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Experimental Study on Pigment Epithelium Derived Factor Combined with Macrophage Conditioned Medium on Rat Retinal Ganglion Cells Axonal Regeneration Influence

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The study of pigment epithelium derived factor (PEDF) combined with macrophage conditioned medium (MCM) could synergetically protect retinal ganglion cells (RGCs) survival and promote axonal regeneration of RGCs purpose, by macrophages and yeast polysaccharide and preparation of culture cell slide and MCM cell of experimental method, draw a conclusion, separate application PEDF cannot protect cultured RGCs survival and axonal regeneration promotion, MCM can protect RGCs survival and promotes axonal regeneration, and PEDF can be synergistic protective RGCs survival and promotes axonal regeneration.

Keywords: Pigment epithelium derived factor, Macrophage conditioned medium, Rat, Retinal ganglion cells, Axons, Regeneration.

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 nervous, must meet two basic conditions: prevention or delay of damaged neuronal death; promote axonal regeneration to a proper destination (Bray *et al.,* 1991; Doster *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.

Retinal cortex system belongs to the central nervous category, retinal ganglion cell (RGCs) with CNS neuron characteristics, the cell bodies located in the retina, optic nerve axons along the extension to reach the brain specific location. This kind of cell polarity structure makes it easy to carry on the retrograde or antegrade marker, the distinction between RGCs cell body and axon (Sapieha *et al.,* 2003; Pernet *et al.,* 2005). And the eye is exposed to the outside, easy operation, protective factor or other substances through the vitreous injection can be easily dispersed into RGC cells.

Neurotrophic factor in impaired RGC cell 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, are thought to influence the early neuronal development in an alternate factor. Subsequent experiments found in the eye in different tissues and cells are expressed, including limbus and non pigmented ciliary epithelial cells (Ortego *et al.,* 1996; Karakousis *et al.,* 2001; Behling *et al.,* 2002; Ogata *et al.,* 2002). In

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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; Tombran *et al.,* 2005). PEDF can inhibit angiogenesis, have neurotrophic and neuroprotective role of (Tombran *et al.,* 2003; Barnstable *et al.,* 2004; Tombran *et al.,* 2005; 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; Houenou *et al.,* 1999; Bilak *et al.,* 1999). In the retina, 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).

PEDF is the first in the human fetal RPE cells conditioned medium was found in, are thought to influence the early neuronal development in an alternate factor. Macrophages can be swallowed by the inhibition of myelin and protection of nerves, promote axonal regeneration after activation, and can also secrete neuroprotective factor. With the CNS injury and disease of the inflammatory response is a double-edged sword. On one hand it can cause further damage, on the other hand, immune mediated inflammatory response can also protect the optic nerve, promote its repair. Research shows that PEDF has anti-inflammatory effect, can reduce inflammation factors on body damage, so this experiment by combined PEDF and macrophage conditioned medium (macrophages conditioned medium, MCM) together in the RGCs, on promoting axon regeneration in vivo.

METHOD

Cell slide preparation

Primary culture of RGCs such as (Ogata *et al.,* 2001; Takita *et al.,* 2003; Li *et al.,* 2010; Fischer *et al.,* 2000) said, the cell suspension inoculated in the preset coverslip culture plate with 24 holes, each hole about 1 ml RGCs suspension, placed 5% CO2 incubator (37! constant temperature and humidity) in culture.

Macrophage conditioned medium preparation

SD rat peritoneal macrophages in culture such as (Ogata *et al.,* 2001; Takita *et al.,* 2003; Li *et al.,* 2010; Fischer *et al.,* 2000) said, the macrophage to 106 /ml vaccination at 24 hole plate. In the 10%FCS and 2%B27 DMEM added in the medium of Yeast Polysaccharides (1.25mg/ml, final concentration), placed 5 %CO2 incubator (37° constant temperature and humidity) in 8 after h culture supernatants were collected,, in order to remove the yeast polysaccharide particles and cell debris, supernatant centrifugal (1500r/ min) 10 min, 8°C save, spare.

Preparation of silicone oil droplets

Silicone oil droplets (vesicle of silicone oil, VOS) using microporous membrane prepared by emulsifying. 5700mPa 0.05mL s silicone oil viscosity at 1 atm emulsion pressure through aperture of 0.45μ m cellulose microporous membrane, dispersed in 10mmol/L 0.95mL PBS solution. Full oscillation, the silicone oil droplets of uniform distribution.

Cells of fetal looking blue staining

Respectively to the preset cell slide 24 orifice plate in the presence of MCM, including PEDF (100 ng/mL) DMEM (2%B27 culture liquid containing), including PEDF (100 ng/mL) MCM and VOS (12.5mL/L, containing the final concentration) medium DMEM (with 2%B27), with 2%B27 DMEM culture liquid control group. Cells continue to culture 1, 3, 5, 7 d after adding fetal cells, for blue staining, calculate the number of live cells. Optical microscope (400), each slide 10 randomly selected fields, counting viable cells. The experiment is repeated at least 3 times.

GAP-43 immunohistochemical staining

In 1, 3, 5 D takes different experimental groups of cells crawling tablet, 0.01mol/L PBS rinse 3 times, fixed in 4% paraformaldehyde 30 min, PBS rinse 3 times, each time 5 min, 3% 5~10 min were incubated with hydrogen peroxide at room temperature, in order to eliminate the endogenous peroxidase activity, double distilled water rinse 3 times LPBS, 0.01mol/ for 5 min. Normal goat serum working fluid to a closed, room temperature were incubated for 10 min, tilt to serum. Join GAP-43 rabbit anti-rat antibody (1:500), 4!overnight. PBS instead of as a negative control. PBS fully rinse 3 times, each time 5min. Join the Sheep anti rabbit biotin labeled two resistance (1:500), room temperature incubation 30min. PBS rinse 3 times, each time 5min. Horseradish peroxidase-labeled

streptavidin avid working fluid room temperature incubation from 10 to 15min. PBS rinse 3 times, each time 5min. DAB light color 10min, double distilled water flushing. Optical microscope, each slide 5 randomly selected fields, each field of view at least count of 30 cells, using the image acquisition and analysis system (Image Pro Plus 6) calculation of the average length of axon. The experiment is repeated at least 3 times.

Statistical analysis

Using the data processing software of SPSS17.0, by t test, two sample, and LSD test, many group of sample, data representation for X±s. P<0.05 for a significant difference.

RESULTS

RGCs survival rate

Cultured RGCs cell fixed on the glass surface. RGCs cultured for 1 D, survive in good condition, the cells were round or oval (Fig. 1), with the extension of time, cells of the state of decline, reducing the number of. Because the cells were cultured for sixth D, cells appeared a lot of death, so in the present experiment, the long count the number of cells cultured for seventh D. Experimental discovery, all experimental groups in cultured 1D, cell number and the control group (group DMEM) had no significant difference. Separate accession to the PEDF group at all time points in RGCs number and the control group was not significant difference. In group MCM cells cultured for third D cell number was 31.4±3.7 (P<0.05), PEDF and MCM group was 34.7±4.2 (P<0.01), and the control group (27.6±2.2) had significant difference. MCM group and PEDF plus MCM group, in third, 5, 7 days, RGCs survival rate than the control group increased significantly (P<0.01). PEDF combined MCM cell number is greater than the MCM group, there was significant difference (P<0.05). Silicone oil droplets and incubated with RGCs, with the extension of time, a substantial reduction in cell number, smaller than that of the control group (P<0.01) (Fig. 2).

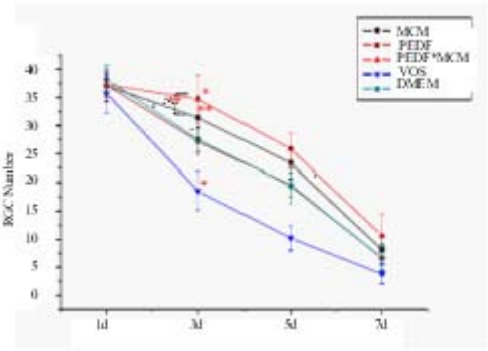


Fig. 2. RGCs cultured in 1, 3, 5, 7 d of each group of cell number, *P<0.01, *P:vs DMEM, **P<0.05, **P:MCM vs PEDF+MCM. MCM, macrophages cultured in liquid; PEDF, pigment epithelium-derived factor; VOS, RGCs, silicone oil droplets; retinal ganglion cells

RGCs axon regeneration

RGCs 1D culture, cells were round or ovoid, cells around out of many short axon (Fig. 1), the immunohistochemical staining of axonal brown yellow, clearly visible (Fig. 3), with the

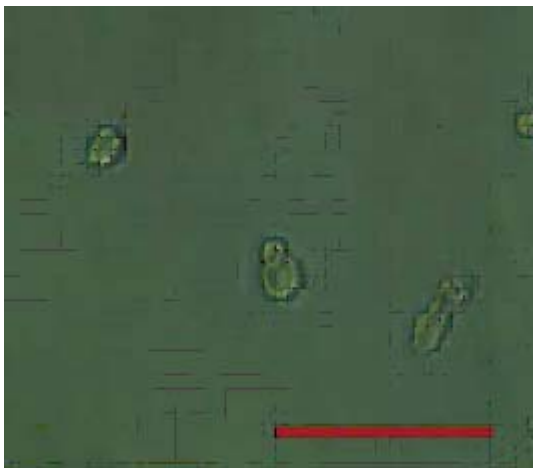


Fig. 1. RGCs after 24 h, visible minority cells extend several short protrusions, Bar=50 μM

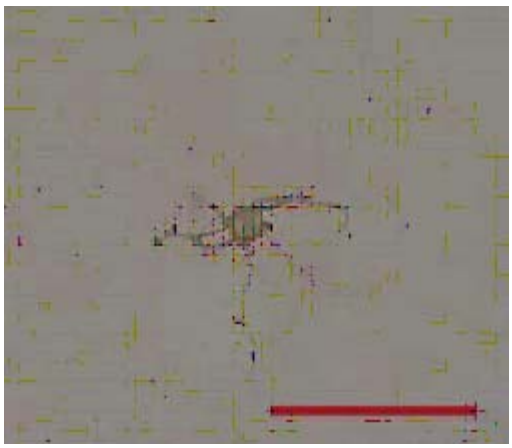


Fig. 3. the RGCs GAP-43 staining, RGCs visible cytoplasmic, membrane and axons can be colored, brown yellow, but the cytoplasm pale, axon clearly visible, Bar=50 μM

extension of time, cell axons gradually elongate, some cells between the axon are connected to each other, even forming a network. Experimental observation of a 1, 3, 5 d cell axonal growth condition is detected, the PEDF group at 1, 3, 5 d, RGCs axon length and the control group (group DMEM) had no significant difference. Cell culture

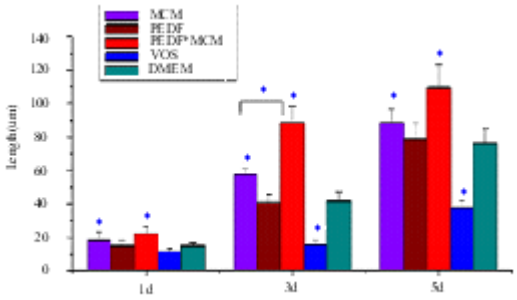
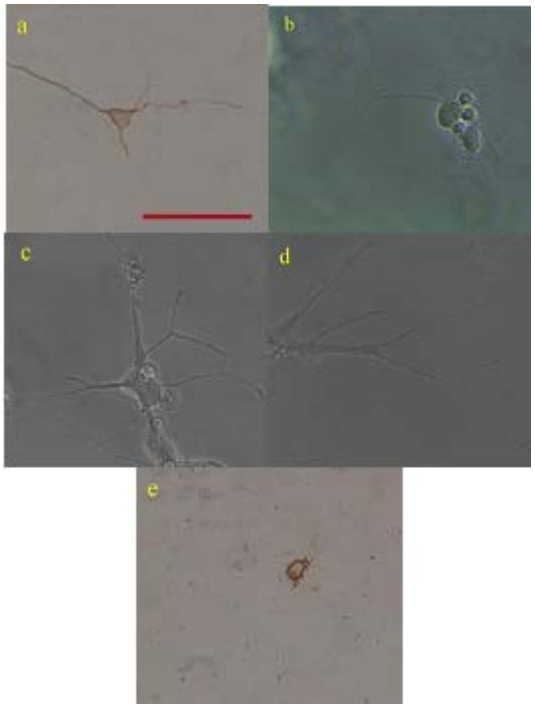


Fig. 4. RGCs cultured in 1, 3, 5 d each group cell axon length, *P<0.01, *P:vs DMEM, *P:MCM vs PEDF+MCM



A. MCM RGCs 3 D of culture; B. PEDF RGCs 3 D of culture; C. PEDF+MCM RGCs 3 D of culture; D. PEDF+MCM RGCs 5 d of culture; E. VOS RGCs D. MCM 3 group training, macrophages cultured in liquid; PEDF, pigment epithelium-derived factor; silicone oil droplets; VOS, RGCs, Bar=50ì m retinal ganglion cells.

Fig. 5. PEDF combined with macrophage conditioned medium on the survival rate of RGCs effect

of article 3D, group MCM cell axon length was (57.6±3.7)μm, PEDF combined MCM cell axon length was (88.6±9.5)μm, and the control group (41.8±5.4)μm were significant difference (P<0.01). PEDF combined MCM axons longer than that in group MCM (P<0.01). Accession to the MCM cells in the experimental group of axons in the 1, 3, 5 d were longer than those in the control group (P<0.01). Cell culture medium for MCM and PEDF experimental group all time points in the cell axon length greater than that of the control group (P<0.01). Cell culture fluid after joining VOS, cell axon length is significantly smaller than that of the control group (P<0.01) (Fig. 4 & 5).

DISCUSSION

As part of the CNS, has long argued that the optic nerve regeneration cannot be (Leon *et al.,* 2000). After optic nerve injury, RGC cells were not completely end bud growth, but not long-distance regeneration. Moreover, if the nerve near the eye, RGC cells soon began to apoptosis. However, by changing the RGCs intrinsic growth state and the offset of the extracellular environment of the inhibitory signals, can be partially reversed the state cannot regenerate.

Recent studies have shown that activation of macrophages, the eye, can promote the regeneration of optic nerve axons. But with the CNS injury and disease of the inflammatory response is a double-edged sword (Leon *et al.,* 2000). On one hand it can cause further damage, on the other hand, immune mediated inflammatory response can also protect the optic nerve, promote its repair. For example, macrophages can secrete one or more unknown factor, can promote the optic nerve and spinal cord sensory axons regeneration (Yin *et al.,* 2003). These factors can not only make us better understand the promotion of axonal regeneration in molecular level mechanism, also for the treatment of optic nerve or spinal cord injury and provide a theoretical basis. Among them, Yin (Yin *et al.,* 2003; Yin *et al.,* 2006; Ramony *et al.,* 1991) first mass analyzer by MCM identified in a macrophage-derived can promote axonal regeneration factor, the factor is a calcium binding protein, carcinoma of calmodulin (calmodulin, OM), the protein with a molecular weight of 10 - 15 kDa.

OM secreted by macrophages, and cyclic adenosine monophosphate (cyclic adenosine 5'-monophosphate, CAMP) combined, can effectively stimulate the regeneration of optic nerve axons. Notable is, join OM can effectively promote the individual cultured RGCs axonal regeneration, culture medium combined with accession to the forskolin (can raise CAMP levels), which promotes axonal regeneration ability raises two times (Ramony *et al.,* 1991). The high intracellular levels of CAMP can make the OM effectively combined in RGC cells. When MCM was removed after OM, even if the intracellular CAMP levels increase, also can promote axonal regeneration, and MCM alone can effectively promote axonal regeneration. So OM MCM and not only can promote axonal regeneration factor.

Study on activation of macrophages to the expression of BDNF and GDNF. GDNF is a glycoprotein, 20% amino acid sequence and neurotrophic factor (NTF) consistent, can contribute to neuronal differentiation, survival, but also can inhibit the ciliary ganglion neurons apoptosis. Cui (Cui *et al.,* 1995) research shows that, NGF can stop after optic nerve axonal degeneration, improve the survive rate of RGCs. PDGF is a mitogenic agent and a chemotactic agent, can promote various types of cell division and proliferation. Oya (Cui *et al.,* 2002) study found that, in the rat optic nerve transection local macrophages and glial cells are observed in PDGF - B expression. In the present experiment, the use of zymosan activation of cultured macrophages, collected supernatant. May stimulate macrophages to secrete more active cytokines, and the original factor synergy, and jointly promote axon regeneration. But what is what factor synergistically this role, the need for further research.

The molecular weight of PEDF was 50 kDa, Serpin gene family members. PEDF is thought to have neurotrophic and anti-angiogenic dual role. It has been a large number of in vivo and in vitro studies confirmed. PEDF can protect cerebellar granule cells, cells in the hippocampus, spinal motor neurons from glutamate mediated apoptosis impairment. PEDF can also prevent glutamate and neurotrophic factor resulted in a reduction in the cytotoxic effect on adult rat RGC cell damage (Pang *et al.,* 2007), reduce local ischemia induced RGC cell loss in (Pang *et al.,* 2007). Like other neurotrophic factor, PEDF should pass and one or more receptor binding specificity or activated to exert their biological effects. However, so far found no specific PEDF receptor. Human retinoblastoma cell line Y-79, rat cerebellar granule neurons, the ganglion cells and bovine photoreceptor cone section has PEDF high affinity binding point (Ogata *et al.,* 2004). Recently, from human retinal epithelial cell isolation and identification of a PEDF binding protein, called PEDF-R. PEDF on this protein has specificity and high affinity. PEDF can bind to and activate the protein, activate the activity of phospholipase A2 (Alberdi *et al.,* 1999). It is unclear whether PEDF-R is the retina PEDF receptor is a subtype of. Although the PEDF receptor not fully understand, but can be identified by NFB and ERK1/2 pathways involved in PEDF process (Tombran *et al.,* 2003).

The present experiment separately joined the PEDF, MCM and combined with PEDF and MCM, observe the effect on RGC cells, the results show that MCM can obviously promote RGC cell survival and axon regeneration, and the control group with significant difference. While individually joins PEDF on cell survival and axonal regeneration in a control group with no significant difference. Combined PEDF and MCM can greatly enhance the effect on RGC cells, and MCM group there was a statistically significant difference between. This may be because macrophages to secrete neurotrophic factors at the same time, secretes some inflammatory factors, and these factors on RGC cell will produce toxic effects of (Leon *et al.,* 2000). Studies have shown, the PEDF is an important endogenous anti-inflammatory factor (Notari *et al.,* 2006). Diabetic proliferative retinopathy patient intravitreal PEDF levels decline, description of the retinal PEDF reduction may promote the development of diabetic retinopathy, may be enhanced through retinal inflammatory reaction caused by (Zhang *et al.,* 2006). Studies have shown that, in DBS/2J mice intravitreal injection of PEDF can reduce RGCs and nerve fiber layer of retina and optic nerve loss, decreased TNF, IL-18 and glial fibrillary acidic protein (glial fibrillary acidic protein, GFAP) expression of (Xiao *et al.,* 2009). So combined with PEDF and MCM, one can play MCM and PEDF in neurotrophic effect, on the other hand can inhibit MCM negative effect,

enhancing effect on RGC cells.

Clinical use of silicone oil tamponade in the treatment of retinal detachment, but silicone oil emulsification effect in long-term application of intraocular silicone oil. Emulsified silicone oil to nearby or distant tissue proliferation, arrive at RGCs and RPE layer. Budde (Budde *et al.,* 2001) on removal of silicone oil eye were observed, the ball can be observed at 9 mm after silicone oil particle. RPE cells are able to secrete promoting axonal regeneration in the RGCs PEDF, in intraocular three may influence each other. To explore this question, the first observation of emulsified silicone oil droplets on cultured RGCs effects, for animal experiments provide cytological basis. The emulsified oil droplet and RGC cells were incubated for 1, 3, 5, 7 d, the results showed that silicone emulsion group cell number and axonal length less than the control group, probably due to the vesicles of silicone oil emulsion prevents RGC cell metabolism. Experiments have shown that emulsified oil droplet can stimulate RPE cell phagocytic activity of (Budde *et al.,* 2001), stimulation of human RPE cell proliferation and (Li *et al.,* 2010), so in the eye may stimulate RPE cells to secrete PEDF. In future studies will be necessary to emulsified silicone oil injection into the vitreous, observation of emulsified silicone oil intraocular can stimulate RPE cell secretion of endogenous PEDF, and promote RGC cell survival and axon regeneration.

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