· Original article ·

Primary cultivation of retinal neurons of neonatal rat

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Abstract

• AIM: To explore an experimental method for primary culture of retinal neurons of neonatal rat.

• METHODS: Retina of postnatal 1-3 days SD rats was dissected into cell suspension by using trypsin digestion and cultured *in vitro* with DMEM/F12. Immunocytochemical methods were used to identify the cultured neurons.

• RESULTS: All cultured cells underwent adherence and some possessed axons, of which some were connected with each other. Most cells were neuron specific enolase (NSE)-positive detected by immunohistochemistry.

• CONCLUSION: Successful culture of retinal neuron cells *in vitro* is helpful in the research of retinal diseases.

• KEYWORDS: cell culture; retinal neuron; trypsin digestion; neuron specific enolase

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INTRODUCTION

In vitro techniques have been widely used in biological research since the advent of the technique almost one century ago, when fragments of neural tube removed from frog, embryo were maintained alive in physiological fluid for several days, allowing real time observation of growing nerve fibres[1]. Without the complexity of the intact animal, *in vitro* approaches could greatly advance the understanding of retina basic properties and environmental adaptive responses. In this *in vitro* system, defined experimental conditions can be readily set and maintained. They offer an excellent balance between accessibility to pharmacologic and molecular manipulation while maintaining complex phenotypic characteristics of intact tissues. Here, we would explore an experimental method for primary culture of neonatal rat retinal neurons, which was based on some successful mothods reported[2-3].

MATERIALS AND METHODS

Animals　Pups of Sprague-Dawley rats were used in all experiments according to the ARVO statement for the Use of Animals in Ophthalmic and Vision Research and were kept under conditions of constant temperature and humidity. Postnatal 1-3SD rats were obtained from the animal center of Qingdao University.

Materials　DMEM/F12 and fetal bovine serum were purchased from Gibco (USA). Rabbit-anti-rat neuron specific enolase (NSE) monoclonal antibody, rabbit-anti-rat glial fibrillary acidic protein (GFAP) polyclonal antibody, goat anti-rabbit IgG and HRP-Streptavidin were purchased from Zhongshan Goldbridge Biotechnology Co., Ltd (Beijing, China). All other reagents were from Sigma Chemical Co. (St. Louis, MO. USA).

Methods

Pre-coating of the tissue culture plates with poly-D-lysine　In order to make the retinal neurons well adhere to the tissue culture plates, the poly-D-lysine was added to the holes of the 24-well plates, where cover glasses was placed in advance. After 10 minutes, the poly-D-lysine was removed, and the hole with coagulated poly-D-lysine was washed three times with sterile distilled water and dried in incubator overnight at 37℃. Then the plate was used immediately.

Retinal dissection　Sprague-Dawley rats of 1-3 days old were anesthetized before decapitation and the eyes were moved into Hank's balanced salt solution. Under light microscopy in aseptic conditions, each eye was opened by puncturing a hole with a hypodermic needle at the limbus margin. Fine scissors and forceps could then be used to extend this opening to remove the cornea from the front of the eye. After lifting out the lens and the bulk of the vitreous humour, the two remaining loosely associated layers of the retina and the pigmental epithelium were gently teased apart, using forceps to pinch the optic nerve thus freeing the retina completely.

Retinal dissociation and culture　The retinas were placed into a solution containing 0. 125% trypsin and incubated for 15 minutes at 37℃. At the end of the designated time, the digestion solution was removed and the tissue was then placed into a solution of Dulbecco's modified Eagle Medium: Nutrient Mixture F-12 (DMEM/F12) containing 10% fetal bovine serum, and filtered with 400 mesh cell sieves. After centrifugation at 1 000rpm for 5 minutes, the cells were reserved and resuspended in DMEM/F12 containing 10% fetal bovine serum by trituration. The cell density could then be examined using a haemocytometer, and plated at a density of 1.0×10^6 cells per cm^2 on 24-well plates, coated with poly-D-lysine (0. 1mg/mL), and cultured in a humidified incubator at 37℃ with an atmosphere of 5% CO_2. The retinal cultures might also be grown on glass cover slips placed within these

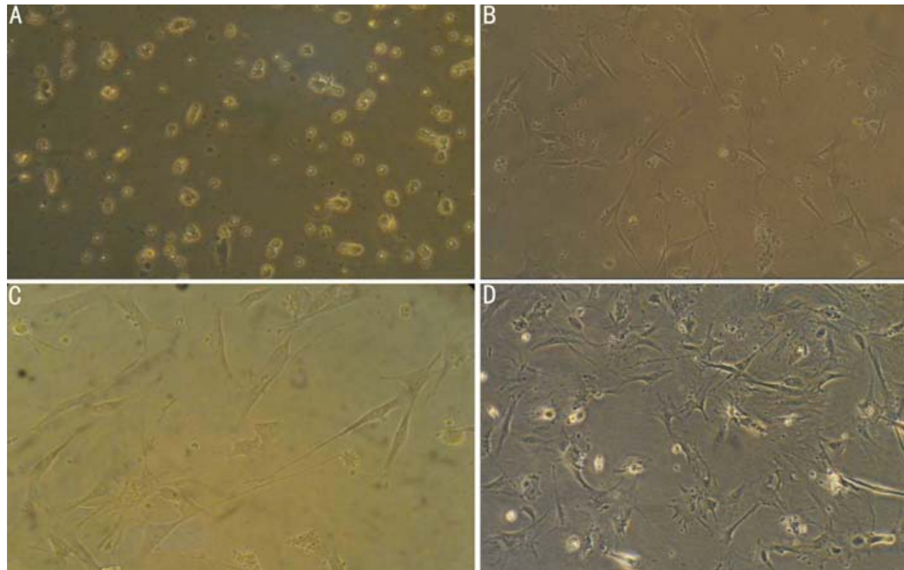

Figure 1 Morphological changes of cultured cells A:24 hours;B:72 hours;C:5 days;D:7 days.

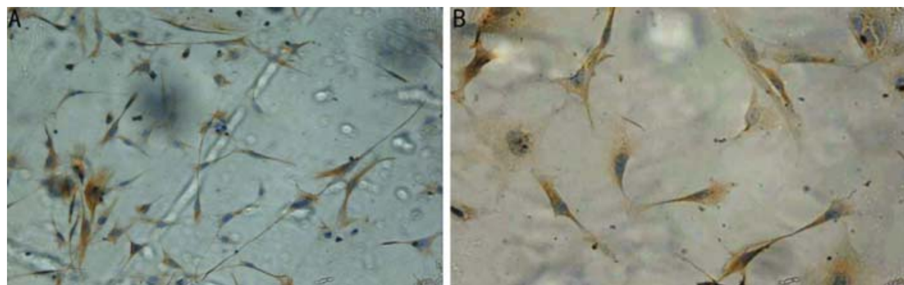

Figure 2 Immunocytochemical staining A: NSE-positive cells; B: GFAP-positive cells.

dishes. After cells were seeded for 24 hours, 5-bromo-2'-deoxyuridine (20μg/mL) was added into the culture media to restrain the non-neurocytes. The medium was replaced after 48 hours. Since then, the medium changed every two days. Cultured cells were observed daily under a phase contrast microscope, and the morphological changes over time were compared.

Identification of the cultured retinal neurons After 3 days *in vitro*, once the cells adhered to the cover slips, the culture medium was discarded and each cover slip washed three times with phosphate buffered saline(PBS). The cultured cells were fixed in 4% paraformaldehyde for 20 minutes, then incubated in 3% hydrogen peroxide for 10 minutes to block endogenous peroxidase activity, washed three times in PBS, incubated with inactivated normal 5% Goat Serum Albumin for 10 minutes at room temperature to block nonspecific binding, and incubated with rabbit-anti-rat NSE monoclonal antibody(1∶50) and rabbit-anti-rat GFAP polyclonal antibody (1∶50) as the primary antibody at 4℃ overnight. After cover slips were washed, they were incubated with biotinylated secondary antibodies(Goat Anti-Rabbit IgG, 1∶100) for 30 minutes at room temperature and then with HRP-Streptavidin for 30 minutes at room temperature. After several washes, color was developed by incubation with 3, 3-diaminobenzidine tetrahydrochloride (DAB) used as a cosubstrate for 5 minutes. For negative controls, the primary antibody was replaced by nonimmune serum. The cover slips were examined with a microscope, and images were recorded by photography.

RESULTS

Morphology At the beginning , the cells were spherical shape, and evenly distributed in the cultured plates. After 4 hours, cultured cells began to adhere. A majority of the cells had adhered after 24 hours, changing from a spherical shape into triangles or polygons, while cells that failed to adhere were removed during media exchange (Figure 1A). Forty-eight hours later, the cells had adhered solidly to the well plate and tended to grow in colonies, showing an evident increase in volume and cytoplasmic projections. Seventy-two hours later, small neurite outgrowth was observed, of which some were connected with each other (Figure 1B). The neurite outgrowth extended and the number of survival cells decreased gradually within the 5-day culture (Figure 1C). After 7 days, the rates of neuronal death began to increase, only a few cells survived (Figure 1D).

Immunocytochemical staining When the cells were cultured for 3 days, most of the survival cells were NSE-positive cells and accounted for 91. 5% , while the GFAP-positive cells only accounted for 2. 4%. NSE-positive cells had a round or elliptical shape and some neurite could be seen, and GFAP-positive cells were flat or fusiform(Figure 2). The negative control showed no positive staining.

DISCUSSION

In vitro, retinal tissues could be cultured either as monolayers, dissociated cells, slices, reaggregates or

explants. Monolayer retinal cultures are quite useful in studying the expression of proteins by individual cells which lack interactions with other cell types. Also, explants could preserve the retinal cytoarchitecture *in vitro*. Reaggregation cultures of retina are of great importance in 3D tissue reconstruction and tissue engineering[4,5]. Though the above-mentioned culture systems have their own significance and advantages, dissociated cell cultures are useful in conducting cell proliferation or apoptotic assays because of their accessibility to abundant isolated cells. The cell culture system of retinal neurons introduced in this paper was established to serve as a model system for toxicological studies representing the retina.

The critical outcome for primary cultures is that the cells must survive and be functional long enough for experiments to be performed. In the process of cultivation, there are some important factors that can influence the results, of which retinal cells separation is the most important one. Enzymatic digestion were commonly used methods to separate retinal neurons. And the most commonly used enzymes were trypsin, but the concentration of trypsin and the time of digestion, which were the key factors impacted on the cells separation, have different reports in several laboratories[6-8]. A suitable trypsin concentration and the time of digestion can produce a relatively higher number of neuronal cells and maintain vitality and function for a prolonged period, whereas it would result in low viability, membrane damage and reduced biosynthetic activity. In the experiment, we used 0.125% trypsin and 0.02% EDTA for 15 minutes at 37℃. And the results show that the effect of the method is satisfactory.

Culture conditions and medium are very important for retinal neuron cells culture, with DMEM/F-12 the most commonly used culture media for neuron cells. DMEM/F-12 contains no proteins, lipids, or growth factors. Therefore, DMEM/F-12 may require supplementation, commonly with 10% Fetal Bovine Serum (FBS), which may be added to the media to improve growth and viability. But there are many uncertain factors in FBS, such as exogenous growth factors and hormones, which resulted in the tremendous uncertainty in the experimental result. So serum-free media for culture of cells should be more scientific and rigorous. In addition, *in vitro* cell culture, the glial cells can provide the nerve cells neurotrophic factors, which maintain the nerve cells survival and promote the neurite outgrowth. But overgrowth of glial cells can inhibit the growth and differentiation of neurons. After cells were seeded for 24 hours, 5-bromo-2'-deoxyuridine (20μg/mL) was added into the culture media to restrain the non-neurocytes and retinal glial cells.

Studies of retinal function and disease occur at many different levels, from epidemiologic and population genetic, to human and whole animal studies, to the role of individual base pairs in retina-specific genes. There are strengths and weaknesses to every approach. We argue that studies utilising isolated cellular systems, particularly primary neuronal cell cultures can be an extremely valuable "level" of inquiry. Successful culture of retinal neuron cells *in vitro* is helpful in the research of retinal diseases.

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大鼠视网膜神经细胞的原代培养

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摘要

目的:在前人建立的方法上优化SD大鼠视网膜神经细胞体外培养的技术和方法,为后续研究提供实验基础。

方法:使用胰酶消化法分离新生1~3d SD大鼠视网膜神经细胞,以DMEM/F12为培养基体外培养,免疫组织化学染色的方法进行鉴定。

结果:观察光镜下培养的细胞贴壁生长,部分细胞伸出突起,且有些突起相互连接。免疫细胞化学染色显示,培养的细胞大多数抗神经元特异性烯醇化酶(neuron specific enolase,NSE)抗体反应阳性。

结论:视网膜神经细胞体外培养成功为进一步进行视网膜疾病的研究创造了条件。

关键词:细胞培养;视网膜神经元;胰蛋白酶消化法;神经元特异性烯醇化酶