These findings provide the evidence suggest the possibility that EPCs transplantation could accelerate the reparative procedure of the neovascularization of OIR. The research aiming at the molecular mechanism of this effect is still ongoing.

ACKNOWLEDGEMENTS

Foundations: Supported by the National Natural Science Foundation of China (No.81400403); the International Science and Technology Cooperation Program of Jilin Province (No.20110733); the Technology Program of Soochow City (No.SYS201375).

Conflicts of Interest: Wang D, None; **Zhang B,** None; **Shi H,** None; **Yang W,** None; **Bi MC,** None; **Song XF,** None; **Zhang C,** None; **Cheng JH,** None; **Hao JH,** None; **Song E,** None.

REFERENCES

1 Campochiaro PA. Ocular neovascularization. *J Mol Med (Berl)* 2013;91(3):311-321.

2 Todorich B, Yiu G and Hahn P. Current and investigational pharmacotherapeutic approaches for modulating retinal angiogenesis. *Expert Rev Clin Pharmacol* 2014;7(3):375-391.

3 Machali ń ska A. The role of circulating endothelial progenitor cells in the development of vascular retinal diseases. *Klin Oczna* 2013;115 (2): 158-162.

4 Rafii S, Lyden D. Therapeutic stem and progenitor cell transplantation for organ vascularization and regeneration. *Nat Med* 2003;9(6):702-712.

5 Medina RJ, O'Neill CL, Sweeney M, Guduric-Fuchs J, Gardiner TA, Simpson DA, Stitt AW. Molecular analysis of endothelial progenitor cell (EPC) subtypes reveals two distinct cell populations with different identities. *BMC Med Genomics* 2010;3:18.

6 Nakagawa Y, Masuda H, Ito R, Kobori M, Wada M, Shizuno T, Sato A, Suzuki T, Kawai K, Asahara T. Aberrant kinetics of bone marrow-derived endothelial progenitor cells in the murine oxygen-induced retinopathy model. *Invest Ophthalmol Vis Sci* 2011;52(11):7835-7841.

7 Caballero S, Sengupta N, Afzal A, Chang KH, Li Calzi S, Guberski DL, Kern TS, Grant MB. Ischemic vascular damage can be repaired by healthy, but not diabetic, endothelial progenitor cells. *Diabetes* 2007;56 (4): 960-967.

8 Miller JW, Adamis AP, Aiello LP. Vascular endothelial growth factor in ocular neovascularization and proliferative diabetic retinopathy. *Diabetes Metab Rev* 1997;13(1):37-50.

9 Abu El-Asrar AM, Struyf S, Verbeke H, Van Damme J, Geboes K. Circulating bone-marrow-derived endothelial precursor cells contribute to neovascularization in diabetic epiretinal membranes. *Acta Ophthalmol* 2011;89(3):222-228.

10 Espinosa-Heidmann DG, Caicedo A, Hernandez EP, Csaky KG, Cousins SW. Bone marrow-derived progenitor cells contribute to experimental choroidal neovascularization. *Invest Ophthalmol Vis Sci* 2003;44(11):4914-4919.

11 Asahara T, Masuda H, Takahashi T, Kalka C, Pastore C, Silver M, Kearne M, Magner M, Isner JM. Bone marrow origin of endothelial progenitor cells responsible for postnatal vasculogenesis in physiological and pathological neovascularization. *Circ Res* 1999;85(3):221-228.

12 Longeras R, Farjo K, Ihnat M, Ma JX. A PEDF-derived peptide inhibits retinal neovascularization and blocks mobilization of bone marrow-derived endothelial progenitor cells. *Exp Diabetes Res* 2011;2012:518426.

13 Song E, Lu CW, Fang LJ, Yang W. Culture and identification of endothelial progenitor cells from human umbilical cord blood. *Int J Ophthalmol* 2010;3(1):49-53.

14 Shi H, Yang W, Cui ZH, Lu CW, Li XH, Liang LL, Song E. Tracking of CFSE-labeled endothelial progenitor cells in laser-injured mouse retina. *Chin Med J* 2011;124(5):751-757.

15 Smith LE, Wesolowski E, McLellan A, Kostyk SK, D'Amato R, Sullivan R, D'Amore PA. Oxygen-induced retinopathy in the mouse. *Invest Ophthalmol Vis Sci* 1994;35(1):101-111.

16 Greenberg KP, Geller SF, Schaffer DV, Flannery JG. Targeted transgene expression in Müller glia of normal and diseased retinas using lentiviral vectors. *Invest Ophthalmol Vis Sci* 2007;48(4):1844-1852.

17 Lutty GA, McLeod DS. A new technique for visualization of the human retinal vasculature. *Arch Ophthalmol* 1992;110(2):267-276.

18 Liu XL, Zhou R, Pan QQ, Jia XL, Gao WN, Wu J, Lin J, Chen JF. Genetic inactivation of the adenosine A2A receptor attenuates pathologic but not developmental angiogenesis in the mouse retina. *Invest Ophthalmol Vis Sci* 2010;51(12):6625-6632.

19 Jonas JB, Witzens-Harig M, Arseniev L, Ho AD. Intravitreal autologous bone-marrow-derived mononuclear cell transplantation. *Acta Ophthalmol* 2010;88(4):e131-e132.

20 Jonas JB, Witzens-Harig M, Arseniev L, Ho AD. Intravitreal autologous bone-marrow-derived mononuclear cell transplantation: a feasibility report. *Acta Ophthalmol* 2008;86(2):225-226.

21 Siqueira RC, Messias A, Voltarelli JC, Scott IU, Jorge R. Intravitreal injection of autologous bone marrow-derived mononuclear cells for hereditary retinal dystrophy: a phase I trial. *Retina* 2011;31(6):1207-1214.

22 Siqueira RC, Jorge R, Messias A, Scott I and Voltarelli J. Transplantation of autologous bone marrow derived stem cells for retinitis pigmentosa. *Invest Ophthalmol Vis Sci* 2011;52:2217.

23 Siqueira R. Stem cell therapy for retinal diseases: update. *Stem Cell Res Ther* 2011;2(6):50.

24 Binder S, Stanzel BV, Krebs I, Glittenberg C. Transplantation of the RPE in AMD. *Prog Retin Eye Res* 2007;26(5):516-554.

25 Machalińska A, Modrzejewska M, Kotowski M, Dziedziejko V, Kucia M, Kawa M, Safranow K, Baskiewicz-Masiuk M, Modrzejewska A, Karczewicz D, Rudnicki J, Machalinski B. Circulating stem cell populations in preterm infants: implications for the development of retinopathy of prematurity. *Arch Ophthalmol* 2010;128 (10): 1311-1319.