·Basic Research·

# Effect of Y-27632 on the cultured retinal neurocytes of rats

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## Abstract

• **AIM: To investigate the effect of Y-27632 on the survival and neurite outgrowth of the cultured retinal neurocytes.**

• **METHODS: After the postnatal day 2-3, Sprague-Dawley retinal neurocytes were cultured for 48 hours, the culture media was replaced with serum-free media (control group) and serum-free media contained 30μmol/L Y-27632 (Y-27632 group), and the cells were continually cultured another 48 hours. The cultured retinal neurocytes were identified with anti-neuron specific enolase (NSE) immunocytochemistry. The survival state of those cells was estimated by MTT assay, and the neurite outgrowth of those cells was evaluated by the computerized image-analysis system.**

• **RESULTS: Compared with the control group, the absorbance values of cells survival in Y-27632 group increased 12.90% and 33.33% respectively after 72 and 96 hours culture. Y-27632 had no significant effect on the diameter of cultured retinal neurocytes. Compared with the control group, Y-27632 induced a stable improvement of neurite outgrowth of retinal neurocytes after 72 and 96 hours culture ($P$=0.001).**

• **CONCLUSION: Y-27632 could promote the survival and neurite outgrowth of the early postnatal cultured retinal neurocytes.**

• **KEYWORDS:** Y-27632; retinal neurocytes; cell culture; neurites

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## INTRODUCTION

Many kinds of eye diseases, such as glaucoma, ischemic optic neuropathy, retinal degeneration, trauma and so on, can cause the injury or death of the retinal neurocytes and subsequently perpetual visual dysfunction. As the mature retinal neurocytes are well differentiated, and the dead neurocytes can't regenerate in normal condition, therefore, it has always been a hot and difficult point in ophthalmic research about how to promote the survival rate of dying retinal neurocyte and its axon outgrowth.

Recent studies have shown that Rho/Rho-associated kinase (ROCK) pathway is an important signal pathway for regulating the survival and axon regeneration of neurons in the central nerve system (CNS), and application of RhoA or ROCK inhibitor can promote the axon regeneration of injured neurocytes in CNS [1-3]. Y-27632 is an inhibitor of ROCK, and several studies have revealed that Y-23762 could promote the axon growth of hippocampal neurons suffered from injury [4,5]. Currently, little is known about the effect of Y-23762 on retinal neurocytes. The aim of this study is to observe the effect of Y-27632 on the survival and axonal growth of cultured retinal neurocytes, and to provide the foundation for subsequently further experiments *in vivo*.

## MATERIALS AND METHODS

**Materials** Animals used in this study were treated in accordance with the Association for Research in Vision and Ophthalmology (ARVO) Statement for the use of animals in ophthalmic and vision research. Pups of Sprague-Dawley rats were used in all experiments, and were kept under conditions of constant temperature and humidity, and fed by their mothers. The day of birth was counted as postnatal day (P) 0, and P2-3 rats were used in our experiment. The rats were killed by cervical dislocation. A total of 48 P2-3 rats were used for this study.

**Experimental reagents** Y-27632 (ROCK inhibitor, Alexis Biochemicals, Switzerland); DMEM (Gibco, New York, USA); fetal bovine serum (Sijiqing biological engineering materials Co. Ltd, Hangzhou, China); 5-bromine-2-deoxidizing urine (Sigma-Aldrich, St. Louis, MO, USA); mouse-anti-rat neuron specific enolase (NSE) monoclonal antibody (Serotec, Oxford, UK); UltraSensitive™ SP Secondary antibody

(Maxin Company, Fuzhou, China); DAB chromogenic agent (Boster Company, Wuhan, China).

**Methods**

**Preparation for rat tail tendon collagen** The rat tail tendon collagen was prepared according to the method previously described [6]. In brief, the tendon of an adult Sprague-Dawley rat was extracted and cut into fragments, the fragments were placed into 150mL dilute acetic acid solution (1:1 000) until they dissolved (reserved in 4℃ for 48 hours, frequently shook). Then the solution was centrifuged at the speed of 4 000r/min, and the supernatant were collected and reserved in -20℃.

Precoating the tissue culture plates with rat tail tendon collagen The method for precoating the tissue culture plates with rat tail tendon collagen was previously described [6]. The collagen supernatant was added into the holes of the tissue culture plates under sterile condition (35μL/hole for 96-well plate, 250μL/hole for 24-well plate), then free ammonium was filled into each hole for 30 minutes. After this, the collagen was coagulated, and the hole was washed 3 times with sterile D-Hanks solution, and dried by airing. After ultraviolet irradiation, the plate can be used immediately.

**Dissociated cell cultures** The suspension of retinal single cells was prepared according to the method previously described [6]. The cell density in the suspension was approximately 1-1.2×$10^6$/mL. Then the cell suspension was added into plate (1mL/hole for 24-well, 200μL/hole for 96-well), and cultured in an incubator at 37℃ with an atmosphere containing 5% $CO_2$. When the cells were cultured for 16 hours, 5-bromo-2'-deoxyuridine (20μg/mL) was added to the culture media to inhibit the nonneurocytes growth. Forty-eight hours later, the culture media was replaced with serum-free DMEM for further culture.

Drug treatment According to the preliminary tests and references, the final concentration of Y-27632 used in this experiment was 30μmol/L [2]. After 48 hours culture, cells were randomly transferred into serum-free DMEM (control group) or serum-free DMEM containing 30μM Y-27632 (Y-27632 culture group) for further culture. The culture was ended after altogether 96 hours culture.

**Immunocytochemistry** Anti-NSE immunocytochemistry was used to identify the purity of mixed retinal neurons in culture. Six samples (including 4 samples from Y-27632 group and 2 samples from control group) were used for identifing each experiment. After 96 hours culture, the cells were fixed in 4% paraformaldehyde for 30 minutes, then washed 3 times with phosphate buffered solution (PBS), then incubated in hydrogen peroxide for 10 minutes to block endogenous peroxidase activity. The cells were washed 3 times with PBS, then incubated with 1% bovine serum albumin (BSA) for 10 minutes. After BSA being threw away, the cells were incubated with mouse-anti-rat NSE monoclonal antibody (1:50) at 4℃ overnight. The cells were washed 3 times with PBS, and incubated with the biotin-labeled secondary antibodies for 30 minutes at room temperature, then washed 3 times with PBS, and then incubated with streptavidin- perosidase (SP) for 20 minutes. After several washes,3,3'-diaminobenzidine tetrahydrochloride (DBA) was used as a co-substrate for 5 minutes, then washed with PBS to stop the immunocytochemistrical process. The primary antibody was replaced by non-immune serum for negative controls. The coverslips were counterstained with hematoxylin and mounted. The cells were examined under microscope. Each experiment was repeated 3 times.

**MTT assay** Briefly, the cells were cultured in 96-well plates, and 20μL MTT labeling solution was added to each well for another 4 hours culture. The supernatant was drained, and 150μL DMSO was added with 10 minutes shaking to dissolve the crystals completely. The absorbance of each sample was measured at a wavelength of 490nm.

**Morphology examination** The morphology of surviving cells were observed under Olympus inverted microscope and recorded by photography.

**Image analysis** The immunocytochemical staining was observed under light microscope. NSE positive cells' body presented large uniform brown granules in different degree, while negative cells' didn't. NSE positive cells with complete membrane were counted in randomly selected microscopic field (magnification ×200). Eight different fields were counted at random in each sample. Then the percentage of positive cells was calculated. The clusters of cells were excluded from the measurement of cell body diameter and neurite length. The cell body diameter and neurite length were assessed using a computerized image-analysis system (Axioskop 2 plus, Carl Zeiss, Oberkochen, Germany). The longest diameter of each cell was selected for measurement, and served as the body diameter of the cell. The longest neurite from each cell was selected for measurement, and the neurite length was defined as the distance between the cell body and the farthest tip of the neurite. Each experiment was repeated in triplicate for statistical analysis.

**Statistical Analysis** Data are expressed as mean ±SD, unless otherwise stated. Statistical analysis were performed using the SPSS11.0 Statistical software package. The normal distribution of the data was proved by the "explore" program. Then, the independent Student's *t*-test was used for comparing data between the 2 groups. $P<0.05$ was considered statistically significant.

**RESULTS**

**Morphology of cultured retinal neurocytes** The cells began to adherent after seeding 4 hours, and almost all cells basically completely adherent after culturing for 24 hours. The cells shaped round or ellipse with round and relatively transparent nucleus, and small neurite outgrowth was

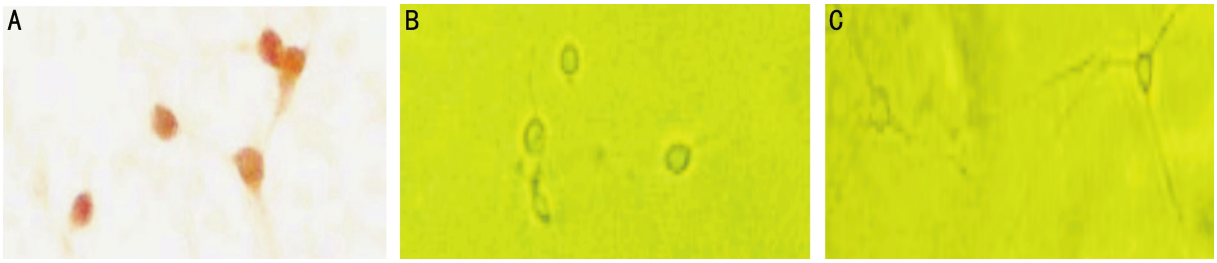


**Figure 1 Neurite outgrowth of retinal cells cultured for 96 hours** A: Anti-NSE immuncytochemistrical staining specifically labeled the neurons; B: retinal neurocytes cultured for 96h under normal culture condition; C: retinal neurocytes cultured for 96 hours with 30μmol/L Y-27632 (magnification ×400).

observed. After culturing for 48 hours, the cells shaped a slightly bigger round or ellipse than before, and the number of neurite outgrowth increased and the neurite extended. After culturing for 96 hours, the number of neurite outgrowth further increased, and some cells extended multiple neurites. There was no significant difference in cell morphology between the Y-27632 group and the control group.

**Cell purity** Immunocytochemical staining showed that NSE positive cells were brown in both body and neurite, and the body shaped round or ellipse. The cells in the negative control group had no brown stain. The percentage of positive cells was (93.02±2.65)%, which meant most cells in this mixed culture system were neurocytes(Figure 1).

**Effect of Y-27632 on the survival of cultured retinal neurocytes** Under the normal culture condition, the number of surviving cells decreased gradually with prolonged culture time. Y-27632 could observably promote the survival rate of cultured retinal neurocytes (Figure 2). The optical density (OD value) of Y-27632 group increased 12.9% and 33.33% respectively at the 72 hours and 96 hours culture compared with the control group ($P$<0.05).

**Effect of Y-27632 on cell body diameter and neutrite length of retinal neurocytes in culture** Within 96 hours culture period, the cell body diameter and the neutrite length of cultured retinal neurocytes increased with prolonged culture time (Table 1). The cell body diameter of retinal neurocytes in Y-27632 group was not significantly different from that of the control group at the culture time of 72 and 96 hours respectively ($P$>0.05, Table 1), which meant that Y-27632 had no significant effect on the cell body diameter. The neutrite of retinal neurocytes in Y-27632 group was significantly longer than that of the control group ($P$=0.001) at the culture time of 72 and 96 hours respectively (Table 1), which meant that Y-27632 could promote the neutrite outgrowth of retinal neurocytes *in vitro*.

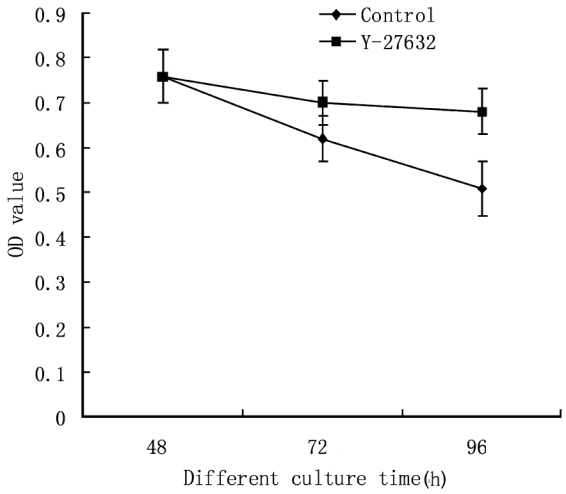


**Figure 2 Survival of retinal neurocytes at different culture time.**

**Table 1 Cell body diameter and neutrite length of retinal neurocytes at the different culture time** ($n$=6, $\bar{x} \pm s$, μm)

| Culture time | Control group | Y-27632 group | $t$ | $P$ |
|---|---|---|---|---|
| Cell body diameter | | | | |
| 48 hours | 9.86±1.37 | 9.72±3.15 | / | / |
| 72 hours | 10.25±1.33 | 10.17±1.16 | 0.115 | 0.911 |
| 96 hours | 10.50±1.38 | 10.67±1.21 | 0.222 | 0.828 |
| Neutrite length | | | | |
| 48 hours | 24.01±10.15 | 24.01±10.15 | / | / |
| 72 hours | 32.50±16.86 | 44.83±20.77[b] | 5.438 | 0.001 |
| 96 hours | 45.33±14.46 | 61.67±26.32[b] | 6.379 | 0.001 |

[b]$P$<0.01 *vs* control group.

## DISCUSSION

RhoA is a member of Rho subfamily of small molecular GTPases superfamily related to Ras. RhoA is considered as a molecular switch on the signal pathway of neuronal cell membrane surface receptor to actin skeleton, which has played a vital role in adjusting neuron development and axon construction. RhoA normally exists in two forms: one is the non-activated form combined with GDP (Rho-GDP), and the other is the activated form combined with GTP (Rho-GTP). RhoA realizes its molecular switch function by transformation between two forms. Several studies showed that the activation of RhoA could result in the growth cone collapse and the neurite outgrowth inhibition, and the inactivation of RhoA could promote axonal regeneration and functional recovery in the injured CNS. Therefore, the Rho/ROCK pathway is closely related to the axon growth after CNS injury[7].

In the adult CNS, due to the presence of inhibitory substances, such as myelin associated glycoprotein (MAG),

Nogo and oligodendrocyte-myelin glycoprotein (OMgp), *etc*, the axon regeneration of damaged neuron is difficult [8,9]. Some studies found that the mechanism of nerve regeneration inhibitory substances for impeding axonal regeneration of the CNS may be through activation of RhoA/ROCK pathway, then lead to the growth cone collapse after the signal transduction [10,11]. Previous studies also showed that the application of RhoA or ROCK inhibitor can promote injury axonal regeneration of the CNS [1-3]. ROCK has two kinds of isomeric forms, namely ROCK1 and ROCK2. ROCK inhibitor Y-27632 can simultaneous inhibit the activity of ROCK1 and ROCK2. Some studies found that Y-27632 could promote the axon growth of PC12 cells (a cell widely used to study axonal regeneration) and hippocampal neuron [4,5,12-14]. Our study also found that Y-27632 could significantly promote the survival and axon growth of cultured rat retinal neurocytes. Ahmed *et al*[15] reported Y27632-induced ROCK inhibition could promote robust disinhibited axon regeneration of adult neurons only when growth promoting factors were added and/or cAMP levels were raised. The different conclusions between Ahmed *et al's* study [15] and the present study may be attributed to the use of different stages of neurons (adult neurons for Ahmed *et al's* study, early postnatal neurons for present study).

In short, the present study found that ROCK inhibitor Y-27632 could significantly promote the survival rate and axon growth of cultured early postnatal rat retinal neurons, which provided a basis for further experiments *in vivo*, and verified the importance of RhoA/ROCK pathway in the axonal regeneration of retinal neurocytes.

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