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Effects of the calcium ion concentration in rats retinal progenitor cells and neural stem cells induced by hypoxia

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缺氧培养下大鼠视网膜祖细胞与脑神经干细胞内钙离子浓度差异的研究

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摘要

目的:比较缺氧培养条件下新生大鼠视网膜祖细胞(retinal progenitor cells, RPCs)与脑神经干细胞(neural stem cells, NSCs)钙离子浓度的差异。

方法:分离新生大鼠 RPCs 及大脑皮质 NSCs,进行无血清体外培养;采用荧光免疫细胞化学的方法进行干细胞鉴定;缺氧培养后,光镜观察细胞形态,采用钙离子荧光探针结合激光共聚焦显微镜测定细胞内钙离子荧光强度。

结果:缺氧培养后,大脑皮质 NSCs 较早出现肿胀、伸出突起等变化,随缺氧时间延长,两种细胞内钙离子荧光强度逐渐增强,缺氧 12h 后 RPCs 内钙离子荧光强度小于脑皮质 NSCs($P<0.05$),差异具有统计学意义。

结论:缺氧培养 12h RPCs 内钙离子浓度低于大脑皮质 NSCs。

关键词:视网膜祖细胞;脑神经干细胞;缺氧;钙离子浓度

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Abstract

• AIM: To compare the difference of the calcium ions concentration between neonatal rats retinal progenitor cells (RPCs) and brain neural stem cells (NSCs) under hypoxia cultivate *in vitro*.

• METHODS: After isolated from the neonatal SD rats and cultured in serum-free medium *in vitro*, RPCs and brain NSCs were identified by fluorescent immunocytochemistry. After cultured under hypoxic condition for different time duration, cell morphology were observed under light microscope, and intracellular calcium fluorescence intensity were measured via laser scanning confocal microscopy (LSCM) and calcium fluorescence probe Fluo-4AM.

• RESULTS: Cultured under hypoxic conditions, brain NSCs swelled and extended protrusions were noticed earlier than RPCs. With increasing time duration of hypoxia, intracellular calcium ion fluorescence intensity of both types of cells gradually increased. After 12 hours cultured under hypoxic condition, calcium ion fluorescence intensity of RPCs showed significant difference lower than the fluorescence intensity in NSCs ($P<0.05$).

• CONCLUSION: Under hypoxic condition for 12 hours, the concentration of calcium ion in RPCs was lower than that in brain NSCs.

• KEYWORDS: retinal progenitor cells; neural stem cells; hypoxia; calcium ion concentration

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INTRODUCTION

Neuroretinal lesions leads to irreversible blindness caused by apoptosis and various neurodegenerative diseases, such as proliferative diabetic retinopathy (PDR), glaucoma, central retinal artery occlusion (CRAO), age-related macular degeneration (AMD) and retinitis pigmentosa (RP). These diseases have high prevalence and poor prognosis[1]. Recently, animal experiments showed that transplanted stem cells can be integrated in host retina, and divided into terminal retinal cells, which can provide new strategies to the therapeutic intervention of these diseases. Until now, there are several types of cells that can be transplanted into retina, including RPCs and brain NSCs[2].

Ischemia, hypoxia, ischemic reperfusion and retinal degeneration lead to pathological changes which produce free radicals free radical and intracellular calcium ion concentration, which finally lead to the cell apoptosis. Proliferation and differentiation of stem cells after transplanted into the injury sites were affected by the local micro-environment; therefore, their tolerance of hypoxia was one of the key factors that decide whether this type of cell was suitable for retinal cell transplantation. Our research showed the changes of morphology and calcium ion fluorescence intensity of RPCs and NSCs under hypoxic conditions, which provide experimental basis and theoretical support for choosing the most suitable stem cell for retina transplantation.

MATERIALS AND METHODS

Materials

Animals Neonatal SD rats, provided by the Laboratory Animal Centre of Xi'an Jiaotong University College of Medicine.

Reagents Serum free complete medium: DMEM/F_{12}, bFGF, EGF, N2 and B27 (Gibco, USA); to rat anti-Nestin antibody (Abcam, USA), to rat anti-Pax-6 antibody (Abcam, USA), Fluo-4 AM and Pluronic F-127(Dojindo, Japan).

Instruments CKX41 inverted microscope, DP71 fluorescence microscope and SZX16 inverted phase contrast microscope (Olympus, Japan), BNA-321D CO_2 Cultivation Cabinet (Espec, England), TCS-Sp2 LSCM and software of Q550-CW for image analysis (Leica, Germany).

Methods

Normal culture of neonatal rats RPCs and brain NSCs The suspension of RPCs and NSCs were collected from retinal neuroepithelial cells and cerebral cortex of newborn rats. These cells were cultured in DMEM/F12 media which containing N2, B27, bFGF and EGF, and then inoculate the concentration of 2×10^5/mL to 50mL culture bottle under 37℃ with 5% CO_2. These cells were passaged every 5-7 days, and observed under inverted phase contrast microscope.

Identification of stem cells by fluorescent immunocytochemistry Stained both kinds of cells by Nestin and Pax-6, and use PBS instead of Nestin and Pax-6 to set up the blank control group, and then observe under fluorescence microscope.

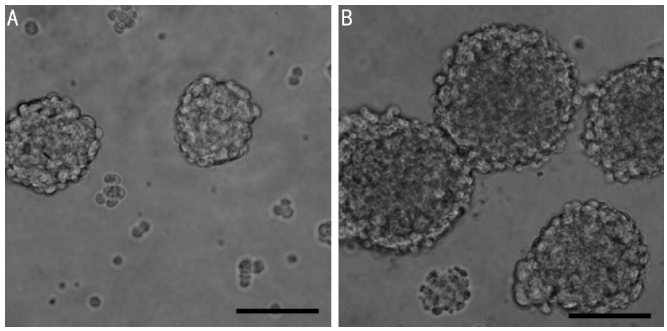

Figure 1 Cell morphology under normal cultivate A: RPCs of P0 Day 3, mulberry-like cell balls with strong refraction; B: NSCs of P0 Day 5, large neural sphere with a weaker refraction in the middle of the cell balls. Scale bars: 50μm.

Hypoxia culture of neonatal rats RPCs and brain NSCs The RPCs and NSCs were grouped respectively according to the cultural time of hypoxia. The hypoxia groups were cultured under the condition of 37℃, 5% CO_2+95% N_2 and constant temperature together with humidity incubator for 1, 2, 4, 8 and 12h. For the control, normal groups were cultured under the same condition except with the normal oxygen concentration of 21%. The cells morphology of every group was observed under inverted phase contrast microscope.

Measurement of intracellular calcium fluorescence intensity The cells of each group were washed by D-Hank's, added Fluo-4AM (3μmol/L) with Pluonic-F127 (0.05%), incubated for 45 minutes, and then washed the not combined Fluo-4AM, lastly, added D-Hank's for another 25 minutes. For the control, added D-Hank's instead of Fluo-4AM with the same procedure. Added the suspension of these cells to the glass slides, and then scanned under LSCM with excitation/emission wavelength of 494/516 -nm. The images were analyzed by Q550-CW software, with randomly measured the average fluoresces of per unit. Three replicas for each group were taken.

Statistical Analysis We used $\bar{x}\pm s$ to express the calcium fluorescence intensity. All the data were analyzed by SPSS 17.0, and used t-test ($P<0.05$) for the significant difference.

RESULTS

Cells morphology of RPCs and NSCs under normal cultivates Both RPCs and NSCs were grown in suspension in the complete serum free medium. First, they were unicellular or 2-5 cells together, and then their volume and cell number increased and they gathered into neural sphere with longer time (Figure 1).

Cells morphology of RPCs and NSCs under hypoxia culture The cells under hypoxia culture for 1h had no significant difference compared with normal groups; In the hypoxia 2h group, the cell boundaries of PRCs inside the cell

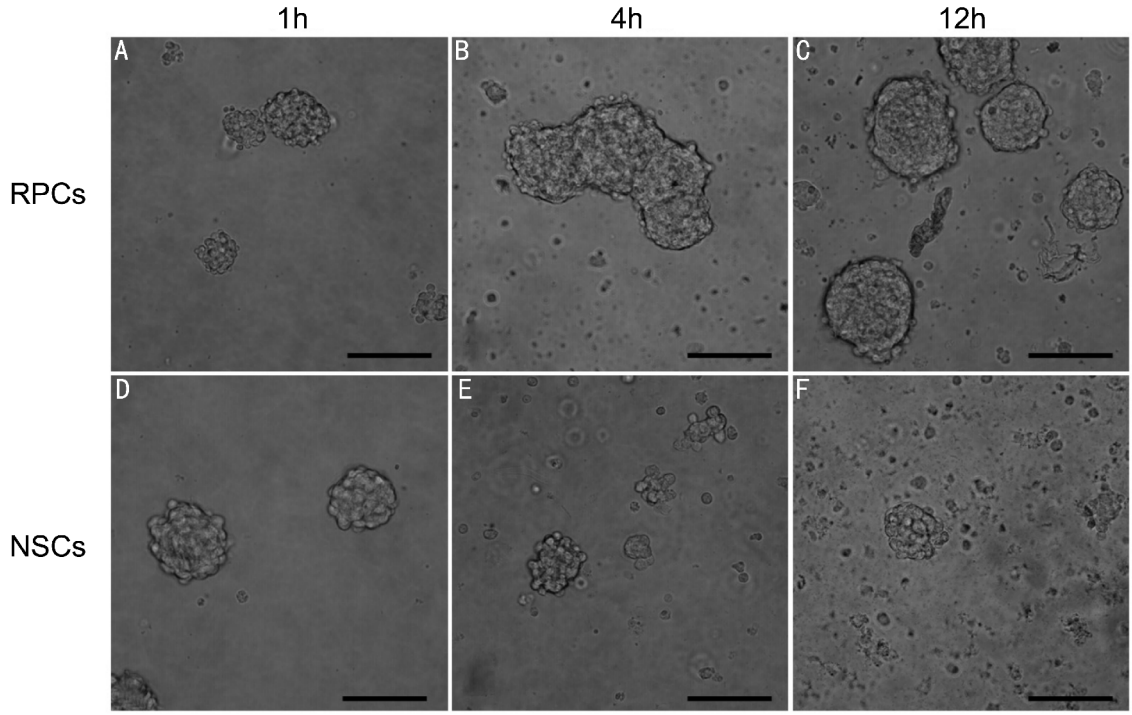

Figure 2 Cell morphology under hypoxia culture A: RPCs under hypoxia for 1h; B: RPCs under hypoxia for 4h, collection of neural sphere with some dead cells; C: RPCs under hypoxia for 12h, irregular cells with swelling, and some fragments of cells were sink to the bottom; D: NSCs under hypoxia for 1h; E: NSCs under hypoxia for 4h, more pronounced swelling and some cells were dead; F: NSCs under hypoxia for 12h, decrease of neural sphere and much more fragments of cells. Scale bars: 50μm.

balls was unclear, and the NSCs show mild swelling; In the hypoxia 4h group, part of the NSCs was adhere to the bottle and stretched out protrusions; In the hypoxia 8h group, the cell balls of the PRCs show aggregation with each other, and a small number of them died, the swelling of the NSCs was more pronounced, partly stretched out protrusions and partly dead; In the hypoxia 12h group, the shape of the RPCs was irregular caused by swelling, and some debris sank to the bottom of the bottle, the cell balls of the NSCs reduced and lot of debris could be seen(Figure 2).

Identification of stem cells Almost all the cells in the visual field under fluorescence microscope showed positive staining of Nestin and Pax-6 (Figure 3), while the control cells showed negative to Nestin and Pax-6 stain.

Calcium fluorescence intensity in RPCs and NSCs and statistical analysis Light fluorescence can be seen in the normal groups of the RPCs and NSCs. In the hypoxia 1h groups, the fluorescence was still weak, the RPCs showed the aggregation state of small cell balls, while the vast majority of the NSCs showed the existence of single cells; In the hypoxia 2h group, the fluorescence of the inside cells of RPCs cell balls was strong than the outside ones, and the volume of the NSCs was increased slightly; In the hypoxia 4h group, the fluorescence was stronger than the hypoxia 2 hours group; In the hypoxia 8h group, the fluorescence continued to enhance mildly, the volume of RPCs was increased, the NSCs showed some irregular shaped and enhanced fluorescence; In the

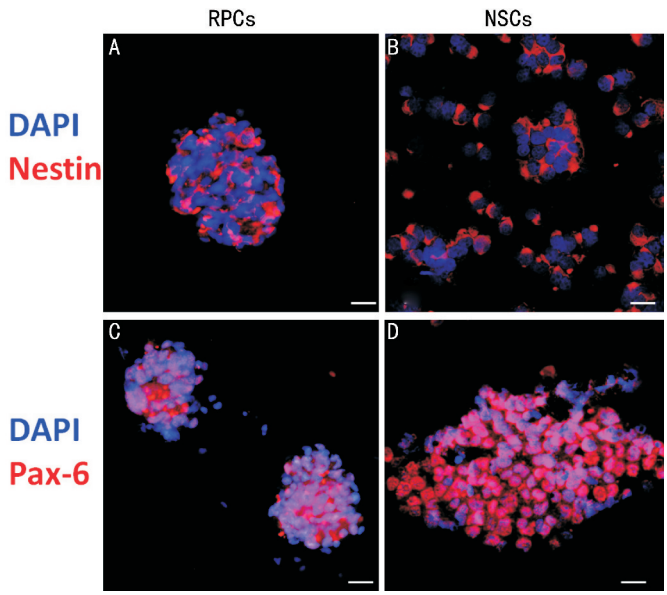

Figure 3 Identification of stem cells A: RPCs, red was cytoplasm with Nestin, blue was nuclear with DAPI; B: NSCs, red was cytoplasm with Nestin, blue was nuclear with DAPI; C: RPCs, nuclear with red Pax-6 and blue DAPI; D: NSCs, nuclear with red Pax-6 and blue DAPI. Scale bars: 20μm.

hypoxia 12h group, both kinds of cells were stained into strong fluorescence, and more irregular shaped, debris-like fluorescence could be seen in the NSCs group(Figure 4). The control groups of both kinds of cells showed no fluorescence during the scan by the LSCM.

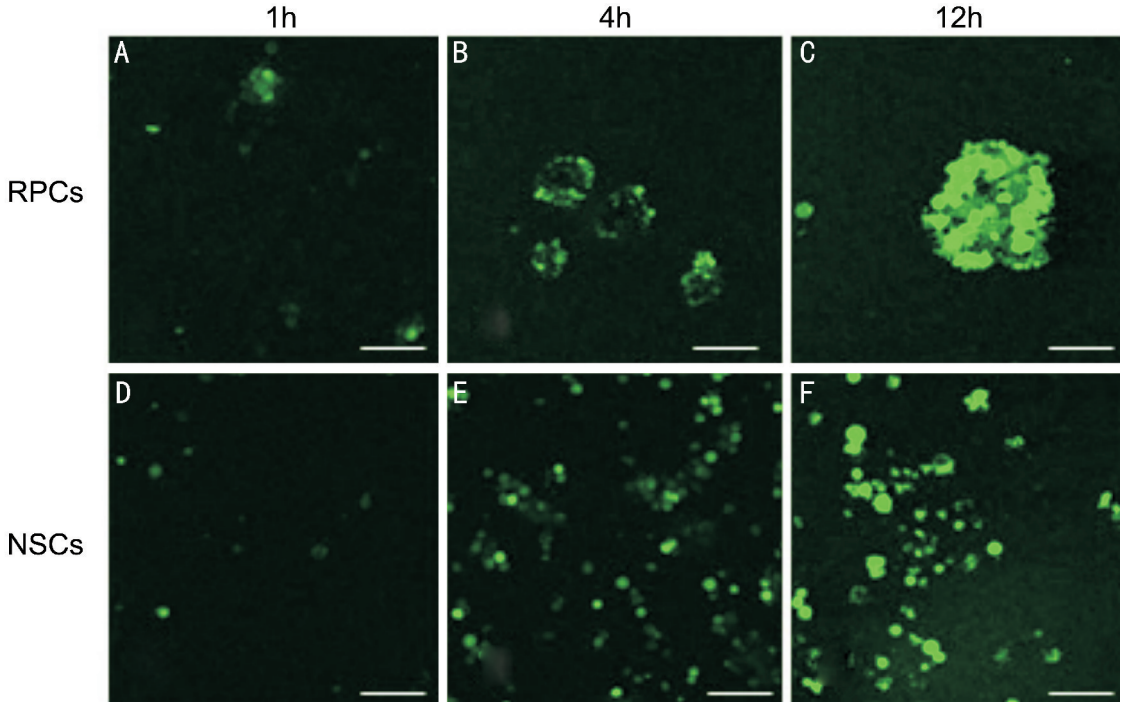

Figure 4 The calcium fluorescence of two kinds of cells A: RPCs under hypoxia for 1h, extremely weak calcium fluorescence; B: RPCs under hypoxia for 4h, small cell balls with weak fluorescence of calcium ion; C: RPCs under hypoxia for 12h, big cell balls with stronger calcium fluorescence; D: NSCs under hypoxia for 1h, extremely weak calcium fluorescence; E: NSCs under hypoxia for 4h, separately cells with weak fluorescence of calcium ion; F: NSCs under hypoxia for 12h, stronger calcium ion fluorescence, with much more irregular and fragments of cells. Scale bars: 75μm.

Table 1 The value of intracellular calcium fluorescence intensity and data analysis by *t*-test ($\bar{x}\pm s$, unite: ROI)

	Normal	Hypoxia 1h	Hypoxia 2h	Hypoxia 4h	Hypoxia 8h	Hypoxia 12h※
NSCs	103.22±8.51	169.80±10.43	183.41±13.36	226.82±15.42	250.34±6.89	255.47±1.00
RPCs	102.55±13.23	164.64±16.65	172.87±16.73	221.02±11.65	253.32±3.28	245.85±15.62
t	0.253	1.622	1.957	1.675	−2.032	3.259
P	>0.05	>0.05	>0.05	>0.05	>0.05	<0.05

※There was significant difference between RPCs and NSCs under hypoxia for 12h.

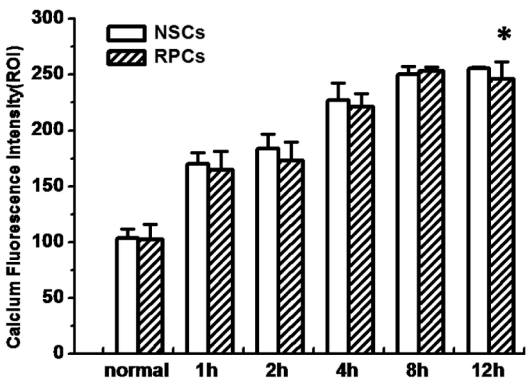

Figure 5 Comparing of intracellular calcium ion fluorescence intensity ※ There was significant difference between RPCs and NSCs under hypoxia for 12h.

The calcium fluorescence intensities of all groups of cells were treated with *t*-test (Table 1, Figure 5). In the hypoxia 12h groups, the intracellular calcium fluorescence intensity of RPCs was less than NSCs, and the difference was statistically significant ($P<0.05$), while no significant differences in the hypoxia groups of 1h, 2h, 4h, and 8h ($P>0.05$).

DISCUSSION

Proliferation and differentiation of stem cells were affected by series of factors. Hypoxia was one of the common reasons, which had significant effects on proliferation, differentiation, apoptosis and transplantation. Hypoxia can lead to the changes in calcium ion concenteration, so, we used the fluorescence intensity of calcium ion as an indicator to study the effects of hypoxia to the culture of RPCs and NSCs *in vitro*.

Identification of NSCs and RPCs Nestin originated in the formation stage of the neural plate, and expressed in pluripotent neuroectodermal cells. It no longer expressed when neuroectodermal cells differentiating into neurons and glial cells. So it is a marker of NSCs[3]. RPCs also derived from neural ectoderm; therefore, Nestin is also a specific marker of RPCs. Pax-6 gene is essential for development of eyeball. This is a marker of RPCs because it first expressed in the preceding neural plate which finally turn into retina[4]. Studies showed that NCSs had the potential to differentiate into retinal cells, so NSCs can express Pax-6, too.

Effects of hypoxiacultivate on cell growth, proliferation and differentiation Researches showed that hypoxia was harmful for the proliferation of cells. Galvni *et al*[5] found that the express of p27kipl inhibited the cell's proliferation of bladder epithelial cells under 1% oxygen concentration, while no apoptosis was found. Vpaoridi *et al*[6] found the cell apoptosis of alveolar epithelial cells under 1% oxygen concentration of 4h cultivate. However, there were also some reports related to the promotion to cell proliferation and directional differentiation under hypoxic condition. Nurse *et al*[7] found that low concentration of oxygen can adjust the mitogen which came from the chromaffin cells of neural crest, and then had an effect on cell proliferation. Yun *et al*[8] found that low oxygen concentration can accelerate the differentiation from bone marrow mesenchymal stem cell to osteoblast and then reduced the differentiation to adiposity. Bartlery *et al*[9] found that the hippocampus of new born rats with unilateral carotid artery ligation showed positive of BrdU in 8% oxygen concentration for 75 min, which indicate that hypoxia can promote the cell proliferation. There is an assumption that oxygen concentration more than 3% will accelerate cell proliferation while less than 3% will speed up the death of stem cells. But the mechanism of hypoxia to regulate and control the proliferation and differentiation of stem cell need deep research in the near future.

According to our research, under extremely hypoxia condition, with the increase of cultural time, the morphology of both RPCs and NSCs swelled, stretched out protrusions and dead gradually, and the intracellular calcium fluorescence intensity increased gradually.

Effects of hypoxia cultivate on calcium ion concentrations As a recognized second messenger, calcium ion have important roles in regulating cell membrane permeability, promoting cell-cell communication, affecting the synthesis and release of neurotransmitters, triggering cell contraction, secretion, regulating enzyme activity, regulating cell cycle, cell division and apoptosis. And also play an important role in retinal development and functional maturation. There is a tremendous difference between the extracellular and intracellular calcium ions concenteration, which can maintenance the mechanism of calcium ion transport. When this mechanism is disrupted, the intracellular free calcium ion concentration will increase abruptly, and then disorder of calcium homeostasis appears. Intracellular energy of cells in severe hypoxia run out gradually, which results in the dysfunction of calcium ion transport mechanism, the overload of intracellular free calcium ion, and the apoptosis of cells. Cells in mild hypoxic micro-environment can maintain the integrity of the cell membrane temporarily by generating energy through anaerobic glycolysis, which via the activation of downstream gene by hypoxia-inducible factor-1α (HIF-1α)[10]. Therefore, under mild hypoxia, overload of intracellular calcium does not occur, with no significant increase in the number of apoptotic cells. While severe hypoxia can lead to outflow of high quantity of calcium, and then cause the complex biochemical reaction process intracellular, finally result in cell damage and death[11]. Once the biochemical and pathological reaction started, it will form a positive feedback, which means even if the hypoxia being corrected, it is difficult to reverse cell damage and death. From this, we know that long time extremely low oxygen concentrations enables the increase in intracellular calcium ion concentration and leads to cell death ultimately.

Ischemia and hypoxia, ischemic reperfusion and degeneration of retina can lead to lesions to retinal neuroepithelium, which can result in large quantities of free radical, increase of excitatory amino acid and overload of intracellular calcium concentration. Increase of intracellular calcium concentration can activate calcium ion - dependent glutamyltransferase activate nuclear transcription factor, which ultimately lead to apoptosis. Because of the change of the microenvironment of damaged tissue (especially due to hypoxia), which had significant effects on the transplanted cells, the research on the relationship between local microenvironment inside the eyeball and the growth of stem cells will have important theoretical significance and widespread application value[12]. Under normal conditions, the concentrations of glutamate and glucose in the vitreous body were significantly higher than in the brain[13]; thus, we speculate that there were differences in hypoxia tolerance between RPCs and NSCs. According to our research, RPCs grow better than NSCs under hypoxic condition, and there is significant difference of hypoxia 12h groups (RPCs showed less calcium fluorescence intensity than NSCs, $P<0.05$), but there is no significant difference of other groups between these two types of cells ($P>0.05$). This indicates that under hypoxic conditions for a short time, there was no significant difference in the hypoxia tolerance of the two types of cells; while in the hypoxia 12h group, hypoxia tolerance of RPCs was stronger than NSCs. However, the most suitable cells for retinal cell transplantation not only depend on the hypoxia tolerance, but also depend on other factors, such as cell proliferation, differentiation, and the ability to integrate into the retina of the transplanted cells in the intraocular injury.

Outlook on cell transplantation of retina The application of stem cells in retina cell transplantation will open up broad prospects to treat the neuroretinal epithelium degenerative diseases. The growth, proliferation, differentiation and integration into the retina of the transplanted stem cells will be the key factors on the success of transplantation. In addition,

the ethics of stem cell's transplantation, the choosing of the most suitable stem cells, the origin of stem cells, the improvement of the micro-environment in eyeball, the tumor-like proliferation of transplanted stem cells, the immunological rejection after transplantation, the regulation of differentiation and integration by gene level, and the large sample analysis of the outcome of the visual function after transplantation still need further research.

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