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Experimental Research on Pigment Epithelium Derived Factor Joint Lens Injury Promote Optic Nerve Regeneration in Rats

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This study investigated whether pigment epithelium derived factor (PEDF) combined with lens injury protection coordination of rat retinal ganglion cells (RGCs) survival and promote RGCs axonal regeneration purposes, using Fluoro-Gold microinjection into adult SD rats and 2 W SD was born in brain of young rats, retrograde labeled RGCs and preparation of optic nerve injury models and crystalline lens injury model, observation number method, draw a result, intravitreal injection of PEDF lens injury can increase RGCs survival number, both of which can be coordinated so that this role increased. Lens injury stimulates axon regeneration, combined with PEDF can enhance the stimulation of regeneration. Lens injury and PEDF can improve GAP-43 mRNA expression, the combination of the two makes this action to strengthen.

Key words: Retinal ganglion cells, Lens injury, Pigment epithelium derived factor, Axon, Regeneration, Opticnerve injury.

The adult mammalian brain or spinal cord injury may lead to neuronal death, cell-cell interaction disappeared, nerve function permanently impaired. Want to repair the damaged central nerve, we must achieve the following two basic conditions: (1) preventing or delaying impaired neuronal death; (2) promote axonal regeneration to a proper destination (Bray *et al*., 1991). So, in the central nervous system (CNS) after injury, must pass through the protection of neuronal activity and stimulate axon regeneration for therapeutic purposes.

Neurotrophic factor in impaired RGC cells plays an important protective role. In these neurotrophic factor, pigment epithelium-derived factor (PEDF) in RGC cells is impaired, the effective protection of cell survival. The first is PEDF from fetal retinal pigment epithelial cells in culture medium is separated from the (Tombran *et al*., 1991), the molecular weight is 50 kDa, subsequently found in intraocular different tissues and cells are expressed, including limbus and non pigmented ciliary epithelial cells (Ortego *et al*., 1996; Karakousis *et al*., 2001). In the brain and spinal cord and nerve tissue, such as epithelial cells and osteoblasts were also found in the PEDF expression of (Tombran *et al*., 2003; Barnstable *et al*., 2004). PEDF is a serine protease inhibitor (serine protease inhibitor, Serpin) gene family members of (Steele *et al*., 1993), however, PEDF doesn't have antiprotease activity (Becerra *et al*., 1995).However, its ability to inhibit angiogenesis, have neurotrophic and neuroprotective role of (Tombran *et al*., 2003; Barnstable *et al*., 2004; Patricia *et al*., 2006). PEDF on many of the neurons and the organization has neuroprotective effects. PEDF can be reduced by glutamate in cerebellar granule cells, neurons in the hippocampus and spinal motor neuron death (Taniwaki *et al*., 1995). In the retina,

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the PEDF can protect oxidative stress in cultured retinal cells survived for (Cao *et al*., 1999). It can also protect photoreceptors from light damage in vivo (Cao *et al*., 2001). Intravitreal injection of PEDF can reduce local ischemia leads to death of RGCs (Ogata *et al*., 2001; Takita *et al*., 2003).

Previous studies confirmed the adult rat lens injury can effectively promote the RGC cell axon regeneration (Fischer *et al*., 2001; Leon *et al*., 2000). If in the optic nerve injury and damage the lens, many RGC cell axons can regenerate into the distal part of the optic nerve. Subsequent experiments confirmed, lens injury can result in macrophages to release certain factor, but the mechanism is still unclear (Yin *et al*., 2003). Therefore, this study aims to investigate when the CNS is impaired when combined, can protect neuronal survival factor (PEDF) and can promote axonal regeneration of injuried lens is able to better protect the survival of RGC cells, and then promote the distal axon regeneration.

METHOD

Fluoro gold retrograde labeling of RGCs

From SD rat (200-250g) 40, intraperitoneal injection of sodium pentobarbital anesthesia (40 mg/kg), treated rats after deep anaesthesia, fixed on the stereotactic frame, disinfection, on the head of median do all the export, the traction sutures in 4, blunt separation of tissue, 30% hydrogen peroxide coated periosteum, exposed calvarial sagittal suture and herringbone stitch, identify the point Bregma. In accordance with the Paxinos rat brain stereotaxic atlas, using dental drill bilaterally in the superior colliculus (Bregma zero point shift of 6.30±0.2 mm, next to 1.40±0.2 mm) and dorsal lateral geniculate nucleus (dorsal lateral geniculate nuclei, dLGN (Bregma) to the zero point after the shift, 4.30±0.2 mm open next to 4.40± 0.2 mm) skull drill open cast. According to the map shows depth (the superior colliculus is 3.50±0.2 mm, lateral geniculate nucleus was 4.60±0.2 mm), 0.5μL 2%FG were injected slowly in bilateral superior colliculus and dorsal lateral geniculate nucleus, the 10 min, FG retrograde labeling of RGCs. Layered suture of fascia and skin. The wound coated erythromycin eye ointment. The operation continued feeding (Figure 1(a), (b)). 1W after optic nerve crush.

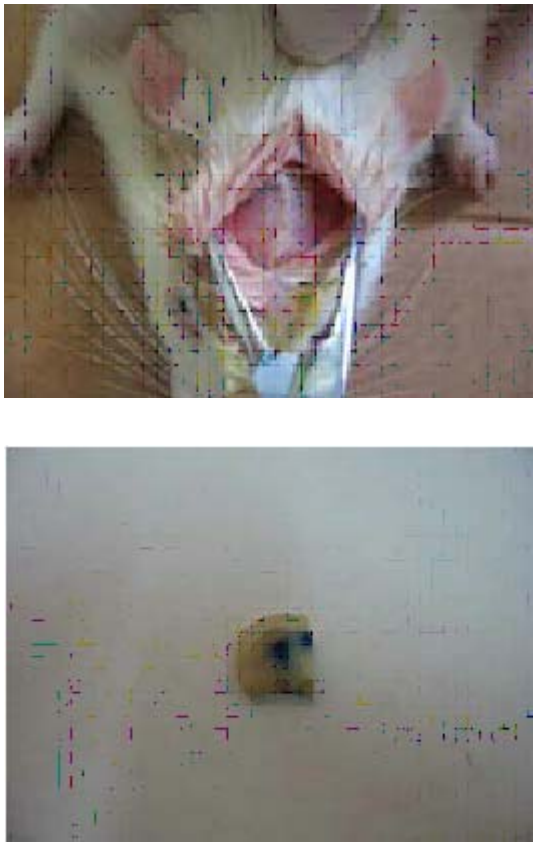

Fig. 1. The rat skull mark point injection point skull projection a, and schematic diagram of the injection point dye marker b

Preparation of optic nerve crush injury model

The rabbits were divided into 2 groups, 60 in total. A group of 1 former w FG retrograde labeling in 40 SD rats, another group did not receive any treatment of 20 SD rats. Intraperitoneal injection of sodium pentobarbital anesthesia (40mg/kg), placed on the operation table, sterilized intraoperative topical ocular, eyes in the lateral canthus to do a 1-1.5 cm incision. Under a dissecting microscope open Tenon capsule, along the temporal scleral surface separating the lateral, longitudinal incision of the optic nerve wrapping the outer layers of myelin, the exposure of the optic nerve, optic nerve peripheral vascular isolation, under direct vision in the eye after 2 mm micro platform tweezers compressive optic nerve 10 s, when the pressure is completely closed platform for microsurgery forceps finish clip trauma, avoid injuring the optic nerve blood vessel. Crush finish

can be seen after clip after optic nerve injury. The first group of 16 randomly selected, second groups of 8 randomly selected at the same time lens damage experiment, the front end of the needle head is bent by 90 degrees, in the cornea and sclera after 2 mm carefully into the needle, to avoid the vortex vein, deliberately punctured lens surface.

Can be directly through the cornea to see the damage to lens, also by 1W lens opacity judgement.Operation operation antibiotic eye drops eye drops to prevent infection.Atropine mydriasis, ophthalmoscope retinal blood supply of the retina of the eye, good blood supply into test.

Vitreous injection

The first group of 8 randomly selected SD rats and 8 lens injury rats, second groups of 4 randomly selected SD rats and 4 lens injury rats, in the optic nerve injury immediately after intravitreal injection of 10 μ L PEDF (1mg/ml), after every 3 D injection time, a total of 3 times. Other optic nerve injury in operation of SD rats after intravitreal injection of 3 d. With 30 needles in the cornea and sclera after 2 mm carefully into the needle, to avoid the vortex vein, intravitreal injection, to avoid hurting the lens, the 30 s, slowly pull the needle. Glass in vivo were injected with 10μ l yeast polysaccharide (12.5mg/ml dissolved in PBS) and to an injection of PEDF lens injury rats intraocular injection of PBS, simple PBS injection as the control group, after every 3 D injection time, a total of 3 times, each consisting of 4 SD rats. The yeast polysaccharide 90°C for 10 min.

Retinal mount count RGCs

The same FG retrograde labeling of RGCs, mean RGCs number obtained.

Frozen section preparation

FG retrograde labeling in the rat, in the vitreous humor after 2W injection,SD rats of depth of anaesthesia, thoracotomy. 300ml PBS after reperfusion, 300ml 4% paraformaldehyde perfusion fixation. Open the skull, removed from the brain, the exposure of the optic nerve and optic chiasma, isolated from peripheral tissues, remove eye with optic nerve, 4% paraformaldehyde fixed (after overnight, 4°C), moved to a 30% sucrose solution (overnight, continuous shaking, 4°C). In the freezing microtome along the optic nerve incision eye direction, will be frozen (thickness of 15μm) in the flat hanging plastic glass slides, -80°C save, spare.

Count the number of RGCs retinal slice

The retinal slice tiled on the slide, in fluorescence microscopy observation and photography. Image acquisition and analysis system (Image-Pro Plus 6) for analysis, calculation of every vision retrograde labeled RGCs number in each slice, random counting 5 view, each of the samples at least count 50 sections, the average demand. All the experiments were repeated 3 times.

Measurement of axon number

Each specimen is cut longitudinally along the optic nerve counting axon regeneration. In fluorescence microscopy observation and photography. Image acquisition and analysis system (Image-Pro Plus 6) measurement, counting distance clip on 250 μ m for each view (400×) fluorescent gold labeled axon number in each slice, random counting 5 view, each of the samples at least count 50 slice. All the experiments were repeated 3 times.

Detection of expression of mRNA RT-PCR GAP-43r

Organization total RNA extraction

Not carried on the FG retrograde labeling of SD rats, in the vitreous humor after 2W injection, SD rats of depth of anaesthesia, remove fresh eye tissue, cut off the cornea, lens and vitreous removal, peeling retinal tissue. Take 20-30mg organization, join 0.8ml TRIZOL homogenate, total RNA extraction.

PCR reaction

Total RNA after reverse transcription, add primers for PCR reaction, experimental set up blank control and GAPDH as the control group. Citing Kato (Kato *et al*., 2003) experimental design, by Shanghai SANGON Biological Engineering Technology Services Limited synthesis. GAP-43: P15-CTCATAAGGCTGCAACCAAA-ATTCAGGC; P25-GGGCATTTCCTTAGGTTTG. GCTTCATC-3, fragmentlength 540bp.

GAPDH: P15-AGGGCATCCTGGGC-TACACT-3; P25-GTTA-TGGGGTCTGGGATGGA-3, fragment length331bp. PCR reaction system including PCR reaction buffer, 0.2mMd NTP, 0.2 μM primer and 1 μ L DNA template, with the final volume of 50μ L. The mixture was heated at 95°C for 1 minutes after the cold, the rapid centrifugal seconds, so that the pipe wall of droplet settling to the bottom, add Taq DNA polymerase (0.5 μl approximately 2.5U), in PCR instrument to complete

the cycle. Denaturation 1min at 95°C, 60°C 30s, 72°C 2 min loop extending, 35 round, PCR. The final round after closing the loop, at 72°C for 10min, the reaction product is amplification fully.

PCR reaction detection

PCR reactant in 1.2% agarose gel electrophoresis of about 40 minutes (80V).Electrophoresis, image scanning analysis. In the experimental group and the gray value of GAPDH gray value ratio to calculate the GAP-43 expression level of mRNA.

Statistical analysis

Using the data processing software of SPSS 17, using t test, two sample, and LSD test, many group of sample, data representation for± s. There were significant differences in P<0.05.

RESULTS

PEDF combined with lens injury promotes regeneration of optic nerve morphology observation

Retinal mount count RGCs

The experimental comparison of PEDF alone and PEDF combined lens damage after optic nerve injury on RGCs survival cell number effect. In order to identify the retina after the optic nerve injury by RGCs, 1W, FG retrograde labeling of RGCs. Lens injury in the implementation of the optic nerve injury, simultaneous intravitreal injection of PEDF. The experimental group consisted of separate injection of PEDF, zymosan, for lens injury or lens injury combined with PEDF intravitreal injection. Separate injection of PEDF or lens injuries can protect RGCs survival. Analysis of the data revealed optic nerve injury after 2W, separate injection of PEDF group RGCs number (672±78 RGC/mm^2) and a separate lens injury group (773±80 RGC/mm^2) in RGCs number has statistics difference (P<0.01). Lens injury to optic nerve protective action and previous reported results consistent with (Fischer *et al*., 2001). Another injection of zymosan, results showed that yeast polysaccharide group RGCs survival number are higher than PEDF group and lens injury group (P<0.01). Then observed the intravitreal injection of PEDF combined with lens injury at the same time points on RGCs survival function. The results show the combination of the two can substantially increase the number of RGCs survival after optic nerve injury. And the separate use of intravitreal injection of PEDF or lens injuries compared, both can increase RGCs overall survival (1411±84 RGC/mm^2), and both have significant difference (P<0.01). These data suggest that PEDF and lens injury to protect RGCs survival synergistic effect (Figure 2, Figure3).

Fig. 2. PEDF combined with lens injury on the retinal preparation RGCs the influence of the number of a. LI b. PEDF c. group; group; group PEDF+LI; d. ZY group; e. PBS group. LI, lens injury; PEDF, pigment epithelium-derived factor; ZY, yeast polysaccharide; RGCs, Bar=100ì m retinal ganglion cells

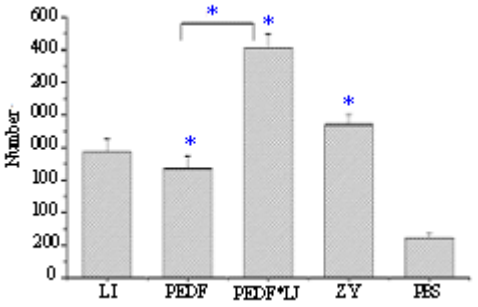

Fig. 3. Retinal mount count RGCs, *P<0.01, *P:vs LI, *P:PEDF vs PEDF+LI. Lens injury; LI, PEDF, pigment epithelium-derived factor; ZY, RGCs, yeast polysaccharide; retinal ganglion cells

Frozen section count RGCs

In order to from another side of observation PEDF alone and PEDF combined lens damage after optic nerve injury on the influence of the number of RGCs cell survival, the specimens were observed under fluorescence microscopy of frozen sections, retrograde labeling of the RGCs survival number. The results show the optic nerve injury after 2W, separate injection of PEDF group RGCs number (4.2±1.4) and separate lens injury group (4.1±1.7) RGCs number similarity. Injection

of zymosan group RGCs survived more than (6.1±1.9) PEDF (P<0.01). Observation of intravitreal injection of PEDF combined with lens injury at the same time points on RGCs survival function, the results show the combination of the two can substantially improve survival after optic nerve injury in RGCs number, were more than two separate intervention (P<0.01). These data also shows that PEDF and lens injury to protect RGCs survival of synergy, and retinal mount count RGCs similar results (Figure 4, Figure 5).

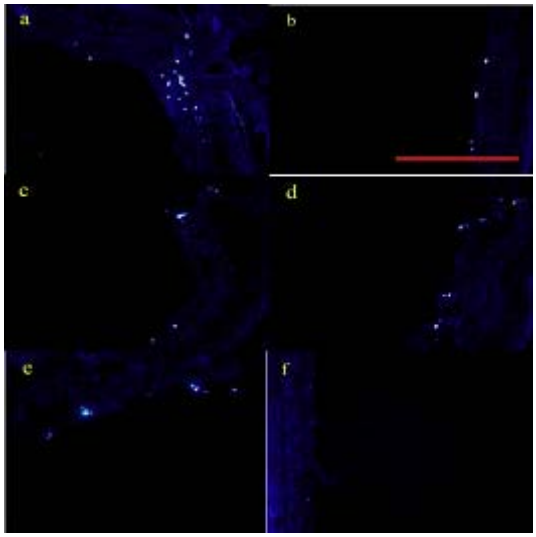

Fig. 4. PEDF combined with lens injury on the retinal slice RGCs number effect.A. optic nerve section; b.LI group; group c.PEDF; d.PEDF+LI; e.ZY group; group f.PBS. LI, lens injury; PEDF, pigment epithelium-derived factor; ZY, yeast polysaccharide; RGCs, Bar=100μ m retinal ganglion cells

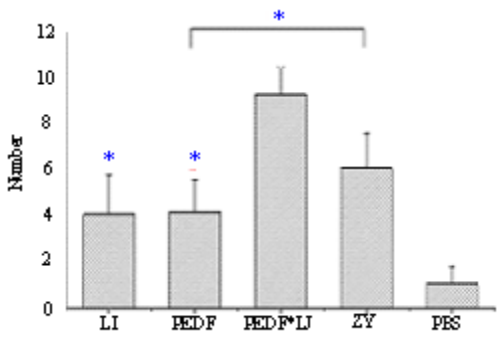

Fig. 5. Frozen sections of count of RGCs, *P<0.01, *P:vs PEDF+LI, *P:PEDF vs ZY

Measurement of axon number

In order to explore the survival neurons increases whether can lead to substantial axonal regeneration, this experiment adopts the model of optic nerve injury. In this model, the injury may be by local formation of the glial scar and clip marks from the judges, 250 μm part to calculate the numbers of regenerating axons. Previous experiments have shown, optic nerve injury can lead to RGCs with prolonging of time of death. The experimental data show that, in the optic nerve injury after 2W, a simple lens injury stimulates distal axonal regeneration in the RGCs, were consistent with previous research (Fischer *et al*., 2001). But the single intravitreal injection of PEDF at the same point in time but not stimulus distal axonal regeneration in the RGCs.

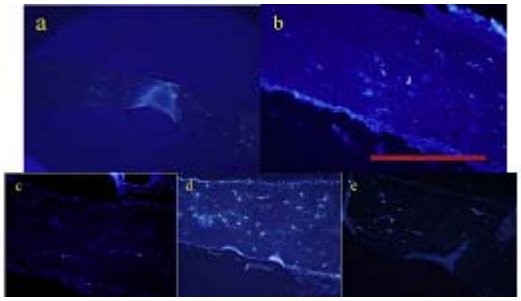

Fig. 6. PEDF combined with lens injury of optic nerve axons number A. optic nerve crush injury; b.LI; c.PEDF; d.PEDF+LI group; group e.ZY. LI, lens injury; PEDF, pigment epithelium-derived factor; ZY, Bar=50ì m of Yeast Polysaccharides

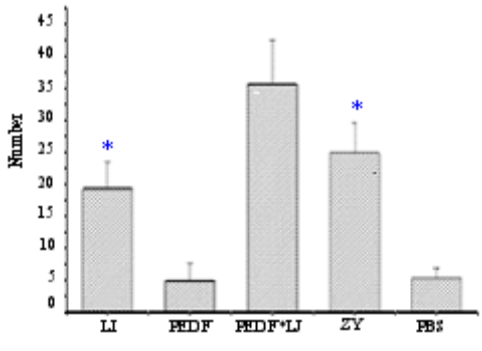

Fig. 7. The regeneration of optic nerve axons, *P<0.01, *P:vs PEDF+LI

The results indicate that axonal count of intravitreal injection of PEDF combined with lens injury to strong stimulation of the distal optic nerve regeneration, and the damage of lens group with significant difference (P<0.01). In order to control other intraocular invasion of macrophages into the simulation, experiments will also yeast polysaccharide vitreous injection, results showed that yeast polysaccharide can stimulate RGCs distal axonal regeneration, but the number of regenerated axons is less than PEDF combined with lens injury group (P<0.01). In conclusion, we think that PEDF alone to promote axon regeneration after optic nerve injury has no obvious effect,but combined with lens injury can markedly stimulate axonal regeneration (Figure 6, Figure 7).

GAP-43 mRNA detection

Expression of GAP-43 in normal optic nerve is very small in volume, after optic nerve injury, the expression of a large number of increase. Comparison of PEDF alone and PEDF combined lens damage after optic nerve injury on the expression of mRNA in GAP-43. Separate injection of PEDF or lens injuries can increase the expression of GAP-43 mRNA. The experimental data show the optic nerve injury after 2W, separate injection group PEDF GAP-43 expression level of mRNA (0.617±0.016) less than a separate lens injury group (0.903±0.028) GAP-43 expression level of mRNA (P<0.01). Another injection of zymosan, the results showed that the GAP-43 expression level of mRNA (0.942±0.021) than in group PEDF (P<0.01).

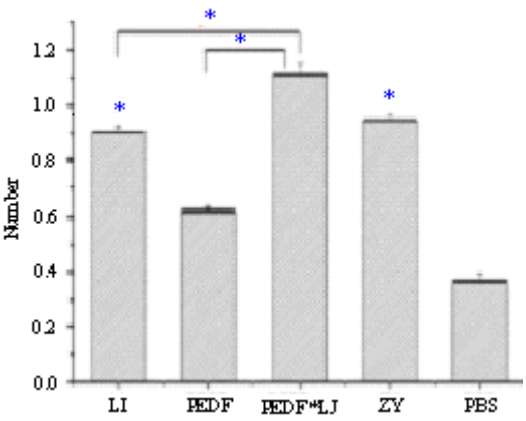

Fig. 8. Retinal tissue GAP-43 expression of mRNA, *P<0.01, *P:vs PEDF; *P:LI vs PEDF+LI; PEDF vs PEDF+LI

Subsequent observation of the intravitreal injection of PEDF combined with lens injury at the same time points on GAP-43 mRNA expression influence. The results show the combination of the two can be greatly improved after optic nerve injury on the expression of mRNA and GAP-43, two separate intervention had significant difference (P<0.01) (Figure 8).

DISCUSSION

In the mature mammalian, RGCs once damaged, soon apoptosis, their axons do not regenerate (Fischer *et al*., 2001). Many clinical diseases will damage the RGC cell, leading to its pathophysiology changes, such as glaucoma and optic nerve injury (Garcia *et al*., 1994). In recent years, studies have shown that following lens injury, the survival rate of RGCs increased, axons are able to regenerate into the distal end of the optic nerve.

In the experiment, the use of a more precise optic nerve crush model. This model can better regeneration of optic nerve axons, superior to the optic nerve transection model. Because the optic nerve was not cut off, the regeneration of axons can residues along the nerve axonal growth, also more close to the clinical situation. But due to the injury site for easy recognition, can accurately count the distal regeneration of axon. Optic nerve transection model with cavity formation and spinal cord contusion is very similar to (Quigley *et al*., 1995; Grafstein *et al*., 1982). But in the following optic nerve axotomy to study axonal regeneration, because nerve transected ends without for axonal regeneration in the matrix. So the optic nerve crush model is more suitable for axonal regeneration research.

Studies have suggested that the lens injury caused by activated macrophages for axon regeneration is achieved. Following lens injury resulting in a large number of macrophages to intraocular invasion, and the activation of macrophages, and inflammatory response does not affect lens promoting axonal regeneration in the RGCs into the optic nerve. Studies show that macrophages are capable of secreting a large number of factors, such as BDNF, GDNF, NGF, PDGF, IL-6, IL-18, TNF(Dougherty *et al*., 2000), can protect the optic nerve, promote axonal

regeneration. In the lens injury a few days later, activated macrophages can play a maximum biological activity, strongly promote axonal regeneration, the lens injury when the role is much better. Lens injury 3 days later, ED-1 positive macrophages has been across the entire retina. This change lasted for 2 weeks, followed by activated macrophages, began to decline, but the changes in RGC cell continues. Separate or combined with intraocular injection of optic nerve injury, not execute lens injury, caused only small amounts of macrophage infiltration, as well as a large number of RGC cell death. In peripheral nerve transplantation experiments, activated macrophages can promote the regeneration of axons rapidly into the distal RGCs. Yeast polysaccharide, is a species of yeast cell membrane suspension, can be activated mannose and CR3 b2- integrin receptor b- dextran lectin binding sites of (Beattie *et al*., 1997), resulting in a large number of macrophages intraocular invasion, site of nerve injury and RGC cells expression of a large number of GAP-43.

In order to further quantify axonal regeneration after optic nerve injury case, application of a reliable measurement of axon elongation, regeneration and plasticity of the markers, GAP-43. GAP-43 in neural development and regeneration of large expression (Xia *et al*., 1999). Moreover, in feeling, movement, CNS neuron nerve fiber damage, GAP-43 expression of (Gianotti *et al*., 1992). So GAP-43 is nerve regeneration or axonal sprouting of good marker. Previous studies showed that GAP-43 in adult rat autonomic ganglia basal expression than other nervous system neurons with high (Stewart *et al*., 1992). Autonomic ganglia tend to have strong plasticity. This may be because the majority of the target organ can be moved, and often subjected to mechanical stress stimulation, so synaptic continuous rearrangement. Mature neurons expressing GAP-43 can induce nerve spontaneous sprouting, upregulation of nerve regeneration, indicating that GAP-43 is axonal regeneration and regeneration of the nervous system plasticity in the very important intrinsic determinant (Benowitz *et al*., 1997). The cells in the experiment, separately joined PEDF cannot increase the RGC cell survival rate; but a separate PEDF injected into the vitreous body can increase the RGC cell survival rate, but can not promote axonal regeneration into the optic nerve in. Lens injury can increase the number of RGC cells and promotes axonal regeneration. In this experiment, observation and PEDF lens injury of optic nerve axons regeneration effect, the result shows that both the joint can be the greatest degree of protection of RGC cell survival, axonal regeneration, increases the expression of GAP-43 mRNA.

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