

Original Article

Effects of ciliary neurotrophic factor on transplantation of iris pigment epithelium into subretinal space

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Abstract: Ciliary neurotrophic factor (CNTF) can facilitate regeneration of impaired neural tissues. Iris pigment epithelium (IPE) can replace retinal pigment epithelium (RPE) for treating retinal degenerative diseases. This study investigated the retinal morphology and CNTF expression after subretinal transplantation of CNTF-transfected IPE. Both healthy and RCS rats (3 weeks old) were transplanted with CNTF gene transfected IPE cells at subretinal space (in treatment group of RCS rats only). At different time points after surgery (3, 5, 7, 9 and 11 weeks), retinal tissues were extracted for observing morphology by HE staining. Enzyme linked immunosorbent assay (ELISA) was used to determine CNTF expression in retinal tissues while TUNEL assay was employed for apoptosis. No significant abnormality or CNTF expression alternation has been found in normal SD rats (control group). Model rats had retinal degeneration with aging, accompanied with decreased CNTF expression ($P < 0.05$). The peak of cell apoptosis occurred at 5 weeks post-surgery. Treatment group had improved retina compared to model rats but without significant change of CNTF expression. The apoptotic peak reached at 9 weeks after surgery. CNTF expression was elevated in treatment group, which also had lower apoptosis compared to model group ($P < 0.05$). No immune rejection was observed. Subretinal transplantation of CNTF gene-transfected IPE cells can retard the progression of retinal degeneration diseases, possibly related with the facilitation of elevated expression of CNTF in retinal tissues.

Keywords: Iris pigment epithelium, retinal pigment epithelium, CNTF

Introduction

Age-related macular degeneration, retinal pigment degeneration and other degenerative diseases are all related with abnormal function of retinal pigment epithelium (RPE), and have no available treatment currently [1, 2]. Retinal transplantation has received certain treatment efficacy in animal study [3, 4]. Some study found that the transplantation of normal RPE into subretinal space can retard the photoreceptor degeneration to some extents. Due to the limited availability and risk of autologous RPE, it is difficult to promote the transplantation [5, 6]. Iris pigment epithelium (IPE) is similar as RPE, but with relatively fruitful sources, and has been proved to be able to replace RPE for treating degenerative diseases by animal studies [7, 8]. The axons of retinal ganglion cells (RGCs) converge at the optical nerve disc,

and form the optic nerve via penetrating sclera. The recovery of optic nerve function is related with RGCs injury, ability of auto-repair and synthesis/transport function of axons [9, 10]. Current studies believed that exogenous neurotrophic factors and cell transplantation could improve local injury microenvironment, and facilitate regeneration of optic nerve [11, 12]. Optic nerve is hard to regenerate, as no completely effective pathway has been found regarding the repair of optic nerve. Some studies showed that neurotrophic factor could facilitate the survival and regeneration of both central and peripheral neurons after axonal injury [13, 14]. Among those factors ciliary neurotrophic factor (CNTF) facilitates the repair and regeneration of injured neurons, and can support the survival of multiple neurons via facilitating axonal growth. Regarding the stimulation of RGCs survival and axonal regeneration, CNTF is the

only neurotrophic factor with such dual functions. The intra-vitreous injection of CNTF can replace targeted neurotrophic factors, thus potentiating the anti-injury potency of RGCs, decrease secondary apoptosis/necrosis of neurons, elevate RGCs regeneration, recover and facilitate axonal transport, and mediate neural nutrient, thus exerting the protective role of RGCs [15, 16]. The repeated intra-vitreous injection of neurotrophic factor, however, can complicate including endophthalmitis and retinal detachment, causing significant risk for clinical use. We thus combined neurotrophic factor with transgenic manipulation, to transplant into subretinal cavity, thus exerting the protective role of neurotrophic factor and facilitating for survival of transplanted cells. This study observed retinal morphology and CNTF expression after subretinal cavity transplantation of CNTF-transfected IPE, in an attempt to investigate the role of CNTF in facilitating subretinal cavity transplantation of IPE.

Materials and methods

Animals

Healthy SD and RCS rats (3 weeks old) were provided by Laboratory Animal Center, Shandong University (Certificate No. SYXK-2013-0025). Animals were kept in an SPF-grade facility with food and water. SD rats were employed as the control group and were divided into 3, 6, 8, 10, 12 and 14 weeks (N=6 each). RCS rats were randomly divided into model or transplant group (N=50). The sub-grouping of ages was the same as in control rats.

Rats were used for all experiments, and all procedures were approved by the Animal Ethics Committee of Qilu Hospital of Shandong University.

Drugs and reagents

Chloral hydrate, sodium pentobarbital, and paraformaldehyde were purchased from Kemiou Chemical (China). CNTF-transfected IPE cells were prepared using AAV-CNTF-IPE vectors following previous records [11] by Gibco (US). Enzyme linked immunosorbent assay (ELISA) kit for CNTF was provided by Jiancheng Bio-engineering (China). TUNEL assay kit was purchased from Roche (US). Rabbit anti-rat GFAP monoclonal antibody was provided by Sigma

(US). Mouse anti-human β -actin monoclonal antibody and goat anti-rabbit fluorescent secondary antibody were provided by Biovision (US). Microplate reader (Varioskan Flash 4.00.51) was provided by Thermo (US).

Subretinal cavity transplantation

CNTF-transfected IPE cells were transplanted into subretinal cavity of RCS rats via external route. After 1% pentobarbital anesthesia, a micro-syringe (30 G needle) was used to implant 2 μ L LAAV-CNTF-IPE into subretinal cavity supratemporally. Antibiotics were then applied to check the condition. Those rats with hemorrhage of vicarious body or large scale retinal detachment were ruled out. The successful transplantation was deduced by the focal extrusion of retinal membrane.

Post-operative examination time points

After 3, 5, 7, 9 and 11 weeks of surgery, rats were sacrificed with the retinal tissue detached under the microscope.

Morphology of retinal tissues

Retinal tissues were fixed in paraformaldehyde and were chosen on the optic nerve plane for the same location. After paraffin embedding, 4 μ m slices were prepared for hematoxylin-eosin staining for observation under the light field microscope.

CNTF expression by ELISA

Retinal tissues were removed from the eye ball and homogenized. After centrifugation, the supernatant was assayed for CNTF expression by ELISA following the manual instruction. Optical density (OD) values were measured at 450 nm.

TUNEL assay

Paraffin slices were prepared from rat eye balls. Following the manual instruction of TUNEL kit, tissue slices were rinsed in xylene, followed by gradient ethanol and PBS. After digestion in proteinase K, TUNEL reaction buffer was used to process the slices, followed by PBS rinsing and DAB development. Hematoxylin was used to counter-stain slices, followed by dehydration in gradient ethanol. Xylene was used to process

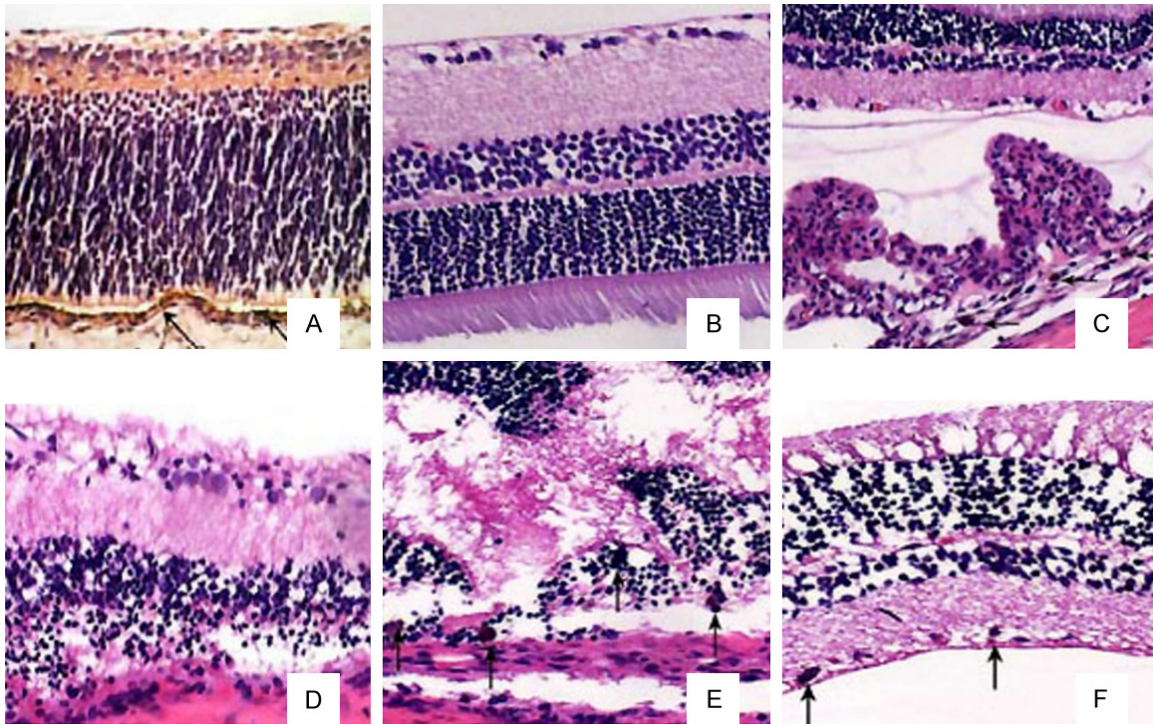


Figure 1. Retinal morphology (HE staining, X400). A. 3 weeks of control group; B. 8 weeks of control group; C. 3 weeks of model group; D. 8 weeks of model group; E. 3 weeks post-surgery (6 weeks) of treatment group; F. 5 weeks post-surgery (8 weeks old) of treatment group.

the slices, which were mounted with coverslips and observed under a light field microscope.

Immunofluorescence

Paraffin-based retinal slices were dewaxed, rehydrated, rinsed and incubated with rabbit anti-rat GFAP antibody (1:2,000), and were observed under fluorescent microscope.

Statistical methods

SPSS20.0 software was used for statistical analysis. Measurement data were firstly tested for normality. Those fitted normal distribution were presented by mean \pm standard deviation (SD). Analysis of variance (ANOVA) was used to compare means across multiple groups. Between-group-comparison was performed in LSD test. A statistical significance was defined when $P < 0.05$.

Results

Subretinal cavity transplantation

During the process of transplantation surgery, 2 rats died due to the over-dose of anesthetics,

and 3 rats died because of inappropriate post-op care. A total of 45 RCS rats successfully received bilateral subretinal transplantation. In a total of 90 eyes, there were 14 cases of large-scale retinal detachment or hemorrhage inside vitreous body, or intraocular inflammation making the successful rate at 82.22% (74 out of 90).

Retinal morphology

HE staining showed no abnormality of retinal tissues across all age groups of SD rats, as regular arrangement of all retinal cell layers without disruption of vacuoles. It can be shown that brown RPE cells had normal arrangement. Model rats lacked pigment cell layer in both retina and ciliary, with only dispersed brown epithelium in ciliary epithelial layer. With aging, retinal tissues showed degeneration, along with disarrangement, vacuoles and atrophy of external nuclear phase. Treatment group had improved retinal morphology after transplantation, as brown epithelium can be seen with thickening of ONL compared to age-matched model rats (**Figure 1**).

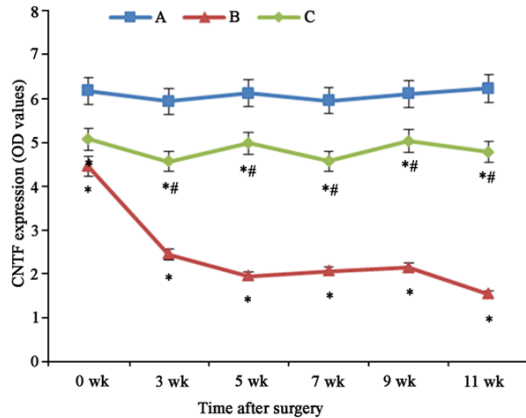


Figure 2. CNTF expression of retina. A. Control group; B. Model group; C. Treatment (transplantation group). *, $P < 0.05$ compared to control group; #, $P < 0.05$ compared to model group.

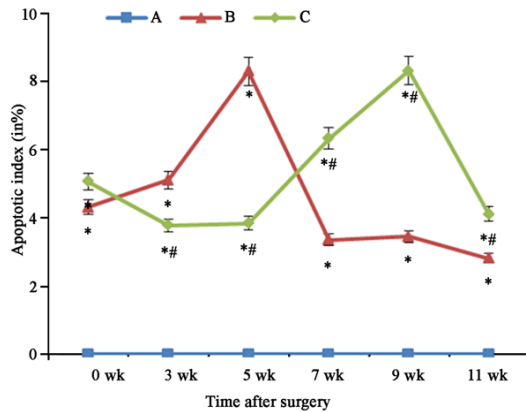


Figure 3. Retinal apoptotic indexes of all rats. A. Control group; B. Model group; C. Treatment (transplantation group). *, $P < 0.05$ compared to control group; #, $P < 0.05$ compared to model group.

CNTF expression of rat retina

CNTF expression was not significantly altered at all time points in control group. In model group, CNTF expression was gradually decreased after 3 weeks old ($P < 0.05$). Treatment group had similar CNTF expression before and after surgery. However, CNTF level in treatment group was significantly higher than that in model group ($P < 0.05$, **Figure 2**).

Retinal apoptotic index

Controlled SD rats had no apoptosis of retinal tissues across all age groups. Model RCS rats had the peak of apoptosis at 5 weeks after surgery (8 weeks age). After cell transplantation,

treatment group rats had peak level of apoptosis at 9 weeks after surgery (12 weeks age), followed by gradual decrease of apoptotic index, suggesting amelioration of retinal apoptosis after cell transplantation (**Figure 3**).

GFAP protein expression in retina

Under normal conditions, Muller cells did not express GFAP protein. The early stage of retinal injury elevated GFAP expression. In controlled SD rats, GFAP proteins were mainly expressed in RGC layer and inner layer. Model RCS rats had strong GFAP expression in Muller cells in out layer of retina. Treatment rats had relatively weak staining in Muller cells, as weak-positive expression of GFAP existed in outer layer (**Figure 4**).

Discussion

Previous studies have shown that electroporation coupled with neurotrophic factor transfection and transplantation onto RPE may retard the progression of rat retinal pigment degeneration [17, 18]. Ultrasonic micro-droplet induced-CNTF gene intraocular transfection could enhance the expression of CNTF gene, protect RGCs in neural injury rats, and facilitate recovery of optical function. Currently external and internal routes for subretinal cavity transplantation are available. Internal route is based on the surgical technique of vicarious body, and can reach the injection site with high precision and direct vision of transplantation area, but requires advanced surgical technique and longer operation time. External route is relatively easy to operate, but may have the risk of retinal puncture due to the blindness of injection area. In this study, juvenile rats (3 weeks old) were employed to transplant gene-transfected IPE cells into subretinal cavity via single channel sclera external route, and reaching an overall successful rate at 82.22%. After surgery, HE staining was used to observe morphology of retinal tissues. In RCS rats, 5 weeks after the surgery, there were still dispersed alive AAV-CNTF-IPE cells inside subretinal cavity, without significant immune reaction or structural damage in retinal tissues, suggesting relatively longer survival time of transplanted cells without immune rejection. In this study, CNTF gene was firstly transfected into IPE cells by molecular approach, followed by cell transplantation into subretinal cavity. No significant change of CNTF

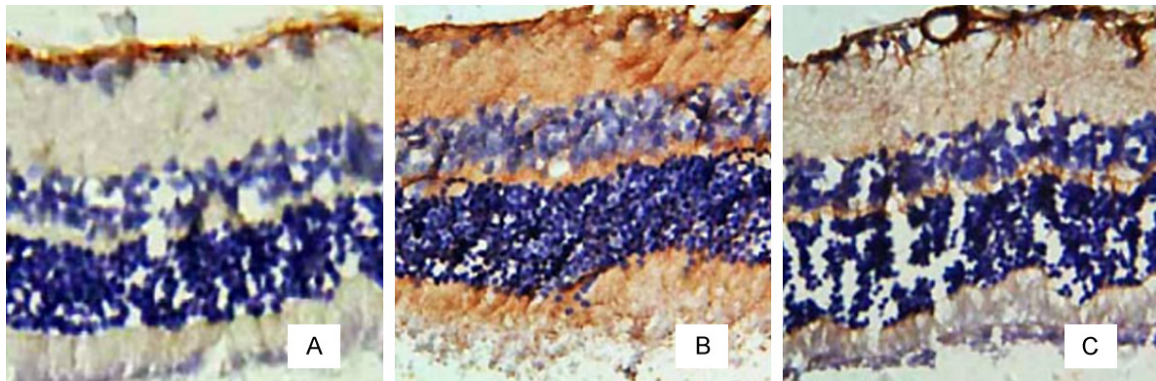


Figure 4. GFAP protein expression (X400). A. Control group; B. Model group; C. 3 weeks after surgery (6 weeks age) in treatment group.

expression was seen in retinal tissues after surgery compared to those before surgery. Such persistently high expression benefits the survival of retinal cells. CNTF expression after surgery in treatment group was also higher than age-matched model animals, which had gradually decreased CNTF expression after 3 weeks age, suggesting that CNTF-gene transfection on IPE cells coupled with subretinal cavity cell transplantation may retard the degeneration of retinal tissues.

Currently no consensus has been reached regarding the mechanism of photoreceptor cell death [19]. Cell apoptosis plays a critical role in the degeneration of retinal pigment cells. No effective method is available for preventing such apoptosis [20, 21]. Therefore the retard or inhibition of retinal photoreceptor cell death benefits the treatment of retinal pigment degeneration. This study performed an examination on the apoptosis of rat retinal tissues and found the degeneration of photoreceptor cells 3 weeks after surgery in RCS rats. With aging of animals, the disease aggravated with more apoptosis of retinal cells. The peak of apoptosis occurred at 5 weeks after surgery (8 weeks old) for model RCS rats. After transplantation of gene-transfected cells, the peak of apoptosis in RCS rats occurred at 9 weeks after surgery (12 weeks age), followed by decrease of apoptotic index. The lag of apoptosis peak value after transplantation, suggested ameliorated apoptosis of retinal cells in treatment group. Therefore, the subretinal cavity transplantation of CNTF-transfected IPE cells retarded the progression of retinal pigment degeneration to some extents, probably related with the facilitation of CNTF expression and retard

of photoreceptor cell apoptosis. Under normal conditions, Muller cells did not express GFAP protein. The early stage of retinal injury elevated GFAP expression. In controlled SD rats, GFAP proteins were mainly expressed in RGC layer and inner layer. Model RCS rats had strong GFAP expression in Muller cells in out layer of retina. Treatment rats had relatively weak staining in Muller cells, as weak-positive expression of GFAP existed in outer layer, suggesting the alleviation of compensatory glial fiber proliferation by the subretinal cavity transplantation of CNTF-transfected IPE cells. In summary, the transplantation of CNTF-transfected IPE cells into subretinal cavity can retard the development of retinal degeneration, possibly due to the elevated expression of CNTF in retinal tissues and retard of photoreceptor cell apoptosis.

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Disclosure of conflict of interest

None.

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