Effect of erythropoietin on the survival of retinal neurocytes in culture upon serum withdrawal

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ABSTRACT

الأهداف: توضيح فيما إن كان الاريثروبين (EPO) يستبدل مكملات المصل في مزرعة الخلايا العصبية الشبكية التي تعاني من سحب المصل.

الطريقة: أجريت الدراسة في معهد شنقهاي لأمراض تقويم العظام والصدمات خلال الفترة من أبريل 2008م ومارس 2009م. استخدم عدد 160 فأر بعد الولادة 3-2 يوم في هذه الدراسة. بعد عمل مزرعة للخلايا العصبية الشبكية لمدة 48 ساعة، استبدلت المستنبتات بمصل خال من أي وسط، وعرضت الخلايا على محلول الاريثروبين (EPO) مقدار 1 وحدة/ملل، و 3 وحدة/ملل، و 6 وحدة/ملل لمدة 24 ساعة أو 48 ساعة إضافية. تم تقيم قطر جسم الخلية باستخدام جهاز تحليل الصورة المقطعي، وتحديد معدلات الحياة، والموت لهذه الخلايا بواسطة مقياس الانتساخ، ومقياس الخلايا. استخدمت الكيمياء المناعية الخلوية لاكتشاف الاريثروبين (EPO)، وظهور مستقبل الاريثروبين (EPOR).

النتائج: وجد الاريثروبين (EPO) ومستقبل الاريثروبين (EPOR) في الخلايا العصبية الشبكية. كانت معدلات الموت الكلي، والمبكر للخلايا العصبية الشبكية مع سحب المصل p=0.002 أعلى من مزرعة الخلايا العصبية التي تحتوي على مصل p=0.049،كانت إمكانية الحياة بعد الولادة للخلايا العصبية الشبكية مختلفة مع سحب المصل بشكل منخفض إحصائياً من الخلايا العصبية مع المصل p=0.047. لا يظهر أي تأثير للاريثروبين في قطر جسم الخلايا لمزرعة الخلايا العصبية الشبكية. كانت معدلات الموت، وإمكانية الحياة بعد الولادة للخلايا العصبية الشبكية مختلفة من المزرعة مسحوبة المصل في تركيزات الاريثروبين (EPO).

خاتمة: ليس في إضافة الاريثروبين (EPO) بعد سحب المصل أي تأثير في منع موت الخلايا العصبية الشبكية بواسطة سحب المصل لا يمكن للاريثروبين (EPO) أن يكون بديلاً لمكملات المصل.

Objectives: To clarify whether erythropoietin (EPO) could substitute for the serum component in cultured retinal neurocytes suffering from serum withdrawal.

Methods: The study was performed in the Shanghai Institute of Traumatology and Orthopedics, Shanghai, China between April 2008 and March 2009. A total of 160 postnatal 2-3 day-old Sprague-Dawley rats were used for this study. After the retinal neurocytes were cultured for 48 hours, the culture media was replaced with serum-free media, and the cells were exposed to 1 U/ml, 3 U/ml, and 6 U/ml EPO for another 24 or 48 hours, the cell body diameter was then assessed using a computerized image-analysis system, and the survival and apoptosis rates of those cells were estimated by method of transcription and translation assay and flow cytometry. Immunocytochemistry was used to detect EPO and erythropoietin receptor (EPOR) expression.

Results: The retinal neurocytes had obvious EPO/EPOR expression. The early (p=0.002) and total (p=0.049) apoptosis rates of retinal neurocytes cultured with serum withdrawal were significantly higher than that of neurocytes cultured with serum, and the cell viability of neurocytes cultured with serum withdrawal was significantly lower than that of neurocytes cultured with serum (p=0.047). The EPO had no effect on the cell body diameter of cultured retinal neurocytes. The cell viability and the apoptosis rates of retinal neurocytes were not significantly different from that of simple serum-withdrawal culture at any EPO concentration.

Conclusion: As the addition of EPO immediately after serum withdrawal had no effect in preventing retinal neurocytes apoptosis induced by serum withdrawal, EPO cannot substitute for the serum component.

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Erythropoietin (EPO) is a traditionally hematopoietic cytokine serving the hormonal role in red blood cell production. Recent studies have shown that EPO is also a neuroprotective factor, particularly in conditions of CNS damage, such as stroke, ischemia, hypoxia, and brain hemorrhage.[1-6] The expression of EPO and EPO receptors (EPOR) in the CNS, and the up-regulation of EPO by hypoxia/ischemia in vitro and in vivo suggests that this cytokine is an important mediator of the brain's response to injury.[7-9] In the retina, EPO and its EPOR interactions exert protective effects against light-induced retinal oxidative injury,[10,11] axotomy-induced degeneration of retinal ganglion cells,[12,13] and retinal ischemia.[14] Previous studies showed that EPO could prevent apoptosis induced by serum withdrawal for P19 teratoma cell lines and motoneurons in vitro, and act as a neuroprotective and neurotrophic factor in primary rat motoneurons.[6] Despite the demonstrated benefits in neuroprotection for retinal/optic nerve injury in vivo,[10,12,14] there are no data available on the survival effect of EPO on the dissociated cultured retinal neurocytes. Therefore, the present study aims to clarify whether EPO could substitute for the serum component as a neurotrophic factor in dissociated cultured retinal neurocytes suffering from serum withdrawal.

Methods. This study was performed in Shanghai Institute of Traumatology and Orthopedics, Shanghai, China between April 2008 and March 2009. 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. A total of 160 rats were used for this study.

Precoating of the tissue culture plates with rat tail tendon collagen. The rat tail tendon collagen was prepared according to the method previously described.[15] In brief, the tendon of an adult Sprague-Dawley rat was extracted and cut into fragments, the fragments were placed into 150 ml dilute acetic acid solution (1:1000) until they dissolved (reserved in 4°C for 48 hours). Then the solution was centrifuged at the speed of 4000 rpm, and the supernatant were collected and reserved in -20°C. The method for precoating the tissue culture plates with rat tail tendon collagen was previously described.[15] The collagen supernatant was added into the holes of the tissue culture plates under sterile condition (35 ul/hole for 96-well plate, 250 ul/hole for 24-well plate, 600 ul/hole for 6-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 under sterile conditions. Then the plate was used immediately.

Dissociated cell cultures. The suspension of retinal single cells was prepared according to the method previously described.[15] Briefly, the eyeballs were enucleated from 2~3-day-old Sprague-Dawley rats, and approximately 20 eyes were harvested for each experiment. The retinas were separated from the enucleated eyeballs and incubated in a solution containing 0.08% trypsin for 30 minutes at 37°C. To yield a suspension of single cells, the retinal tissue was then triturated sequentially through a narrow-bore Pasteur pipette in a solution of Dulbecco's modified eagle medium (DMEM [Gibco Company, New York, NY, USA]) containing 20% new-born calf serum (Gibco Company, New York, NY, USA). After centrifugation at 600 rpm for 5 minutes, the cells were resuspended in DMEM containing 20% fetal calf serum (Gibco Company, New York, NY, USA). Then the dissociated cells were seeded at density of approximately 1-1.2 x 10^6 cells/ml on the culture plate preconditioned as above described, and cultured in a humidified incubator at 37°C with an atmosphere of 5% CO_2-95% O_2. After the cells were seeded for 16 hours, 5-bromo-2'-deoxyuridine (20 ug/ml [Sigma, St. Louis, MO, USA]) was added to the culture media to restrain the non-neurocytes. Thirty-two hours later, the culture media was replaced with DMEM containing 20% fetal calf serum, or serum-free, or drug-supplemented media for further culture.

Identification and the EPO and EPOR expression of the cultured retinal neurons. A total of 30 rats were used for cell identification and the EPO and EPOR expression of the cultured retinal neurons. After 96 hours in vitro, the cultured cells were fixed in 4% paraformaldehyde for 30 minutes, then incubated in 3% hydrogen peroxide for 10 minutes to block endogenous peroxidase activity. The cells were washed 3 times with phosphate buffered solution (PBS), then incubated with 1% bovine serum albumin (BSA) for

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20 minutes at room temperature to block non-specific binding, and incubated with mouse-anti-rat neuron-specific enolase (NSE) monoclonal antibody (1:25 [Serotec Company, Oxford, UK])/ rabbit-anti-rat glial fibrillary acidic protein (GFAP) polyclonal antibody (1:200 [Neomarker Company, Fremont, CA, USA])/ rabbit-anti-rat EPO polyclonal antibody (1:200 [Santa Cruz Biotechnology, Santa Cruz, CA, USA])/ rabbit-anti-rat EPOR polyclonal antibody (1:100 [Santa Cruz Biotechnology, Santa Cruz, CA, USA]) at 4°C overnight. The cells were washed 3 times with PBS, and incubated with the biotinylated secondary antibodies (1:400 [Santa Cruz Biotechnology, Santa Cruz, CA, USA]) for 30 minutes at room temperature, and then with avidin-biotin complex (ABC solution [Santa Cruz Biotechnology, Santa Cruz, CA, USA]) for 30 minutes at room temperature. After several washes, 3,3-diaminobenzidine tetrahydrochloride (DAB [Sigma-Aldrich, St Louis, MO, USA]) was used as a co-substrate for 5 minutes. The coverslips were counterstained with hematoxylin and mounted. The primary antibody was replaced by non-immune serum for negative controls. The cells were examined with a microscope (Nikon Company, Tokyo, Japan), and images were recorded by photography. Each experiment was repeated 3 times.

Drug treatment. The EPO (R&D Systems, Minneapolis, MN, USA) was prepared by dissolving 10 ug in 200 ul (8.3 ug=1000 U EPO) distilled water containing 0.1% BSA (Gibco, New York, NY, USA). After the cells were cultured for 48 hours, the culture media was replaced with serum-free media, and 1 U/ml, 3 U/ml, 6 U/ml EPO were diluted in serum-free medium and incubated with the cells for another 48 hours. The vehicle control was serum-free medium containing 0.1% BSA. After co-incubation for 48 hours, the survival and apoptosis rates of those cells were examined by the method of transcription and translation (MTT) assay and fluorescein isothiocyanate (FITC)-annexin V/propidium iodide (PI) flow cytometry as previously described.[15] Each experiment was repeated in triplicate for statistical analysis.

Evaluation of the cell body diameter. A total of 40 rats were used for the evaluation of the cell body diameter. After the cells were cultured for 48 hours (EPO, 0 hour administration), 72 hours (EPO, 24 hours administration) and 96 hours (EPO, 48 hours administration), the cell body diameter was assessed using a computerized image-analysis system (Axioskop 2 plus [Carl Zeiss, Oberkochen, Germany]). The clusters of cells were excluded from the cells diameter measurement, and 120 dissociated cells in each sample were randomly chosen for measurement. The longest diameter of each cell was selected for measurement, and served as the body diameter of the cell. Each experiment was repeated in triplicate for statistical analysis.

MTT assay. A total of 40 rats were used for MTT assay. Survival of retinal neurocytes was estimated by using the MTT assay according to the manufacturer's directions (Shanghai Analytical Instrument Factory, Shanghai, China). Briefly, the cells were cultured in 96-well plates, and the 2 mg/ml MTT labeling solution was added to each well in the culture medium. After the cells were incubated for 3 hours at 37°C in the incubator, the absorbance of each sample was measured at a wavelength of 490 nm. Each experiment was repeated in triplicate for statistical analysis.

Flow cytometry. A total of 50 rats were used for flow cytometry. The cultured cells were digested in 0.125% trypsin for one minute, the enzymatic reaction was stopped by DMEM (Gibco Company, New York NY, USA) containing 20% new-born calf serum, and the cells were pelleted gently. The flow cytometry was performed according to the method as previously described.[15] Briefly, the freshly collected cells were centrifuged at 1500 rpm for 5 minutes, and resuspended in the binding buffer solution with the cells density of approximately 10^6cells/ml. A 100 microliters of the cells suspension were transferred into another tube, then 5 microliters of FITC-annexin V (Sigma, St. Louis, MO, USA), 5 microliters of PI (Sigma, St. Louis, MO, USA) and 400 microliters buffer solution were added to the tube. The cell suspension was incubated for 15 minutes at room temperature in the dark, then checked by flow cytometry (FACS Calibur [Becton, Dickinson and Company, Franklin Lakes, NJ, USA]). Each experiment was repeated in triplicate for statistical analysis.

Statistical analysis. Data are expressed as mean ± SD, unless otherwise stated. Statistical analyses were performed using the Statistical Package for Social Sciences for Windows version 10.0 software package (SPSS Inc, Chicago, IL, USA). The independent student t-test was used for comparing data between the 2 groups. One-way analysis of variance (ANOVA) followed by the least significant difference (LSD test [homogeneity of variance]), or Games-Howell test (non homogeneity of variance) was used for comparing data among 3 or more groups. $P<0.05$ was considered statistically significant.

Results. ***Identification and EPO/EPOR expression of cultured cells.*** After seeding for 24 hours, the cultured cells shaped round or ellipse, and small neurite outgrowth was observed (**Figure 1a**). The cell neurites were extended, and the number of survival cells decreased gradually within the 5 days culture (**Figures 1b & c**). The cultured cells almost died away at the 6~7 days culture. After the cells were cultured for 96 hours, most of the surviving cells were NSE-positive cells (**Figure 1d**), and accounted for 93.015 ± 2.649%, and an obvious EPO/EPOR immunoreactivity staining was observed in those cells (**Figures 1e & f**).

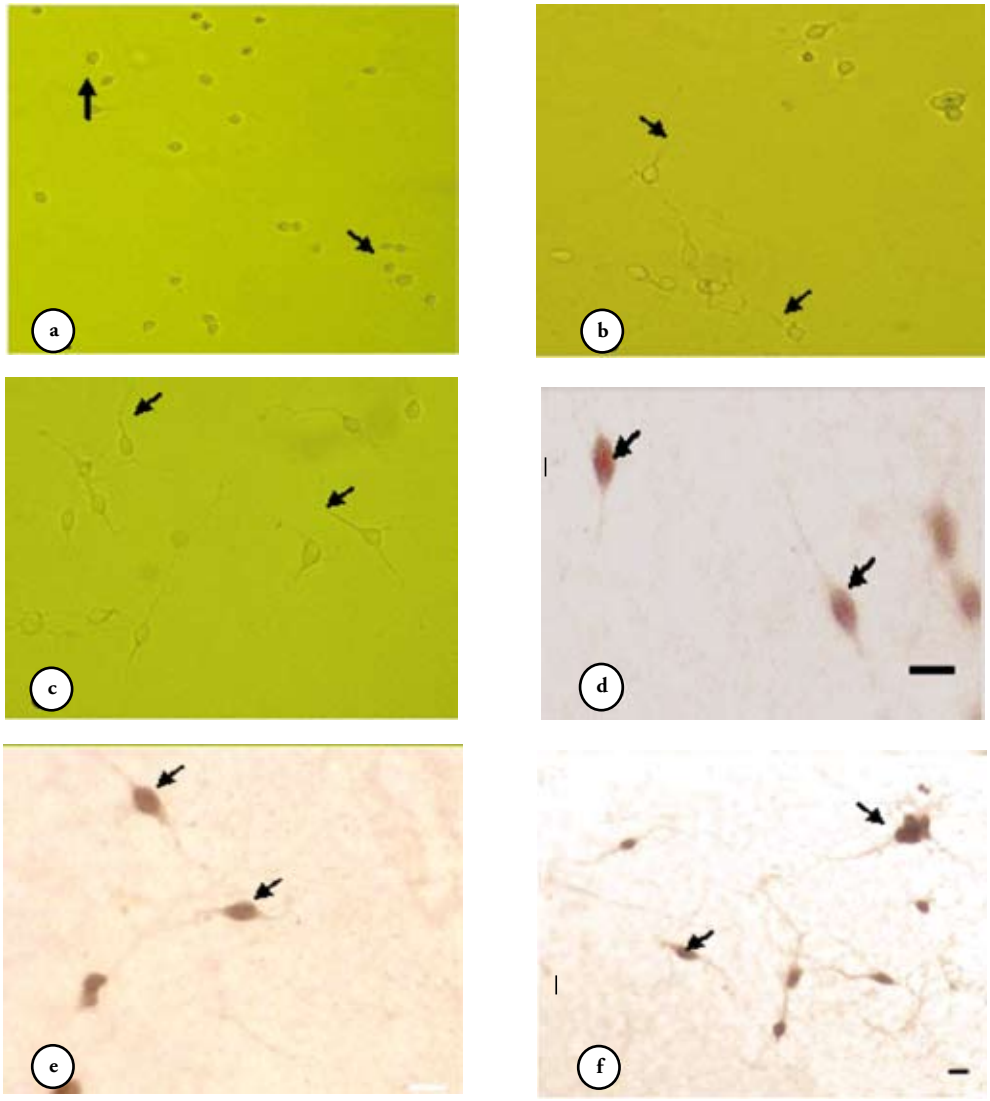

Figure 1 - Retinal cells culture and immunocytochemical staining images, showing: **a**) 24 hours culture, the cells shaped round or ellipse, and small neurite outgrowth was observed. **b**) 48 hour culture, the cells shaped round or ellipse, and the obvious neurite outgrowth was observed. **c**) 96 hour culture, obvious neurite outgrowth was also observed. **d**) 96 hour culture, the majority of survival cells were neuron-specific enolase positive cells, the cells shaped round or ellipse and the neurites could be seen. **e & f**) Obvious erythropoietin (EPO) immunoreactivity staining was observed in those cultured cells (Figures 1a-c: magnification x200; Figures 1d-f: immunocytochemical staining, magnification x400, bar=10 μm)

Table 1 - The apoptosis rates of retinal neurocytes cultured for 96 hours with serum, or 48 hours serum withdrawal (n=18).

Variable	Serum culture	Serum withdrawal culture	t value	*P*-value
	Mean ± SD			
Early apoptosis rate (%)	4.98 ± 0.95	14.63 ± 1.24	7.101	0.002
Late apoptosis rate (%)	6.06 ± 3.21	6.99 ± 3.74	0.328	0.759
Total apoptosis rate (%)	11.05 ± 3.64	21.62 ± 5.43	2.181	0.049
MTT absorbance value	0.799 ± 0.06	0.679 ± 0.11	2.255	0.047

MTT - method of transcription and translation, SD - standard deviation

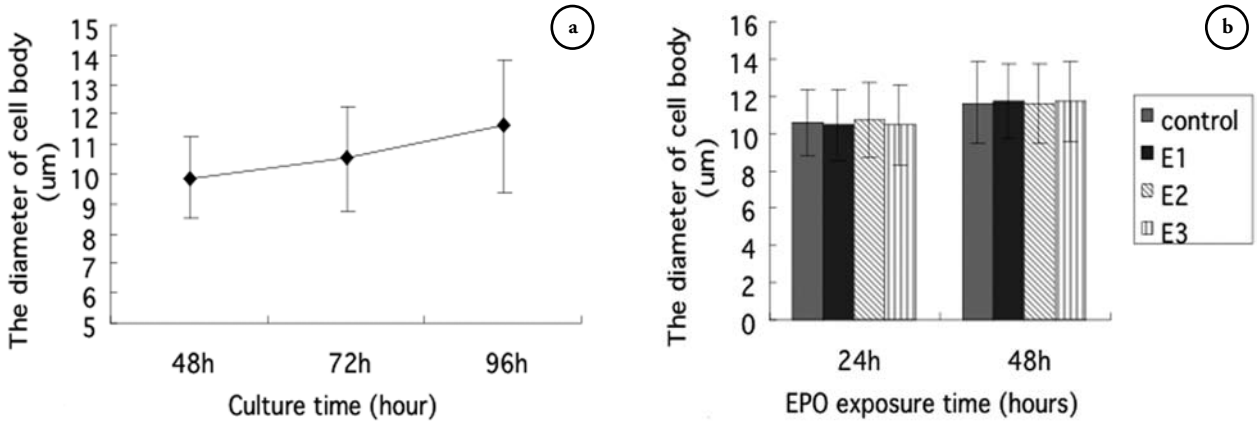

Figure 2 - Effect of erythropoietin (EPO) on cell body diameter, showing: **a**) The cell body diameters of retinal neurocytes in the vehicle control gradually increased with the elongation of the culture time. **b**) The cell body diameters of retinal neurocytes was not significantly different from that of vehicle control at any EPO concentration. (E1: 1 U/ml EPO, E2: 3 U/ml EPO, E3: 6 U/ml EPO)

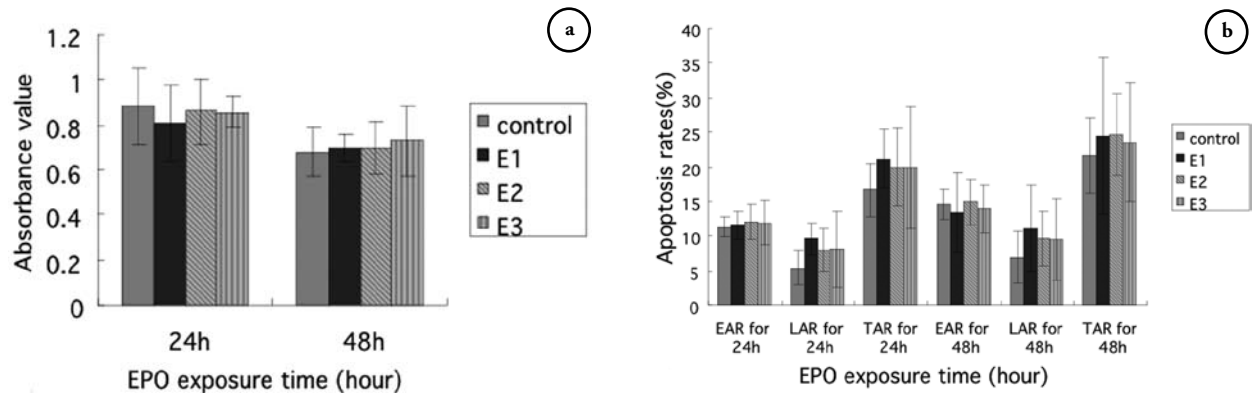

Figure 3 - Effect of erythropoietin (EPO) on cell survival and apoptosis of retinal neurocytes, showing: **a**) methods of transcription and translation assay of cell viability in retinal neurocytes exposed to various concentrations of EPO for 24 hours or 48 hours, the cell viability of retinal neurocytes was not significantly different from that of the vehicle control at any EPO concentration. **b**) fluorescein isothiocyanate-annexin V/propidium iodide assay of cell apoptosis rates in retinal neurocytes exposed to various concentrations of EPO for 24 hours or 48 hours, the early apoptosis rate (EAR), late (LAR), and total (TAR) apoptosis rates of retinal neurocytes were not significantly different from that of the vehicle control at any EPO concentration. (E1: 1 U/ml EPO, E2: 3 U/ml EPO, E3: 6 U/ml EPO)

The survival state comparison of retinal neurocytes between the serum culture and the serum withdrawal culture. **Table 1** showed that the apoptosis rates and cell viability of retinal neurocytes cultured for 96 hours with serum or 48 hours serum withdrawal. The early apoptosis rate and the total apoptosis rate of retinal neurocytes cultured with serum withdrawal media were significantly higher than that of retinal neurocytes cultured with serum media. However, the late apoptosis rate of retinal neurocytes cultured with serum withdrawal media was not significantly different from that of retinal neurocytes cultured with serum media. The cell viability of retinal neurocytes cultured with serum withdrawal media was significantly lower than that of the retinal neurocytes cultured with serum media.

Effect of EPO on cell body diameter of retinal neurocytes in culture upon serum withdrawal. Within the 96 hour culture period, the cell body diameters of retinal neurocytes gradually increased with the elongation of the culture time (**Figure 2a**). When EPO was added to the culture medium during the serum-withdrawal period, the cultured retinal neurocytes were exposed to 1 U/ml, 3 U/ml, and 6 U/ml EPO for 24 hours (72 hour culture period) or 48 hours (96

hour culture period), the cell body diameters of retinal neurocytes was not significantly different from that of the vehicle control at any EPO concentration (**Figure 2b**).

Effect of EPO on cell survival and apoptosis of retinal neurocytes in culture upon serum withdrawal. When EPO was added to the culture medium during the serum-withdrawal period, the cultured retinal neurocytes were exposed to 1 U/ml, 3 U/ml, and 6 U/ml EPO for 24 hours (72 hour culture period) or 48 hours (96 hour culture period), the cell viability of retinal neurocytes was not significantly different from that of the vehicle control at any EPO concentration (**Figure 3a**), meanwhile, the early, late, and total apoptosis rates of retinal neurocytes were not significantly different from that of the vehicle control at any EPO concentration (**Figure 3b**).

Discussion. To gain a deeper understanding of the nature of EPO-dependent neuroprotection, we studied its biological effects on dissociated cultured retinal neurocytes suffering from serum withdrawal. Our results suggest that the cell viability of retinal neurocytes cultured with serum withdrawal media was significantly lower than that of retinal neurocytes cultured with serum media, and the early apoptosis rate and the total apoptosis rate of retinal neurocytes cultured with serum withdrawal media were significantly higher than that of retinal neurocytes cultured with serum media. When EPO was added to the culture medium during the serum-withdrawal period, the cell viability and the apoptosis rates of retinal neurocytes was not significantly different from that of simple serum-withdrawal culture at any EPO concentrations, indicating that EPO could not completely substitute for a serum component.

Whether EPO can support survival of neurons in serum-withdrawal medium has been a disputed question. Some findings showed that EPO promoted survival of cholinergic neurons, primary motoneurons ,and P19 teratoma cell lines.[6,16] However, other studies did not provide clear evidence that EPO can support survival of hippocampal and cerebrocortical neurons in serum-withdrawal medium,[17,18] which is consistent with our findings. This dispute may be owing to different cell types, or experimental condition and methods. In the P19 teratoma cell line, only pretreatment with EPO was effective in preventing apoptosis induced by serum withdrawal, in contrast, addition of EPO immediately after serum withdrawal had no effect, and a continued presence of EPO did not improve the protection conferred by pretreatment.[6] These neural-like cells behave similarly to other culture systems reported in which a gene expression program is initiated by EPO but requires >4 hours for completion.[15]

Prior studies showed that EPO may participate in the protection of retinal neurons from hypoxia, ischemia, degeneration, or experimental glaucoma.[10,12-14,19-24] Multiple mechanisms may explain how EPO protects neurons from acute injury. The EPO could significantly reduce terminal deoxyribonucleotidyl transferase mediated dUTP nick-end labelling (TUNEL)-positive cells in the ischemic penumbra after focal cerebral ischemia, which suggests an antiapoptotic effect.[6] Similarly, the ischemic retinas that had received EPO exhibited a marked decrease in TUNEL labeling.[14] The EPO may inhibit neuronal apoptosis by up-regulating B-cell lymphoma-extra large (Bcl-xL) and Bcl-2.[25] The EPO also was shown to inhibit apoptosis of cultured cortical neurons deprived of growth factors or exposed to kainic acid by activating mitogen-activated protein kinase and phosphatidylinositol 3-kinase/AKt pathways.[6] The EPO may also exert an antiapoptotic effect by recruiting nuclear factor-kappa B,[17] which translocates to the nucleus and can activate neuroprotective genes such as superoxide dismutase or inhibitors of apoptosis proteins. However, the present experiment found that addition of EPO immediately after serum withdrawal had no effect in preventing retinal neurocytes apoptosis induced by serum withdrawal, which may be attributed to no EPO pretreatment and inactivation of those antiapoptotic pathways.

In the present study, the cultured retinal neurocytes had EPO and EPOR expression. These findings are consistent with prior studies that the retina has the expression of EPO and EPOR,[11] which suggests that EPO has the effects of autocrine and paracrine in the retina. There were a few limitations in our study. First, the present study did not provide any cytokine or agent able to increase cell viability under the condition of serum withdrawal. Second, the study did not provide any new insight on the potential mechanism of EPO action in neural cells. Further investigations are needed to demonstrate the potential mechanism of EPO action in retinal neurocytes.

In conclusion, our results suggest that addition of EPO immediately after serum withdrawal had no effect in preventing retinal neurocytes apoptosis induced by serum withdrawal, indicating that EPO could not completely substitute for a serum component.

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ETHICAL CONSENT

All manuscripts reporting the results of experimental investigations involving human subjects should include a statement confirming that informed consent was obtained from each subject or subject's guardian, after receiving approval of the experimental protocol by a local human ethics committee, or institutional review board. When reporting experiments on animals, authors should indicate whether the institutional and national guide for the care and use of laboratory animals was followed. Research papers not involving human or animal studies should also include a statement that approval/no objection for the study protocol was obtained from the institutional review board, or research ethics committee.