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BASIC SCIENCE

Protective effects of human umbilical cord blood stem cell intravitreal transplantation against optic nerve injury in rats

Tantai Zhao · Yunqin Li · Luosheng Tang · Yuehua Li · Fang Fan · Bing Jiang

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Abstract
Background The majority of studies addressing traumatic optic neuropathy (TON) have focused on drugs, proteins, cytokines, and various surgical techniques. A recent study reported that transplantation of human umbilical cord blood stem cells (hUCBSCs) achieved therapeutic effects on TON, but the exact effects on optic nerve injury are still unknown, and the mechanisms underlying nerve protection remain poorly understood.
Methods A total of 135 healthy Sprague–Dawley adult rats were randomly assigned to three groups: sham-surgery, model and transplantation, with 45 rats in each group. TON was induced in the model and transplantation groups via optic nerve crush injury. The crush injury was not performed in the sham-surgery group. Seven days after the injury, 10^6 hUCBSCs were injected into the rat vitreous cavity of transplantation group, and an equal volume of physiological saline was administered to the model and sham-surgery groups. Pathological observation of rat retina tissues was performed by hematoxylin–eosin (H&E) staining at days 3, 7, 14, 21 and 28 post-surgery. The number of retinal ganglion cells (RGCs) and mRNA expression levels of brain-derived neurotrophic factor (BDNF) and glial cell line-derived neurotrophic factor (GDNF) were assessed by the Fluorogold (FG) retrograde labeling and reverse transcriptase–polymerase chain reaction (RT–PCR) methods, respectively.
Results The number of labeled RGCs and the expression of BDNF and GDNF mRNA obviously increased, and pathological injury was significantly ameliorated in the transplantation group compared to the model group ($P<0.05$).
Conclusions Via intravitreal transplantation, the hUCBSCs resulted in a significant increase in the survival of the RGCs, and improved pathological changes in the rat retina, following TON. The protective mechanism is correlated with the continuous secretion of BDNF and GDNF in vivo of retina in optic nerve injury rats by the transplanted hUCBSCs.

Keywords Human umbilical cord blood stem cells (hUCBSCs) · Traumatic optic neuropathy (TON) · Brain-derived neurotrophic factor (BDNF) and glial cell line-derived neurotrophic factor (GDNF) · Neural regeneration

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T. Zhao · L. Tang (✉) · Y. Li · F. Fan · B. Jiang
Department of Ophthalmology, Second Xiang Ya Hospital,
Central South University,
No. 139, Renmin Road,
Changsha, Hunan Province 410011, People's Republic of China
e-mail: welovetls57@126.com

Y. Li
Department of Ophthalmology,
Second People's Hospital of Yunnan Province,
No. 176, Qingnian Road,
Kunming, Yunnan Province 650021, People's Republic of China

Introduction

Traumatic optic neuropathy (TON) is one of the most common neuropathy, affecting more and more people worldwide and leading to the loss of neural cells of the eyes. Although recent advances in treatment can now slow

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down its progression, many individuals with TON still suffer an irreversible loss of vision [1, 2]. Research is currently focused on developing new treatment strategies to rescue damaged photoreceptors and retinal ganglion cells (RGCs), and to replace lost cells through transplants. The neuroprotective and regenerative potential of stem and progenitor cells from a variety of sources has been explored in models of retinal disease and ganglion cell loss. It can be concluded that stem cells can potentially be used for both neuroprotection and cell replacement [3–5].

Numerous studies have also demonstrated the use of neural stem cells, embryonic stem cells and peripheral nerve transplantations as treatments for optic nerve injury. Due to the limited sources of embryonic stem cells, and ethical concerns, studies and clinical application using embryonic stem cells are greatly restricted [6].

Knudtzon reported in 1974 that human umbilical cord blood contains hemopoietic stem cells [7]. Since then, several studies have revealed the existence of stem cells in umbilical blood [8]. Human umbilical cord blood stem cells (hUCBSCs) are considered to be an optimal source for cell transplantation therapy of nervous system diseases, because of the abundance, availability, high quality, inferior immunogenicity, and lack of ethical implications.

Umbilical cord blood stem cells have been shown to directly engage in neural tissue repair and regeneration by replacing damaged cells and regulating the local microenvironment [9–11]. However, the protective ability of hUCBSCs in traumatic optic neuropathy (TON) has not been investigated yet. Moreover, it remains unknown how umbilical cord blood stem cells can provide neuroprotection.

The current study aimed to investigate the therapeutic effects of transplanted human UCBSCs (hUCBSCs) in rat models of traumatic optic neuropathy (TON), to determine brain-derived neurotrophic factor (BDNF) and glial cell line-derived neurotrophic factor (GDNF) mRNA levels in retina tissues, and to explore the protective mechanisms with hUCBSCs transplantation in TON.

Materials and methods

Umbilical cord blood stem cells

Umbilical cord blood stem cells were provided by Beike Biotech Shenzhen, China.

Animals and transplantation

Principles of laboratory animal care were followed according to the OPRR Public Health Service Policy on the Humane Care and Use of Laboratory Animals and the U.S. Animal Welfare Act, as amended.

A total of 135 healthy Sprague–Dawley adult rats, weighing 230–270 g and irrespective of gender, were randomly assigned to three groups: sham-surgery, model, and transplantation, with 45 rats in each group. TON was induced in the model and transplantation groups using optic nerve crush injury. Rats were anesthetized in a prone position via an intraperitoneal injection of 10% chloral hydrate (3.5 ml/kg). The temporal eyelid incision was performed to expose the intraorbital optic nerve, which was clamped by a special designed clip with 40-gram holding force constantly pressed for 30 seconds and then sutured. The crush injury was not performed in the sham-surgery group. Seven days after the injury, 10^6 hUCBSCs were injected into the rat vitreous cavity of the transplantation group, and an equal volume of physiological saline was administered to the model and sham-surgery groups.

Tissue specimen preparation

Nine rats were selected from each group at days 3, 7, 14, 21 and 28 post-surgery and the retinas were removed. Three of the retinas were used for frozen sections and hematoxylin–eosin staining. Three of the fresh retinas were used for RT–PCR. And three were fixed by paraformaldehyde after being labeled by fluorogold (FG) for the RGC counting and RGC rate.

The labeled RGC counting and RGC rate in the retina, as demonstrated by FG retrograde labeling

The number of RGCs was counted after retrograde labeled by fluorogold dye, using a slight modification of the method of Peinado-Ramon et al. [12]. Briefly, 4 days before killing, anesthetized rats were placed in a stereotactic frame and the head was immobilized. The skin was incised and two small holes (1.5×1.5 mm) were drilled through the skull bilaterally above both superior colliculi. Three microliters of 5% fluorogold retrograde tracer was injected with a micropipette at different depths into the superior colliculi. After wound closure, animals were allowed to recover before being returned to the cage.

Morphological evaluation in the retina, as detected by hematoxylin–eosin staining

Sections were dipped into gradient ethanol, stained with hematoxylin for 10 minutes, differentiated in 1% (v/v) hydrochloric acid alcohol for 5–10 seconds, incubated in saturated lithium carbonate solution for 5–10 seconds, and stained with eosin for 10 minutes. The sections were rinsed with distilled water for each interval step. The sections were then dehydrated with gradient ethanol, cleared with xylene, mounted with neutral gum, dried, and observed using light microscopy [13].

Retina BDNF and GDNF mRNA levels, as determined by reverse transcriptase–polymerase chain reaction (RT–PCR)

Assay of BDNF mRNA and GDNF mRNA expression was detected by reverse transcriptase–polymerase chain reaction. At days 3, 7, 14, 21 and 28 after intravitreal injection, the sham rats (control group), model rats (n=3 per time point) and transplantation rats (n=3 per time point) were anesthetized and sacrificed. The entire retina was removed from each rat immediately, and snap-frozen in dry ice. Tissue samples were stored at −80°C until use. Total RNA from retina was harvested using Trizol reagent according to the manufacturer's instructions. First-strand cDNA was synthesized by reverse transcriptase (Fermentas RevertAid first Strand cDNA Synthesis Kit) at 42°C using 1 μg total RNA extract. RT-PCR was conducted in 0.2 ml domed-cap pcr tubes (Axygen) with a total reaction volume of 25μl containing 1μl first-strand reaction product, 0.2 nM specific upstream and downstream primers, and 12.5 μ2×Taq polymerase buffer (TianGeng, China). Amplification of cDNA fragments and analysis were carried out. Amplification of the housekeeping gene GADPH mRNA transcript, which served as a normalization standard, was carried out. The primers and the conditions of RT-PCR are summarized in Table 1

Statistical analyses

Statistical analysis was performed through the use of SPSS 13.0 software, and data were expressed as mean±SD. Results were analyzed using one-way analysis of variance, and comparison among groups was performed using the LSD method. A level of $P \leq 0.05$ was considered statistically significant.

Results

Quantitative analysis of experimental animals

A total of 135 rats were included in this study. Some rats showed agitation for a short time following cell transplantation, and then recovered. No abnormal appearance or rejection occurred, and all rats survived.

Evaluation of transplantation effects

The labeled RGC counting and the labeled RGC rate in the retinas

In order to assess the protective effects of transplanted stem cells into rat retina, labeled RGC counting was used. A mean RGCs count labeled by FG was 197.52±39.25 in normal retina (Fig. 1a). In the sham-surgery group, a mean cell count was 195.76±36.12 (Fig. 1b), and there was no significant change compared with normal retina (P>0.05). We choose two points at days 7 and 28 for illustration. At day 7 post-surgery in the model group, the number of RGCs was obviously reduced, with a mean count of 111.19±20.32 (Fig. 1c, P<0.05), compared with the sham-surgery group. At day 7 post-surgery in the transplantation group, the number of RGCs was obviously increased after intravitreal injection, compared with the model group, with a mean count of 150.97±21.07 (Fig. 1d, P<0.05). At day 28 post-surgery in the model group, the number of RGCs was further reduced, with a mean count of 94.86±18.26 (Fig. 1e, P< 0.05), compared with the sham-surgery group. At day 28 in the transplantation group, a mean cell count was 113.23± 17.19 (Fig. 1f). It was obviously reduced compared with the sham-surgery group (P<0.05), but the survival rate was higher than that of the model group (P<0.05), indicating a protective effect to RGCs by hUCBSC transplantation.

The columns in Fig. 2 presents the number of RGCs among the sham, model and transplantation groups. Results are statistically significant as follows: (1) sham group vs model group — at days 3, 7, 14, 21 and 28 (all with P< 0.05), (2) transplantation group vs model group — at days 3, 7, 14, 21 and 28 (all with P<0.05). There was significant difference between any two groups. These results indicated that the survival rate in the transplantation group was higher than that of the model group, but still lower than that of the sham-surgery group.

Figure 3 illustrates the time-course of the number of RGCs in the sham, model and transplantation groups. Similar protective effects of RGCs by transplant cells into the retinas were observed at days 7, 14, 21 and 28 post-

Table 1 Primers and the conditions of RT-PCR

Gene	Primer sequence(5′-3′)	Product size	Temp.(°C)
BDNF	Forward: CAGTATTAGCGAGTGGGTCA Reverse: CCGAACATACGATTGGGT	223 bp	58
GDNF	Forward: TCACCAAAACAAATGGCAGTG Reverse: GGACGACTCAGATACCACACCTTTTAG	377 bp	55
GADPH	Forward: AACAAGCAACTGTCCCTGAGC Reverse: GTAGACAGAAGGTGGCACAGA	451 bp	61

BDNF=brain-derived neurotrophic factor,
GDNF=glial cell line-derived neurotrophic factor

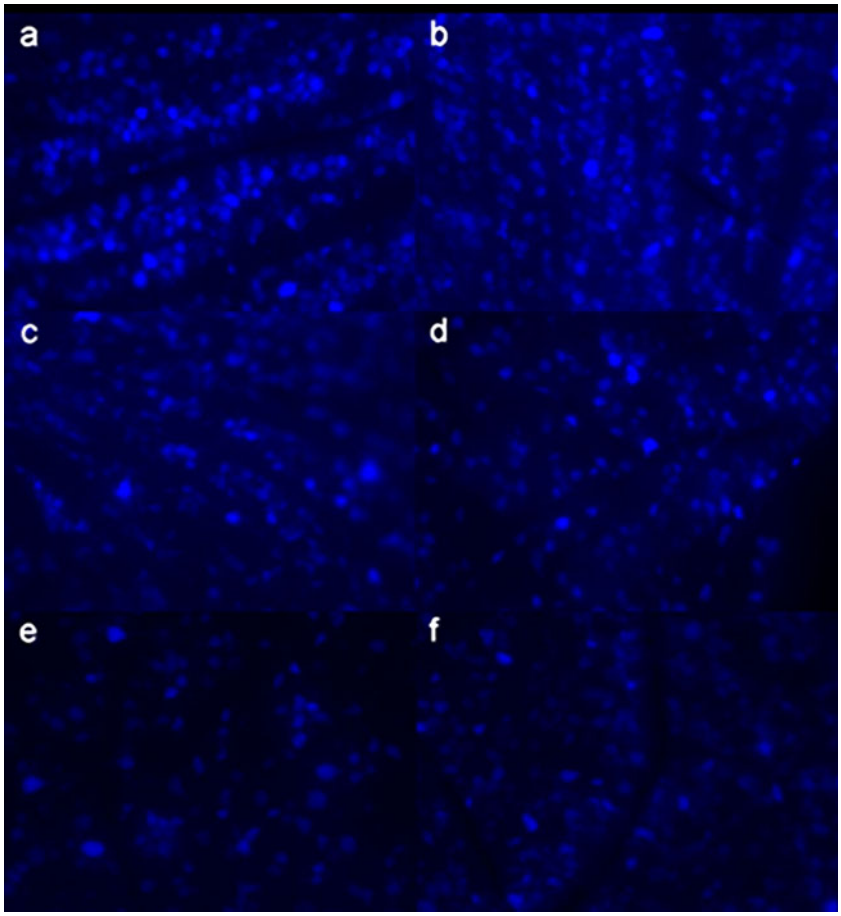

Fig. 1 RGCs labeled by FG in retinas (200× magnification). **a** Normal retina. **b** Sham-surgery group, no significant change compared with normal retina. **c** At day 7 post-surgery in the model group, the number of RGCs was obviously reduced. **d** At day 7 in the transplantation group, the number of RGCs was significantly increased, compared with the model group. **e** At day 28 in the model group, the number was further reduced. **f** At day 28 in the transplantation group, the number of RGCs was obviously reduced compared with normal retina, but was higher than that of the model group

surgery as noted in Fig. 2. However, transplanted stem cells into the retina did not reach full protection to normal level for RGC survival, as happened in the control group.

Pathologic assessment of retina tissues

To further determine the neuroprotective effects of transplanted stem cells into retina, we examined the morphological changes of retinas. Retinas were collected and subjected to hematoxylin–eosin (H&E) staining. As seen in sections from the sham-surgery group, all layers are normally organized, in which retinal ganglion cells are neatly arranged, with prominent nucleoli (Fig. 4a). There was no significant difference between normal retina and sham-surgery retina at days 7, 14, 21 and 28.

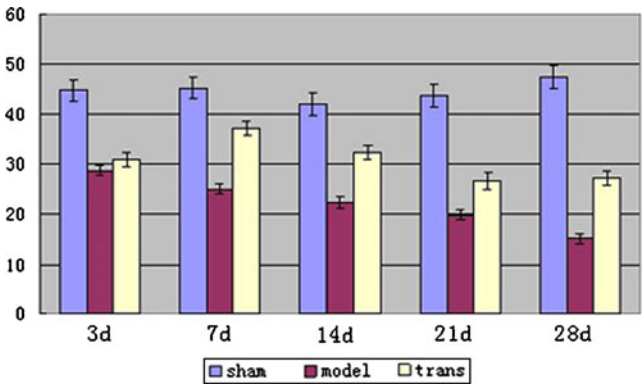

Fig. 2 Number of RGCs among the sham, model and transplantation groups ($x \pm SD$). There was significant difference between any two groups

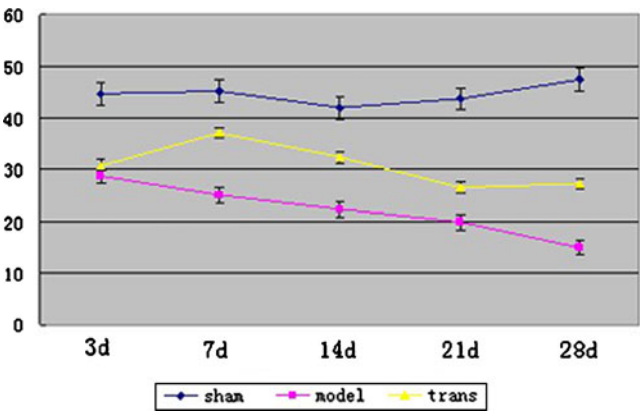

Fig. 3 Time-course of the number of RGCs. The number of RGCs in the transplantation group is higher than that of the model group, but lower than that of the sham-surgery group

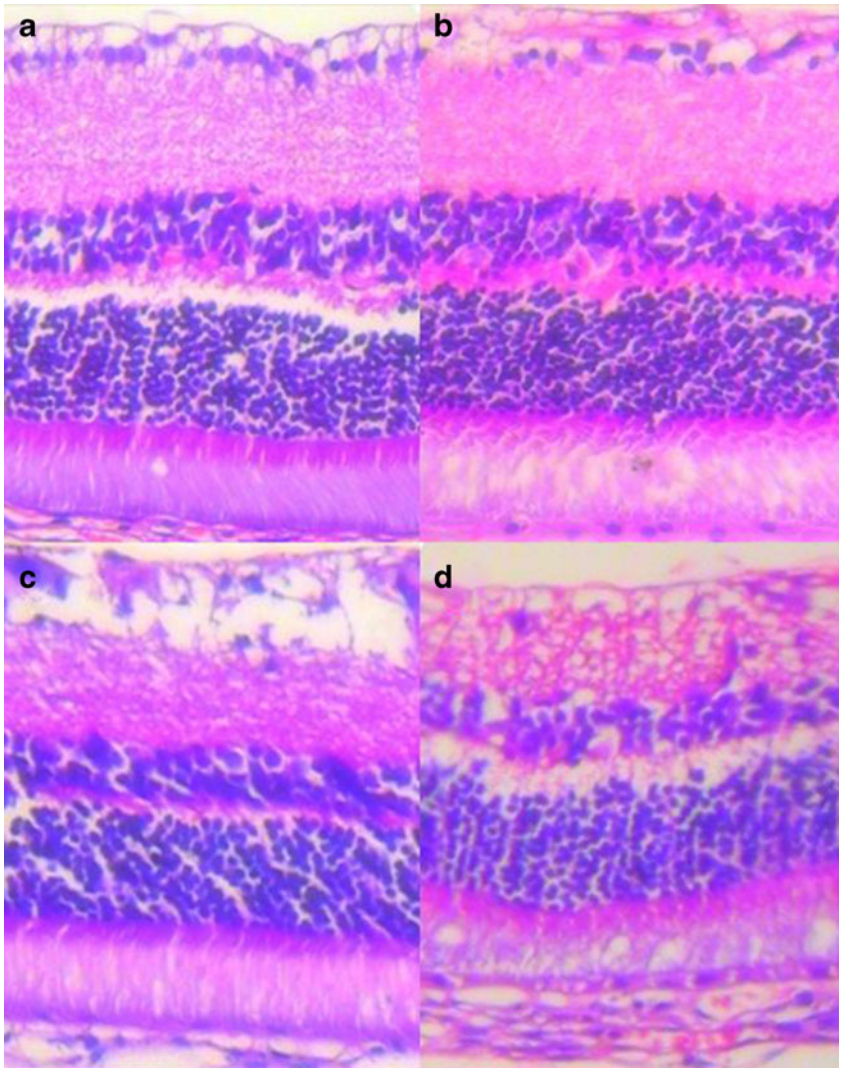

Fig. 4 Morphological changes in rat retinas (H&E staining, ×200). **a** sham-surgery group, all layers show normal patterns. **b** At day 7 in the model group, all layers reveal slight disarray, and ganglion cells display a little nuclear pyknosis, loss of nucleoli, and cytoplasmic vacuolation in a subtle degree. **c** At day 28 in the model group, layers turn thinner, with a vague outline, with more nuclear pyknosis, loss of prominent nucleoli, and cytoplasmic vacuolation. **d** At day 28 in the transplantation group, the degree of pathological changes is greatly improved compared with the model group

At day 7 post-surgery in the model group, all retina layers became slightly disarrayed and ganglion cells started to show a little nuclear pyknosis, loss of nucleoli, and cytoplasmic vacuolation in a subtle degree (Fig. 4b). At day 28 post-surgery in the model group, all retina layers turned thinner, with an unclear outline. Nuclear pyknosis, loss of prominent nucleoli, and cytoplasmic vacuolization of ganglion cells were much worse (Fig. 4c). In addition, the number of ganglion cells was reduced at days 7 and 28 post-surgery in the model group in H&E sections compared with the sham-surgery. At day 28 post-surgery in the transplantation group, although the structure was disarrayed, nuclear pyknosis and decreased ganglion cells were still visible (Fig. 4d); the degree of morphologic changes was greatly improved compared with the model group.

BDNF and GDNF levels in the rat retinas

We sought to further determine potential mechanisms of hUCBSC transplantation in protection of retinal ganglion loss; BDNF and GDNF mRNA expression levels by RT-PCR were investigated in all three groups.

In the retina tissue of transplantation rats, the expression of both BDNF and GDNF mRNA increased in a time-dependent manner (Fig. 5), and had a much higher level than that of the control group (the sham-surgery group). However, a small amount of BDNF and GDNF mRNA expression was detected in the model group.

The level of BDNF mRNA constantly increased in a time-dependent manner in the transplantation group (Fig. 6), most obviously after day 14 (1.45±0.15). The level of BDNF mRNA in the control rats was 0.67 ±0.13, In the model group, it reached a peak at days 21 (0.96± 0.17), then gradually decreased, and had a lower level than that of the control group at day 28 (0.58±0.11).

The level of GDNF mRNA was constantly elevated in a time-dependent manner in the transplantation group (Fig. 7), then reached a peak at day 21 (1.77±0.20), and slightly decreased at day 28 (1.31±0.32). In the control rats, there was minimal detectable GDNF mRNA. In the

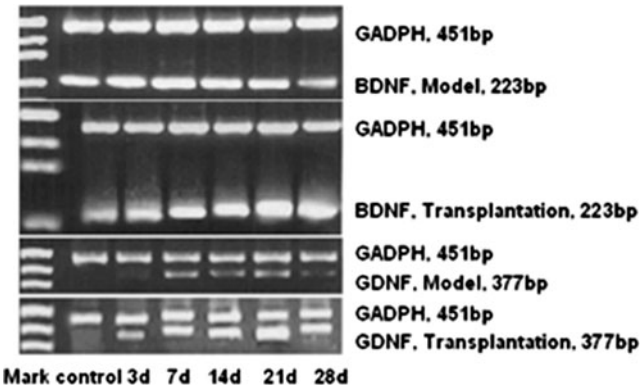

Fig. 5 BDNF and GDNF mRNA expression by reverse transcription–polymerase chain reaction (RT–PCR). Both BDNF and GDNF mRNA levels increased in a time-dependent manner in the transplantation group, and were higher than those of the model group. Control indicating the sham-surgery group and GADPH indicating internal control

model rats, it started to have an increase at day 3 (0.21± 0.14), then reached a peak at day 21 (0.53±0.09), and slightly dropped at day 28 (0.42±0.0.13).

Discussion

Traumatic optic neuropathy (TON) refers to any injury to the optic nerve secondary to trauma, and is an important cause of severe visual loss following penetrating or blunt head and eye trauma [14]. The pathophysiology of TON is complex, and may be multifactorial. Retinal ganglion cells (RGCs) are clusters of specific cells in the optic nerve, and play a major and direct role in transmitting information from the eye to the vision centers within the brain. Upon damage and shearing of RGCs following trauma, RGCs develop apoptosis, necrosis and degeneration. RGC regeneration after injury is limited, and may be due to multiple

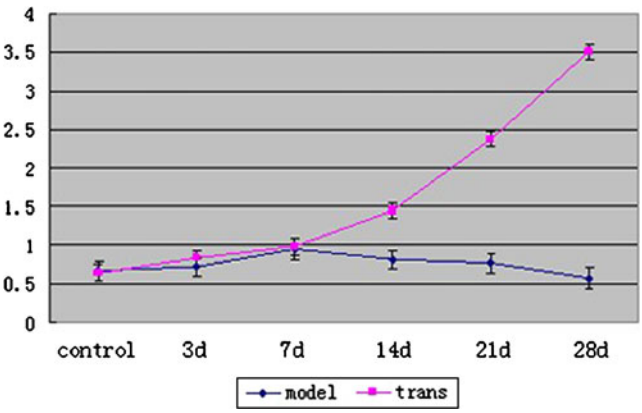

Fig. 6 Time-course of BDNF mRNA expression in the model and transplantation groups by RT–PCR. The level of BDNF was constantly elevated in the transplantation group, and was higher than that of the model group

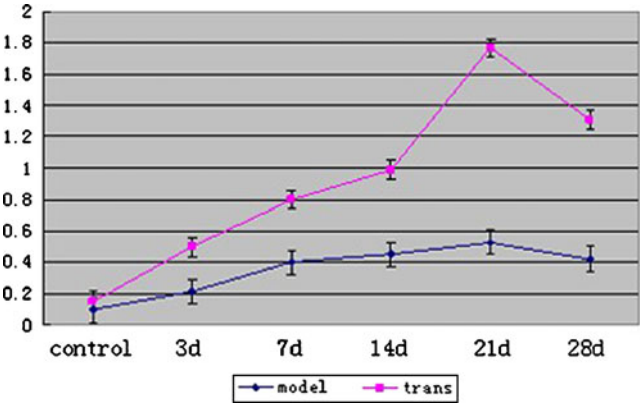

Fig. 7 Time-course of GDNF mRNA expression in the model and transplantation groups by RT-PCR. The level of BDNF constantly increased in the transplantation group, and was higher than that of the model group

inhibitory effects which include neurotrophic factors, inflammatory cytokines and cells, and surrounding angiogenesis [2, 14, 15]. Previous studies were focused on drug treatment (all kinds of neuroprotection drugs), and surgical intervention (optic nerve decompression surgery). But the effects are not satisfactory [16–19]. The need for more effective and safe treatment has led to searching for different therapeutic strategies to restore retinal function. Stem cell transplantation is a promising and attractive cell-based treatment modality for repairing damaged neural cells and photoreceptors. Embryonic stem cells, a leading candidate in stem cell research, have been used experimentally in the therapy of Parkinson's disease, spinal cord injury, stroke, diabetes, cardiac disease, and amyotrophic lateral sclerosis, because of their high and pluripotent self-renewal capacity [6]. However, its use has been precluded due to ethical and political issues, inaccessibility, and technical difficulty. Similarly, neural and bone marrow-derived stem cells have been used for stem cell therapies in many diseases, and also have encountered similar problems. In contrast, human cord blood stem cells have become increasingly popular because of their abundance, accessibility, non-invasive harvests, and low ethical and political concerns [20, 21]. The hUCBSC cells are immune naive, and they subsequently cause less graft rejection, graft-versus-host disease, post-transplant reaction [22]. They have pluripotent capacity to differentiate into other cells, including the neural lineage. Canque reported that CD34-/CD45- non-hematopoietic mononuclear cells fraction of hUCBSCs can generate neural-stem-like cells, and differentiate into the neural-like cells [23]. They also have the ability to produce several neurotrophic factors and moderate immune and inflammatory reactions. Human umbilical cord blood transplantation has been applied in the therapeutic treatment of more than ten diseases since it was successfully used in a patient with Fanconi syndrome

by Bacigalupo in 1988 [24]. However, little has been reported regarding the treatment of TON by hUCBSCs. In addition, hUCBSC transplantation is a novel biotherapy approach for traumatic optic neuropathy, and may directly provide a source of neurons and interstitial cells to the lesional areas, and promote survival and regeneration of RGCs.

The unique aspects of our study are the observation that the hUCBSC grafts not only resulted in a significant increase in RGC survival, but also showed a persistent increase in BDNF and GDNF mRNA expression, as determined by hematoxylin–eosin staining, FG labeling and reverse transcriptase–polymerase chain reaction (RT–PCR). Following trauma, RGCs may die through apoptosis, necrosis and degeneration. RGC loss from the above death mechanisms is determined by morphology and Fluorogold retrograde labeling. RGC may die by apoptosis after traumatic optic nerve damage. In earlier reports, Fluorogold retrograde labeling has shown a good correlation with apoptosis as detected by TUNEL and/or caspase-3 methods [1, 2, 25]. Our experiment demonstrated an increase in RGC survival through grafted hUCBSCs. Thus, we conclude that intravitreal transplantation of hUCBSCs resulted in a reduction in traumatic optic damage to nerve function in rats, suggesting that transplanted hUCBSCs may provide protection against traumatic injury in the retina in human beings.

The protective mechanisms for TON by grafted hUCBSCs are unknown, and may be complex. Dasarie et al. reported axonal remyelination after transplantation of hUCBSCs in a rat spinal cord injury model, and found out the differentiation of hUCBSCs into neural cells, and elevated levels of the mRNA expression of NT3, BDNF and others [26]. Intravitreal delivery of neurotrophic factors, such as BDNF and GDNF, slowed down photoreceptor degeneration in rat models of traumatic optic neuropathy, but the effect was temporary [27]. Slow-release preparations and gene therapy approaches to induce retinal cells to secrete neurotrophic factors are two ways to induce longer-term effects [28–30]. A third option, which might be the most important, is to use stem cells as long-term delivery agents to do so. Interestingly, there was a small amount of BDNF and GDNF mRNA expression in the model group, indicating that the retina itself following traumatic injury could secrete BDNF and GDNF at a low level to confer minimal protective effects. However, in the transplantation group, BDNF and GDNF levels were significantly higher than in the model group. Our data demonstrated that the protective mechanisms of transplanted hUCBSCs are associated with continuous secretion of BDNF and GDNF in vivo of retina of optic nerve injury rats. Elevated levels of BDNF and GDNF by grafted stem cells may promote differentiation of the grafted cells into neural cells, inhibit apoptosis, improve angiogenesis, suppress an inflammatory infiltrate, and maintain grafted cell survival [31].

In summary, as our study demonstrated, hUCBSCs indeed had protective effects against optic nerve injury in rats undergoing intravitreal transplantation. However, translation of these findings into clinical therapies has been little achieved. Therefore, more investigations should be performed to guarantee better results and the success of future clinical application of hUCBSCs. With more understanding of hUCBSCs, it is certain that hUCBSCs will be a good candidate to restore function after traumatic optical injury.

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