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Recombinant human erythropoietin protects long-term cultured ageing primary nerve cells by upregulating the PI3K/Akt pathway

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Objective **Previous studies have found that recombinant human erythropoietin (rhEPO) protects long-term cultured ageing primary nerve cells by enhancing the endogenous antioxidant capacity of cells; however, its signalling pathways are not clear. This study aimed to explore the relationship between the rhEPO and PI3K/Akt pathways in the protection of senescent nerve cells at the cellular level.**

Methods **Primary nerve cells were cultured for 22 days to mimic the natural ageing process of nerve cells. rhEPO and LY294002 were administered as an intervention on the 11th day of culture. Western blot, immunochemistry, 3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyltetrazolium bromide, immunofluorescence double-labelling staining, Annexin V-FITC/PI double-labelling flow cytometry, and SA-β-gal staining experiments were used to observe the expression levels of erythropoietin receptor (EPOR) and phosphorylated Akt (p-Akt) protein and the related indices of nerve cell senescence.**

Results **Western blot experiments showed that in ageing long-term cultured primary neurons, the EPOR and p-Akt decreased and rhEPO upregulated the expression levels of EPOR and p-Akt protein. The rest showed that the PI3K/Akt pathway blockade reduced the antioxidation capacity, cell viability, cell morphology, and ratio of apoptotic cells and senescent cells of rhEPO on ageing long-term cultured primary nerve cells.**

Conclusions **This study explored the relationship between the rhEPO and PI3K/Akt pathways in the protection of ageing nerve cells at the cellular level and found that rhEPO protects long-term cultured ageing primary nerve cells by upregulating the PI3K/Akt pathway. These findings provide a theoretical basis and experimental evidence for the antiaeging mechanism of EPO in the nervous system. *NeuroReport* 33: 186–198**

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Keywords: ageing, erythropoietin, long-term culture, PI3K/Akt pathway, primary nerve cells

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Introduction

Animal and cell studies have suggested that erythropoietin (EPO) has a protective effect against a variety of nervous system injuries. Previous studies have found that recombinant human erythropoietin (rhEPO) protects long-term cultured primary nerve cells by enhancing the endogenous antioxidant capacity of senescent nerve cells [1]; however, the signal-related pathways involved require further research. The phosphatidylinositol-3-kinase (PI3K)/protein kinase B (Akt) pathway is a key pathway with a wide range of biological effects on the nervous system, especially its protective effect [2–4], but it is not clear whether it is involved in the protection of senescent nerve cells by rhEPO.

Ageing differs by cell type. Specifically, nerve cells are terminally differentiated and consequently no longer divide and replicate. Thus, they undergo nonreplicative ageing. During long-term culture, protein carbonyls and Aβ proteins accumulate in primary nerve cells, and cell mitochondrial membrane potential and antioxidant capacity decrease, which ultimately increases the number of apoptotic and ageing cells. These changes approximate the changes of nerve cells in the central nervous system during ageing. A number of studies have used long-term cultured primary nerve cells to study naturally ageing-associated mechanisms in the nervous system at the cellular level [1,5].

In this study, we also used long-term cultured primary nerve cells to establish a nerve cell model of natural ageing. This study aimed to observe the effects of rhEPO on PI3K/Akt pathway-related proteins and changes in the protective effect of rhEPO on senescent nerve cells after PI3K/Akt pathway intervention, to explore the relationship between the rhEPO and PI3K/Akt pathways in the

protection of senescent nerve cells at the cellular level, and to find new therapeutic targets for antiageing research.

Materials and methods

Long-term primary culture of neurons

One-day-old neonatal Sprague–Dawley rats (Animal Experiment Centre of the Biofavor Biotech Service Co., Ltd., Wuhan, China; No. 42000600012249) were sacrificed by cervical dislocation, and the brain cortex was isolated and digested with 0.125% trypsin. The digested mixture was filtered through a 200-mesh filter, and the filtrate was centrifuged for 5 min at 800 rpm to harvest the cells. Neurobasal (Gibco BRL, Gaithersburg, Maryland, USA) + B27 medium (Gibco BRL) was used to prepare the cell suspension solution, which was seeded into a polylysine-coated culture plate and incubated overnight at 37 °C with 5% CO_2 in a saturated humidity atmosphere. Cells were cultured for 22 days [1,5]. Half of the medium was changed every 2 days, and the size and morphology of the cells were observed under an inverted microscope. All experiments were performed at the Experimental Centre of the Second Affiliated Hospital of Xi'an Jiaotong University, China. This study was carried out in strict accordance with the recommendations of the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The animal use protocol was reviewed and approved by the Institutional Animal Care and Use Committee of Xi'an Jiaotong University.

Experimental grouping

Cells in the control group were cultured for a total of 22 days and represented aged nerve cells. In the EPO group, starting on day 11, the culture medium was supplemented with EPO (final concentration, 100 μ/ml; PeproTech, Rocky Hill, Connecticut, USA). In the EPO + LY294002 group starting on day 11, the culture medium was supplemented with EPO (final concentration, 100 μ/ml; PeproTech) and LY294002 (final concentration, 5 μM; vehicle: DMSO, the final concentration of DMSO was 0.05%; Selleck, Houston, Texas, USA). In the LY294002 group, on day 11, the culture medium was supplemented with LY294002 (final concentration, 5 μM; Selleck).

Immunofluorescence labelling of nerve cells to observe the erythropoietin receptor and phosphorylated protein kinase B

Isolated cells were seeded into polylysine-treated 12-well plates at a density of 1.0×10^6 cells/ml, and 1 ml of medium was added to each well. The cells were cultured overnight at 37 °C with 5% CO_2 and saturated humidity. The coverslips covered with cells were washed with PBS, and the cells were fixed with 4% paraformaldehyde. The cells were permeabilized with 0.5% Triton X-100 at room temperature for 20 min. After being washed with PBS, the cells were blocked with normal goat serum at room temperature for 30 min. The cells were then incubated with the following antibodies: erythropoietin receptor (EPOR) (1:100, rabbit; Abclonal, Wuhan, China) or p-Akt (1:100, rabbit; CST, Boston, Massachusetts, USA) at 4 °C overnight, followed by incubation with fluorescent (Cy3) goat anti-rabbit immunoglobulin (IgG) secondary antibody (1:200; Boster Biological Technology Co. Ltd., Wuhan, China) for 1 h at 37 °C in a humidified atmosphere. PBS containing Tween was used to wash the slides three times for 3 min each. Subsequently, 4′6-diamidine-2′-phenylindole dihydrochloride (DAPI) was added dropwise and incubated for 5 min in the dark to counterstain the nuclei. The slides were sealed with a mounting medium containing an antiquenching agent and then observed under a fluorescence microscope to capture images. The CY3 produced red fluorescence at an excitation wavelength of 550 nm and emission wavelength of 570 nm, and all manipulation steps were conducted in the dark.

Western blot analysis of erythropoietin receptor and phosphorylated protein kinase B

The cells were homogenized and after protein determination, each sample (40 μg/lane) was subjected to 8% SDS-polyacrylamide gel electrophoresis. The membranes were incubated with antibodies against the active forms of EPOR (1:1000, rabbit; Abclonal) and p-Akt (1:1000, rabbit; CST). After being washed, the membranes were incubated with a horseradish peroxidase-conjugated secondary antibody (goat anti-rabbit IgG, 1:2000; Boster Biological Technology Co. Ltd.). Antibody-reactive bands were detected by chemiluminescence.

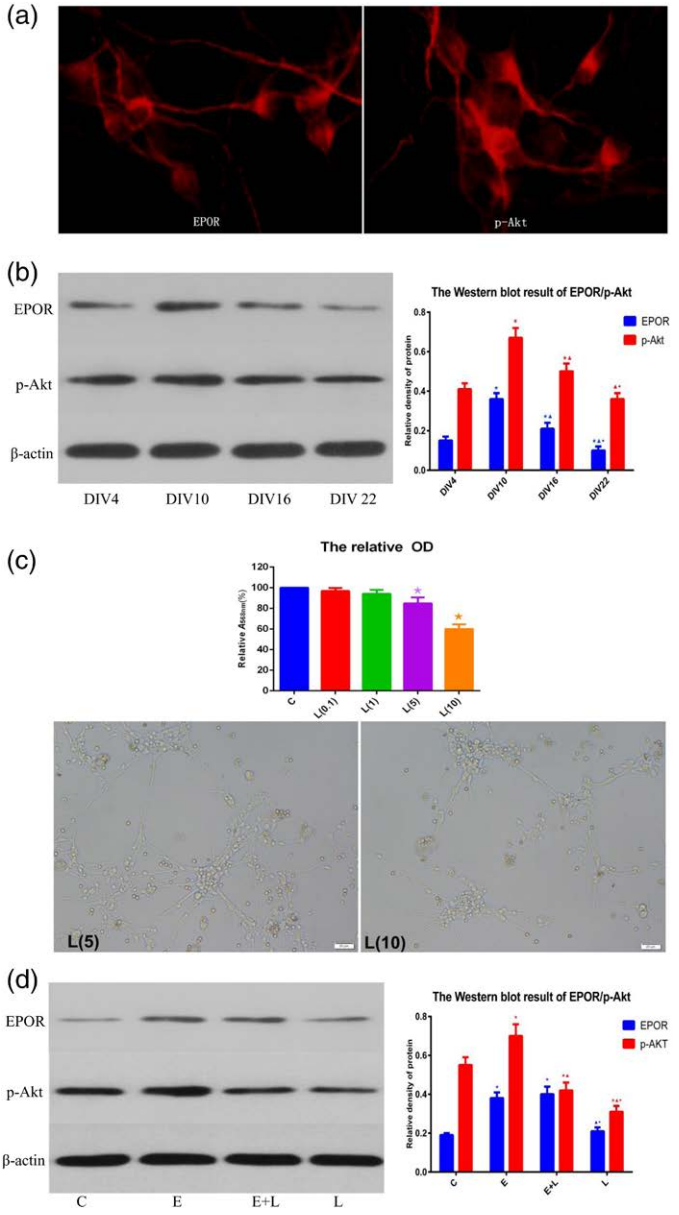
Observation of the related indices of nerve cell senescence

Nerve cells were labelled by double immunofluorescence of microtubule-associated protein (MAP)-2 antibody (1:100, rabbit; Proteintech Group Inc., Wuhan, China) and glial fibrillary acidic protein (GFAP) antibody (1:100, mouse; Santa Cruz Biotechnology, Santa Cruz, California, USA) to determine the purity of the cells. Nerve cell activity was determined by the MTT assay (Sigma, Santa Clara, California, USA). The apoptotic cell ratio was determined by flow cytometry using an Annexin V-FITC/propidium iodide (PI) apoptosis detection kit (Nanjing KeyGen Biotech Co. Ltd., Nanjing, China). The ratio of aged cells was determined by SA-β-gal staining (Nanjing KeyGen Biotech Co. Ltd.). The superoxide dismutase (SOD) activity and glutathione (GSH) and malondialdehyde (MDA) contents were determined using an SOD assay kit (Jiancheng Bioengineering, Nanjing, China), GSH detection kit (Jiancheng Bioengineering), and MDA assay kit (Jiancheng Bioengineering).

Statistical analysis

All data were analysed using SPSS (version 23.0) software. All experiments were performed in triplicate, and the data were presented as the mean ± SD. Data were also analysed using one-way analysis of variance. The level of statistical significance was set at $P < 0.05$.

Fig. 1



Expression of EPOR and p-Akt protein of long-term cultured primary neural cells after different treatments. (a) Immunofluorescence staining of EPOR and p-Akt of nerve cells in DIV22 of the control group (five positive nerve cells which were magnified 800 times). The results showed that both EPOR and p-Akt were expressed in the long-term cultured aging primary nerve cells. (b) Western blotting results of EPOR and p-Akt protein at different time points in the control group. Significantly higher levels of EPOR and p-Akt were observed in cells at DIV10 than at DIV4. However, as the culture time increased, the EPOR and p-Akt expression levels decreased successively at DIV16 and DIV22. (c) Changes of long-term cultured ageing primary nerve cells after different concentration of LY294002 treatments. L(0.1), L(1), L(5), and L(10): the cell on day 22 treatment with different concentrations (0.1, 1, 5, and 10 μM) of LY294002 starting on day 11. An MTT assay was used to measure the OD value of the long-term cultured nerve cells in the control group, which was used as the reference value. The absorbance value (A568 nm) was measured using a microplate reader. Changes in the morphology of nerve cells were observed under an inverted microscope (scale bar = 20 μm). With increasing inhibitor concentration, cell viability gradually decreased. When the concentration of LY294002 was increased to 5 and 10 μM, the decrease in neuronal cell viability was significantly different. The morphology of neuronal cells after exposure to 5 and 10 μM interventions showed more neuronal cells after treatment with 5 μM LY294002, and some synapses were intertwined into a network. Finally, the concentration of LY294002 used in the follow-up experiment was 5 μM. (d) Expression of EPOR and p-Akt protein after different treatments. Western blotting results showed that the expression of EPOR and p-Akt protein in senescent long-term cultured primary neural cells increased significantly after rhEPO intervention, and the expression of p-Akt protein decreased significantly after intervention with LY294002, whereas the expression of EPOR did not change significantly. Representative images of the control group are shown. Data are shown as mean ± SD values obtained from three separate experiments. Statistical analysis was performed according to one-way ANOVA. ★$P < 0.05$ vs. DIV4, ▲$P < 0.05$ vs. DIV10, ♦$P < 0.05$ vs. DIV16. ANOVA, analysis of variance; C, control group; DIV4, 4 days *in vitro*; DIV10, 10 days *in vitro*; DIV16, 16 days *in vitro*; DIV22, 22 days *in vitro*; E, EPO group; E + L, EPO + LY294002 group; EPO, erythropoietin; L, LY294002 group; MTT, 3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyltetrazolium bromide; OD, optical density; p-Akt, phosphorylated protein kinase B; rhEPO, recombinant human erythropoietin.

Results

Erythropoietin receptor and phosphorylated protein kinase B expression levels decreased in senescent long-term cultured primary neural cells

EPOR is the basis of the biological function of EPO, and Akt phosphorylation is a sign of PI3K/Akt pathway activation. To observe expression of both EPOR and p-Akt of senescent long-term cultured primary neural cells, immunofluorescence staining was used to show that long-term cultured primary neural cells at 22 days *in vitro* (DIV22) expressed EPOR and p-Akt (Fig. 1a). Western blotting was used to detect changes in the expression levels of EPOR and p-Akt in nerve cells at different time points (Fig. 1b). The results showed that significantly higher levels of EPOR and p-Akt were observed in cells at DIV10 than at DIV4 (EPOR: $P < 0.001$; p-Akt: $P = 0.0015$). However, as the culture time increased, the EPOR and p-Akt expression levels decreased successively at DIV16 (EPOR: $P = 0.0036$ vs. DIV10; p-Akt: $P = 0.01$ vs. DIV10) and DIV22 (EPOR: $P = 0.0062$ vs. DIV16; p-Akt: $P = 0.0083$ vs. DIV16).

Determination of the concentration of the PI3K/Akt pathway blocker intervention

The long-term culture of primary neuronal cells requires strict culture conditions, and the cells are highly sensitive to intervention conditions. This study used the 3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) method to determine the viability of long-term cultures of primary neurons after intervention with different concentrations of LY294002 and to determine the appropriate concentration of this inhibitor. With increasing inhibitor concentration, cell viability gradually decreased. When the concentration of LY294002 was increased to 5 and 10 μM, the decrease in neuronal cell viability was significantly different (5 μM: $P = 0.0099$ vs. control group; 10 μM: $P < 0.001$ vs. control group) (Fig. 1c). Further observations of the morphology of neuronal cells after exposure to 5 and 10 μM interventions showed more neuronal cells after treatment with 5 μM LY294002, and some synapses were intertwined into a network. After treatment with 10 μM LY294002, the number of neuronal cells was reduced and cells aggregated; the neural network basically disappeared (Fig. 1c). Finally, the concentration of LY294002 used in the follow-up experiment was 5 μM.

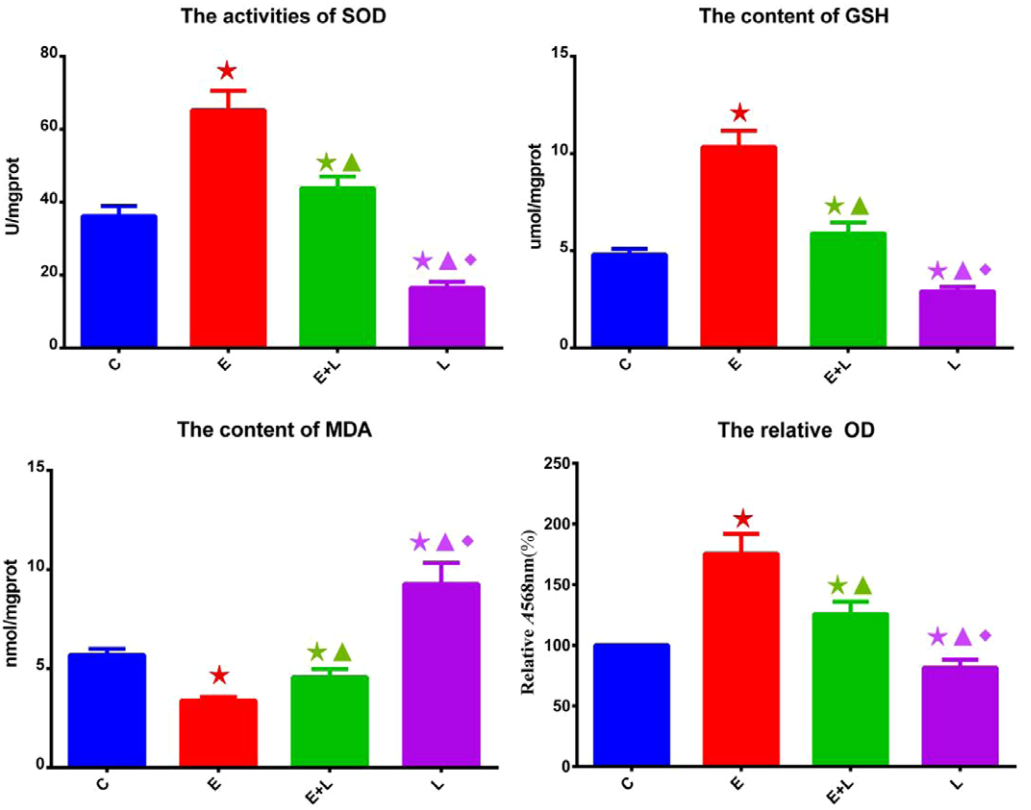
Recombinant human erythropoietin upregulates erythropoietin receptor and phosphorylated protein kinase B expression levels in senescent long-term cultured primary neurons

To clarify the effect of rhEPO on the PI3K/Akt pathway of senescent long-term cultured primary neural cells, western blotting was used to detect changes in the expression levels of EPOR and p-Akt in nerve cells after rhEPO intervention (Fig. 1d). The results showed that the control group had EPOR protein expression, and the EPOR protein expression level of the EPO group with rhEPO intervention was significantly higher than that of the control group ($P = 0.001$). The EPOR protein level of EPO + LY294002 group with the PI3K/Akt pathway inhibitor LY294002 and rhEPO cointervention showed no significant change compared with the EPO group ($P = 0.527$ vs. control group). The EPOR protein level of the LY294002 group which was only given the intervention of LY294002 and control group were at similar levels ($P = 0.196$ vs. control group), but it was significantly lower than that in the EPO group and EPO + LY294002 group ($P = 0.001$ vs. EPO group; $P = 0.002$ vs. EPO + LY294002 group). The p-Akt protein level of the control group was maintained at a moderate level, and it was significantly higher in the EPO group than in the control group ($P = 0.022$) and significantly lower in EPO + LY294002 group than in the EPO group ($P = 0.003$). However, in the LY294002 group, the p-Akt protein level was lower than that in the other three groups ($P = 0.016$ vs. control group; $P = 0.003$ vs. EPO group; $P = 0.019$ vs. EPO + LY294002 group).

Recombinant human erythropoietin enhances the antioxidant capacity of senescent long-term cultured primary nerve cells and improves cell viability by activating the PI3K/Akt pathway

Enhancing cellular antioxidant capacity is an important mechanism for the protection of aged cells in long-term cultured primary nerve cells. To observe the role of the PI3K/Akt pathway in this mechanism, immunochemical methods were used to determine the SOD activity, GSH content, and cellular peroxidation product MDA content after rhEPO intervention and LY294002 intervention (Fig. 2). The results showed that the SOD activity ($P = 0.0013$ vs. control group) and GSH content ($P < 0.001$ vs. control group) of cells in the EPO group treated with rhEPO intervention were significantly higher than those in the control group, whereas the MDA content was significantly reduced ($P < 0.001$ vs. control group). After LY294002 treatment, the SOD activity ($P = 0.0050$ vs. EPO group) and GSH content ($P < 0.001$ vs. EPO group) of cells in the EPO + LY294002 group were significantly lower than those in the EPO group, whereas the MDA content was significantly increased ($P = 0.0107$ vs. EPO group), but each index was significantly better than those of the control group (SOD: $P = 0.0394$ vs. control group; GSH: $P = 0.0461$ vs. control group; MDA: $P = 0.0278$ vs. control group). In addition, the SOD activity ($P < 0.001$ vs. control group; $P < 0.001$ vs. EPO group; $P < 0.001$ vs. EPO + LY294002 group) and GSH content ($P = 0.001$ vs. control group; $P < 0.001$ vs. EPO group; $P = 0.0011$ vs. EPO + LY294002 group) of LY294002 group treated with LY294002 alone was significantly lower than that in the other three groups, whereas the MDA content was significantly increased ($P = 0.0045$ vs. control group; $P < 0.001$ vs. EPO group; $P = 0.002$ vs. EPO + LY294002 group).

Fig. 2



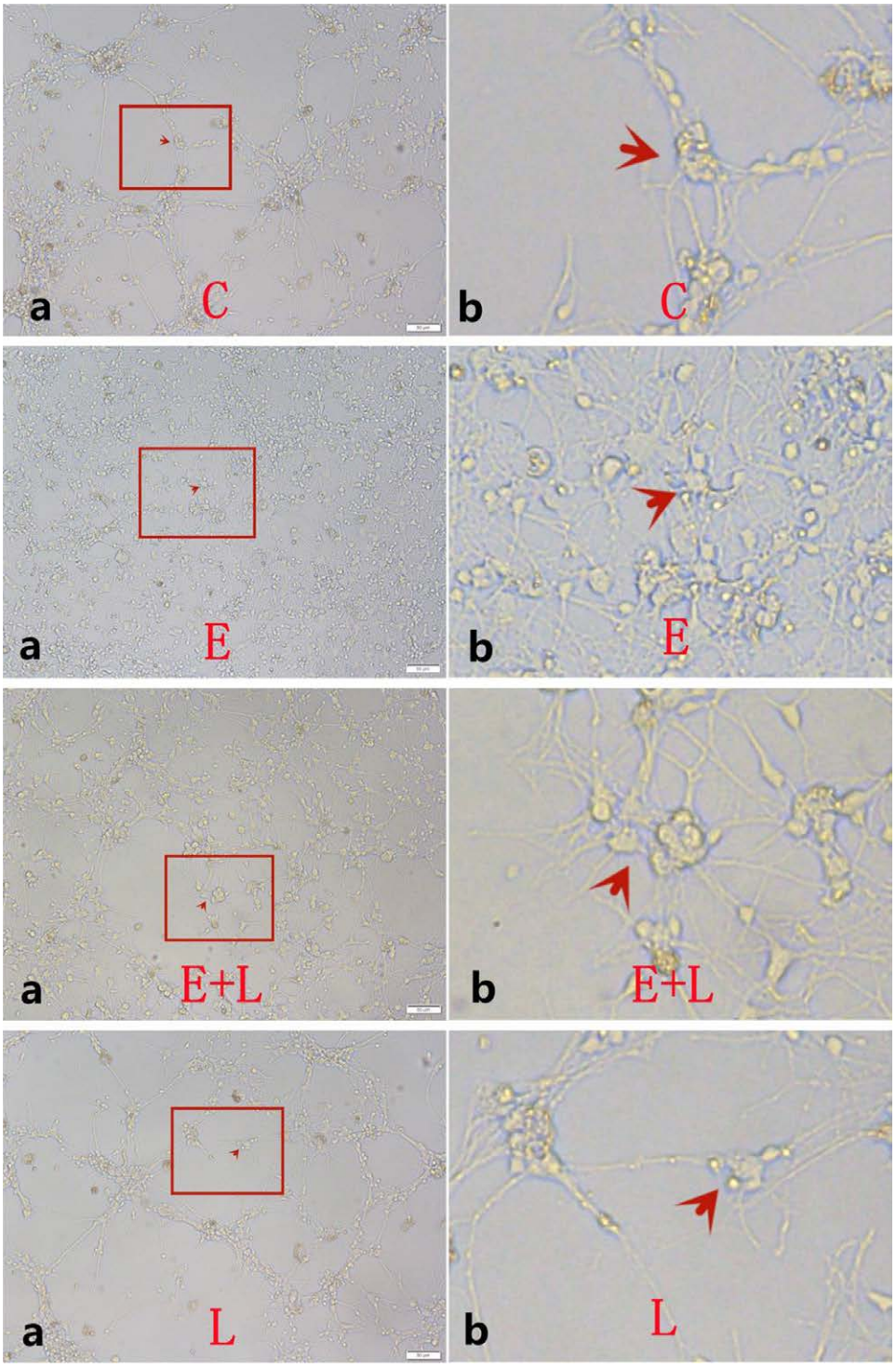
Changes in SOD activity, GSH content, MDA content, and cell viability in each group of cells. Immunochemical methods were used to determine cell antioxidant enzyme SOD activity, GSH content, and cell peroxidation product MDA content. An MTT assay was used to measure the OD value of the long-term cultured nerve cells in the control group, which was used as the reference value. The absorbance value (A568 nm) was measured using a microplate reader. The results showed that after rhEPO intervention, SOD activity of the senescent long-term cultured primary nerve cells was significantly enhanced, GSH content was significantly increased, MDA content was reduced, and cell viability was significantly increased. After the intervention of LY294002, SOD activity decreased, GSH content decreased, intracellular MDA increased, and cell viability decreased. Data are shown as mean ± SD values obtained from three separate experiments. Statistical analysis was performed according to one-way ANOVA. ★$P < 0.05$ vs. control group, ▲$P < 0.05$ vs. EPO group, ♦$P < 0.05$ vs. EPO + LY294002 group. ANOVA, analysis of variance; C, control group; E, EPO group; E + L, EPO + LY294002 group; EPO, erythropoietin; GSH, glutathione; L, LY294002 group; MDA, malondialdehyde; MTT, 3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyltetrazolium bromide; OD, optical density; rhEPO, recombinant human erythropoietin; SOD, superoxide dismutase.

Decreased cell viability is one of the manifestations of senescence in long-term cultured nerve cells. To observe whether rhEPO can improve the vitality of ageing long-term cultured nerve cells through the PI3K/Akt pathway, we used the MTT method to observe the changes in the vitality of nerve cells in each group after the intervention with rhEPO and LY294002. The results showed that the cell viability of the EPO group was significantly higher than that of the control group ($P = 0.0013$ vs. control group), and the cell viability of EPO + LY294002 group was significantly lower than that of the EPO group ($P = 0.0117$ vs. EPO group), but it was still higher than that of the control group ($P = 0.011$ vs. control group). The cell viability of the LY294002 group was lower than that of the other groups ($P = 0.0089$ vs. control group; $P < 0.001$ vs. EPO group; $P = 0.0032$ vs. EPO + LY294002 group) (Fig. 2).

Recombinant human erythropoietin activates the PI3K/Akt pathway to improve the morphology of ageing long-term cultured nerve cells

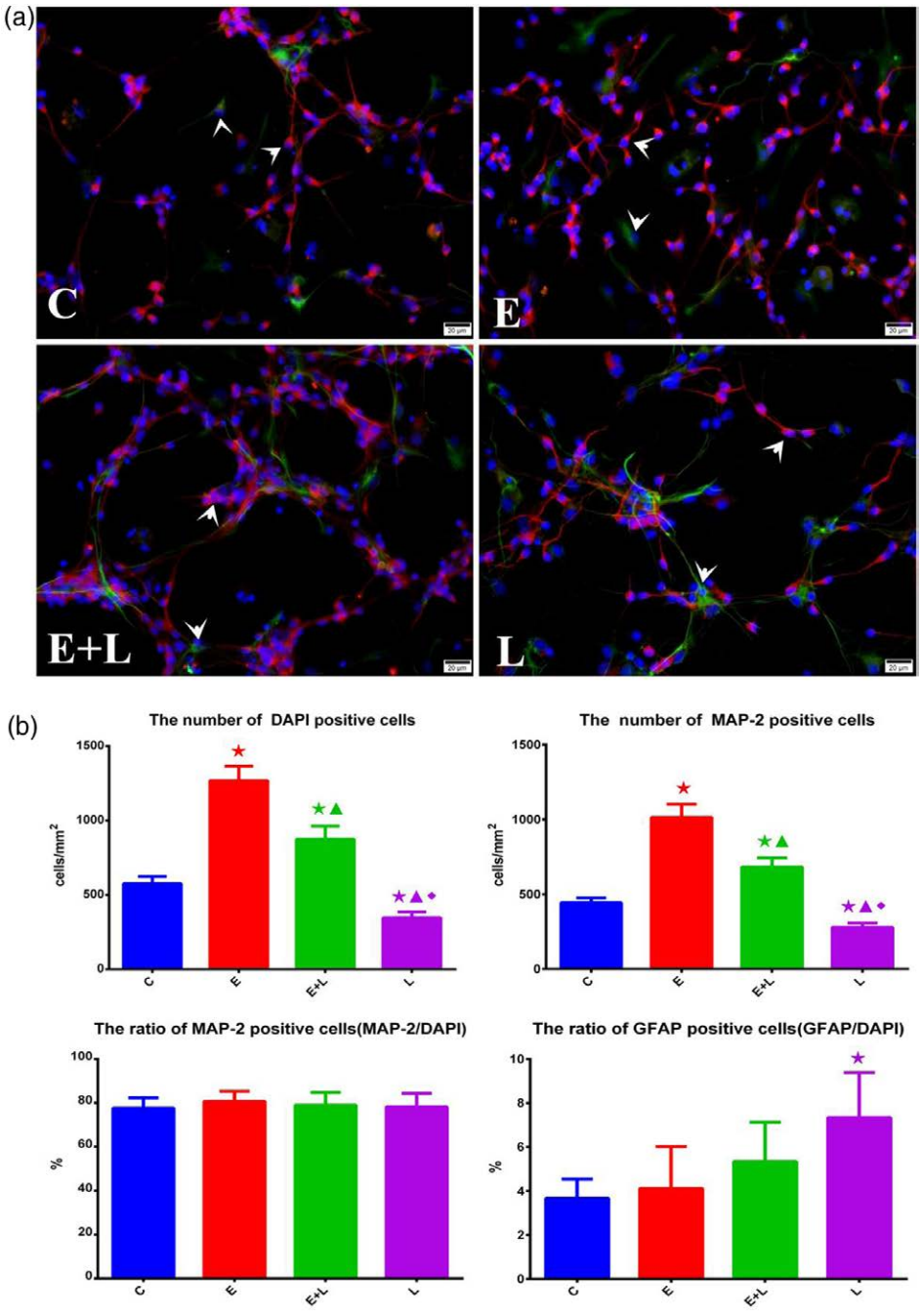
Morphological changes in nerve cells are a manifestation of cell senescence. To observe whether rhEPO can improve the morphology of ageing long-term cultured nerve cells by activating the PI3K/Akt pathway, we used an inverted phase-contrast microscope to observe the changes in the morphology of nerve cells in each group after the intervention with rhEPO and LY294002. The results showed that in the control group, senescent neuronal cells degenerate, some cells are clustered together, and the neural network is sparse. The number of neuronal cells in the EPO group increased significantly, the cell body was relatively intact, and the neural network was relatively well developed. The neural network in the EPO + LY294002 group was sparser than that in the EPO

Fig. 3



Changes in the morphology of long-term cultured ageing primary nerve cells after different treatments, as observed under an inverted microscope (a: scale bar = 50 μm; b: ×4 magnification of the part in the red box of artwork a). The red arrow indicates senescent long-term cultured primary neuronal cells. Representative images of each group are shown. In the control group, senescent neuronal cells degenerate, some cells are clustered together, and the neural network is sparse. The number of neuronal cells in the EPO group increased significantly, the cell body was relatively intact, and the neural network was relatively well developed. The neural network in the EPO + LY294002 group was sparser than that in the EPO group. Most of the neuronal cells in the LY294002 group accumulated into clusters, the cells were degenerated, and the neural network was extremely sparse. C, control group; E, EPO group; E + L, EPO + LY294002 group; EPO, erythropoietin; L, LY294002 group.

Fig. 4



Changes in the count and purity of senescent long-term cultured nerve cells in each group (immunofluorescence double-labelled staining). (a) Changes in the morphology of long-term cultured primary nerve cells in various groups as observed by immunofluorescence microscopy (scale bar = 20 μm). Neuronal marker MAP-2 staining: the cytoplasm of positively stained cells fluoresced red. Glial cell marker GFAP staining: the cytoplasm of positively stained cells fluoresced green. DAPI: counterstained the nuclei fluoresced blue and represented the total number of cells. Representative images of each group are shown. (b) Statistical analysis of the ratio of DAPI-positive cells of each group to MAP-2-positive cells and GFAP-positive cells. Data are shown as mean ± SD values obtained from three separate experiments. Statistical analysis was performed according to one-way ANOVA. The results showed that the total number of cells and nerve cell counts were significantly higher after EPO intervention than those of the control group, whereas the total number of cells and nerve cell counts were significantly reduced after LY294002 intervention. There was no significant difference in the ratio of nerve cells in each group, and they were maintained at about 80%. The proportion of glia increased with both EPO and LY294002 intervention, but only the LY294002 group was statistically significant. ★$P < 0.05$ vs. control group, ▲$P < 0.05$ vs. EPO group, ◆$P < 0.05$ vs. EPO + LY294002 group. ANOVA, analysis of variance; C, control group; DAPI, 4′6-diamidine-2′-phenylindole dihydrochloride; E, EPO group; E + L, EPO + LY294002 group; EPO, erythropoietin; GFAP, glial fibrillary acidic protein; L, LY294002 group.

group. Most of the neuronal cells in the LY294002 group accumulated into clusters, the cells were degenerated, and the neural network was extremely sparse (Fig. 3).

Recombinant human erythropoietin activates the PI3K/Akt pathway to increase the count of ageing long-term cultured nerve cells

The number of senescent, long-term cultured nerve cells decreased significantly with increasing culture time. To observe whether rhEPO increased the number of senescent, long-term cultured nerve cells through the PI3K/Akt pathway, we performed immunofluorescence double-labelling staining to count the cells in each group after the intervention with rhEPO and LY294002 (Fig. 4a). The results showed that the total number of cells and nerve cell count in the EPO group were significantly increased compared with the control group (DAPI: $P < 0.001$ vs. control group; MAP-2: $P < 0.001$ vs. control group); the total number of cells and nerve cells in EPO + LY294002 group was significantly lower than those in EPO group (DAPI: $P = 0.007$ vs. EPO group; MAP-2: $P = 0.0057$ vs. EPO group), but still significantly higher than those in the control group (DAPI: $P = 0.0069$ vs. control group; MAP-2: $P = 0.0032$ vs. control group). The total number of cells and the number of nerve cells in the LY294002 group were significantly lower than those in the other three groups (DAPI: $P = 0.0028$ vs. control group, $P < 0.001$ vs. EPO group, $P < 0.001$ vs. EPO + LY294002 group; MAP-2: $P = 0.0012$ vs. control group, $P < 0.001$ vs. EPO group, $P < 0.001$ vs. EPO + LY294002 group). The ratio of nerve cells in each group was maintained at approximately 80%, with no statistical difference. The proportion of glia ($GFAP^+$ cells) increased with both EPO and LY294002 intervention, but only the LY294002 group was statistically significant ($P = 0.0471$ vs. control group) (Fig. 4b).

Recombinant human erythropoietin activates the PI3K/Akt pathway to reduce the apoptosis of long-term cultured ageing nerve cells

The increased proportion of apoptotic cells is an important manifestation of nerve cell senescence [1,6]. To observe whether rhEPO reduces the apoptosis of senescent, long-term cultured nerve cells through the PI3K/Akt pathway, we used Annexin V-FITC/PI dual-label flow cytometry to detect the ratio of apoptotic cells in each group after the intervention with rhEPO and LY294002 (Fig. 5). The results showed that the percentage of apoptotic cells in the EPO group was significantly lower than that in the control group ($P < 0.001$ vs. control group). The apoptosis rate in EPO + LY294002 group was significantly higher than that in the EPO group ($P = 0.0123$ vs. EPO group), but it was still significantly lower than that in the control group ($P = 0.0148$ vs. control group). The percentage of apoptotic cells in the LY294002 group was more than 70%, which was significantly higher than that in the other groups ($P = 0.0363$ vs. control group; $P < 0.001$ vs. EPO group; $P = 0.0017$ vs. EPO + LY294002 group).

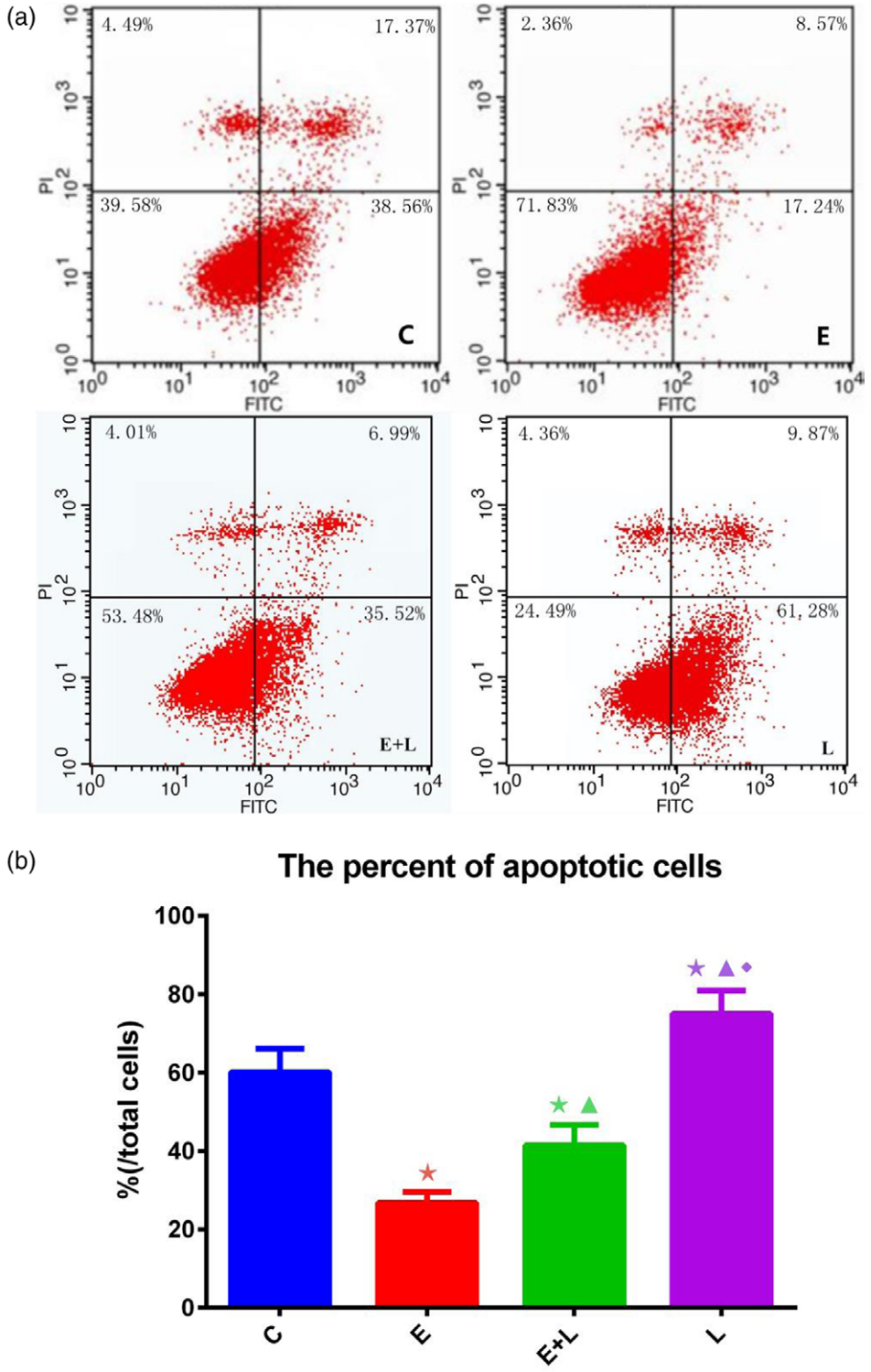
Recombinant human erythropoietin activates the PI3K/Akt pathway to reduce the ratio of senescent cells in long-term cultured nerve cells

The increase in the ratio of senescent cells directly reflects the senescence of long-term cultured nerve cells. To observe whether rhEPO reduces the ratio of senescent cells in long-term cultured nerve cells through the PI3K/Akt pathway, we used SA-β-gal staining to detect the changes in the ratio of senescent cells in each group after the intervention with rhEPO and LY294002 (Fig. 6). The results showed that the ratio of senescent cells in the EPO group was significantly lower than that in the control group ($P < 0.001$ vs. control group), whereas the ratio of senescent cells in the EPO + LY294002 group was significantly higher than that in the EPO group ($P = 0.0040$ vs. EPO group), but still lower than that in the control group ($P < 0.001$ vs. control group). The percentage of senescent cells in the LY294002 group was approximately 88%, which was significantly higher than that in the EPO group ($P < 0.001$ vs. EPO group) and EPO + LY294002 group ($P < 0.001$ vs. EPO + LY294002 group), but there was no significant difference compared to control group ($P = 0.572$ vs. control group).

Discussion

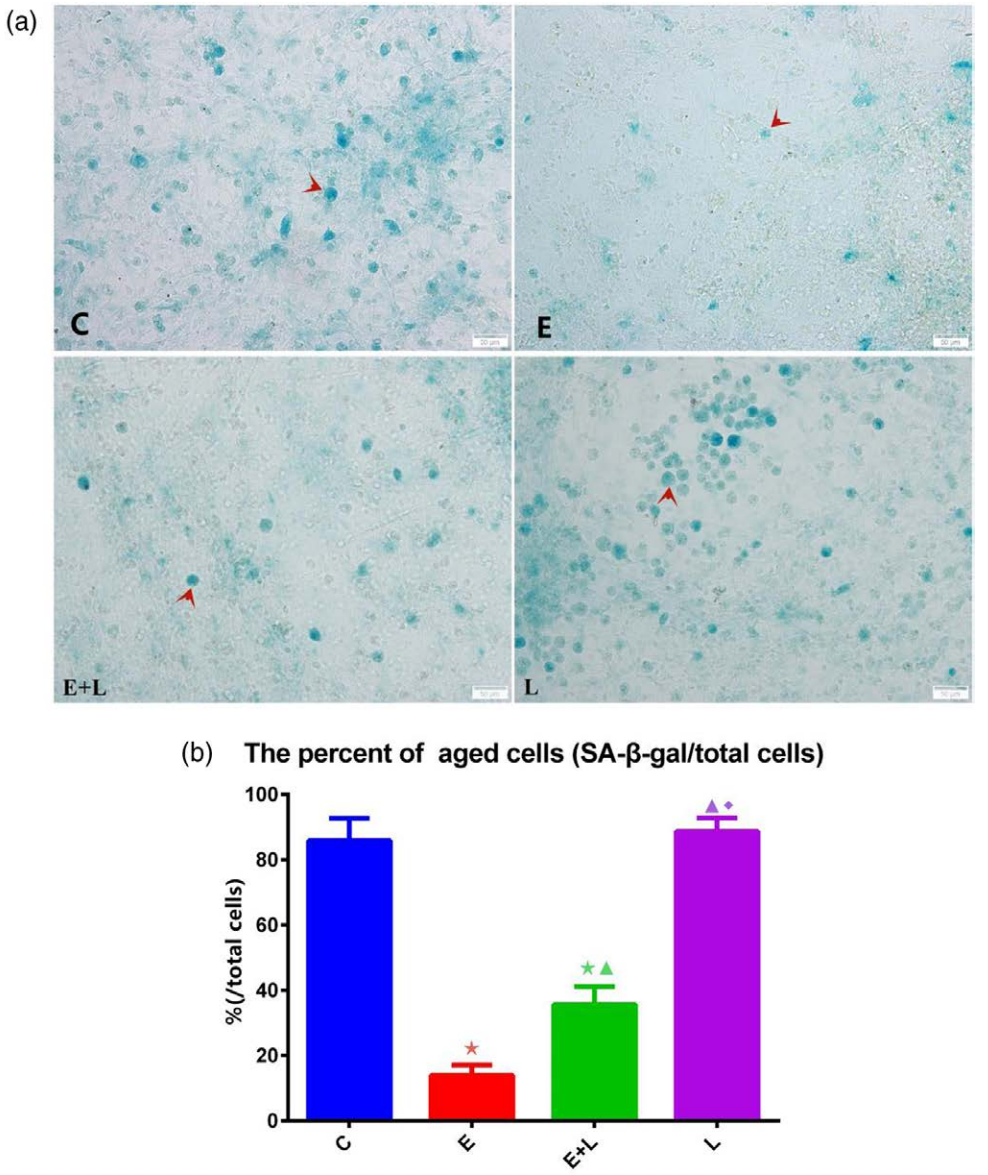
Cell culture is a good method to study ageing at the cellular level. Due to the highly differentiated state of nondividing cells of the neural cell line, ageing research based on the dividing cell model does not fully reflect the characteristics of the ageing of the nervous system, and thus ageing research based on 'neuronal culture' has become the focus of exploration. In the past 20 years, substantial progress has been achieved in the primary culture of neuronal cells. The introduction of 'Neurobasal' medium and 'B27 growth factor' has significantly prolonged the survival time of neurons *in vitro* and maintained high neuronal cell purity throughout the entire culture process [7]. A number of previous studies have used the abovementioned culture system for the primary culture of neuronal cells and found that the survival rate is still more than 50% by the 4th week of culture, whereas the proportion of astrocytes is less than 20% [7–9]. Primary neuronal cells undergo three stages of development, maturation and stability, senescence, and degeneration in long-term culture. The mitochondrial membrane potential of primary neurons entering the degenerative stage of ageing decreases, their antioxidant capacity decreases, protein carbonyls and Aβ accumulate, and the expression of apoptosis-related proteins changes. Finally, the sensitivity of neurons to cell death caused by various stresses increases [9]. This process is similar to the ageing-induced changes in neuronal cells in the central nervous system and may be a good cell model for studying the natural ageing of

Fig. 5



Apoptosis of nerve cells in each group (detected by AnnexinV-FITC/PI double-labelled flow cytometry). (a) Scatter diagram of apoptotic cell inspection; (b) statistical analysis diagram of the ratio of apoptotic cells in each group. Representative images of each group are shown. Data are shown as mean ± SD values obtained from three separate experiments. Statistical analysis was performed according to one-way ANOVA. The results showed that the apoptotic cell ratio of long-term cultured ageing primary nerve cells after EPO intervention was significantly lower than that of the control group, whereas the apoptotic cell ratio increased significantly after LY294002 intervention. ★$P < 0.05$ vs. control group, ▲$P < 0.05$ vs.EPO group, ♦$P < 0.05$ vs. EPO + LY294002 group. ANOVA, analysis of variance; C, control group; E, EPO group; E + L, EPO + LY294002 group; EPO, erythropoietin; L, LY294002 group; PI, propidium iodide.

Fig. 6



Senescence of nerve cells in each group. (a) Aged cells in long-term cultured primary nerve cells in each group as observed under an ordinary light microscope (scale bar = 50 μm). SA-β-gal immunohistochemical staining; SA-β-gal-positive cells that were stained blue represent aged cells. Representative images of each group are shown. (b) Statistical analysis of the proportion of SA-β-gal-positive cells in each group. Data are shown as mean ± SD values obtained from three separate experiments. Statistical analysis was performed according to one-way ANOVA. The results showed that the ratio of senescent cells in the long-term cultured primary neural cells after EPO intervention was significantly lower than that in the control group, whereas the ratio of senescent cells was significantly increased after LY294002 intervention. ★$P < 0.05$ vs. control group, ▲$P < 0.05$ vs. EPO group, ♦$P < 0.05$ vs. EPO + LY294002 group. ANOVA, analysis of variance; C, control group; E, EPO group; E + L, EPO + LY294002 group; EPO, erythropoietin; L, LY294002 group.

the nervous system. However, reports on the time points when primary neuronal cells enter the senescence and degeneration stages are not uniform, and they are considered closely related to the cell source, medium, and culture conditions [10,11]. In the past, we used cerebral cortical neurons from 1-day-old SD rats to establish long-term cultures of primary neurons in the Neurobasal + B27 culture medium. Dynamic observations showed that the neural network of the primary cultured neurons was the most developed on the 10th day. The rate of apoptotic cells was 7%, and the rate of senescent cells was only 2%, consistent with the performance of mature and stable cells. Neuronal cells cultured for 22 days showed typical signs of senescence: the neural network was significantly degraded, the cell activity decreased to 50%, the rate of apoptotic cells exceeded 60%, and the rate of senescent

cells exceeded 80% [1]. This result is basically consistent with previous studies, suggesting that the cerebral cortical neuronal cells of 1-day-old SD rats entered the senescence stage after 22 days of primary culture and can be used as a model for ageing research and time nodes related to ageing.

EPO is an important glycoprotein hormone in the body. Early studies proposed that EPO is only a haematopoietic factor in the blood, and the cells from which it is secreted are renal tubular endothelial cells, liver cells, and macrophages. In recent years, animal and human neuronal cells and astrocytes have also been shown to secrete EPO [12]. It is widely distributed in the central and peripheral nervous systems. EPO binds EPOR to participate in the development of the nervous system and exerts a neuroprotective effect on pathological processes such as ischaemia and hypoxia, damage caused by exogenous toxins, and primary or secondary inflammatory reactions. Some researchers have even stated that 'EPO proposes a new concept for neuroprotection' [13]. Ageing-induced damage to the nervous system is the result of a combination of many factors. The relationship between EPO and this injury is not entirely clear. A number of previous studies have shown a decreasing trend for EPO expression in the nervous system of elderly animals and humans with increasing age [14]. Supplementation with exogenous EPO can delay the occurrence and development of neurodegenerative diseases such as Alzheimer's disease, neurodegenerative disease, amyotrophic lateral sclerosis, and Huntington's disease [15,16], reduce the oxidative stress and D-galactose-induced damage to the brain tissue in ageing rats, and improve their learning and memory functions [17]. Based on these studies, EPO potentially exerts a protective effect on ageing-induced damage to the nervous system from both positive and negative aspects. In the past, we used long-term cultures of primary nerve cells to establish a natural ageing model. At the cellular level, researchers have suggested that exogenous rhEPO delays the ageing of neuronal cells by enhancing the antioxidant capacity of cells, and it has the potential to become an anti-nervous system ageing drug, but its specific downstream mechanism remains unclear [1]. Therefore, the purpose of this study was to confirm that the PI3K/Akt pathway is one of the key pathways by which rhEPO protects senescent neuronal cells.

The PI3K/Akt pathway is an important pathway for the intracellular transduction of cell membrane receptor signals, and the phosphorylation of Akt is a sign of activation of this pathway. The activated PI3K/Akt signal transduction pathway plays a key role in the maintenance of cell survival and inhibition of apoptosis [18,19]. Several studies have shown that the PI3K/Akt signalling pathway in the nervous system plays a key role in the survival of nerve cells [20–22]. LY294002 is a specific inhibitor of the PI3K/Akt pathway. It inhibits the activation of PI3K and the phosphorylation of Akt by inhibiting the combination of ATP and PI3K-P85, thereby affecting the transduction of the P13K/Akt pathway [23,24]. LY294002 has similar PI3K/Akt inhibitory efficacy in all cells, but different cell models and different intervention methods have significant differences in tolerability [25,26]. In this study, 5 μM LY294002 was used to continuously inhibit the PI3K/Akt signal transduction pathway, and it was found that the p-Akt level in senescent long-term cultured primary neurons was significantly reduced, which achieved the effect of inhibiting this pathway. A further comparison between the control group and LY294002 group revealed that after LY294002 intervention, the long-term cultured primary nerve cells had reduced antioxidant capacity and accumulated MDA in the cells, whereas nerve cells had more obvious clusters and sparser neural networks, and the total number of cells and the number of nerve cells decreased significantly, and the ratio of apoptotic cells increased significantly. The above results showed that simply inhibiting the PI3K/Akt pathway aggravates the ageing damage of long-term cultured primary nerve cells, suggesting that, like other nerve injury models, the PI3K/Akt pathway is a protective pathway in the ageing process of nerve cells, and its mechanism may involve upregulation of internal antioxidant capacity, reduction of cell apoptosis, and so on. Therefore, it is speculated that drug intervention for the PI3K/Akt signal transduction pathway may be an effective clinical treatment for anti-neural system ageing. Furthermore, this study found that the total number of cells and neuronal cell counts in the LY294002 group were significantly reduced compared with those in the control group, whereas the proportion of GFAP-positive cells increased significantly. Elevated GFAP expression is a hallmark of the response of the central nervous system to injury. We speculated that the increase in the proportion of GFAP-positive cells after the LY294002 intervention is due to inhibition of the PI3K/Akt pathway that aggravated the damage to senescent neuronal cells and astrocytes, causing GFAP expression in the culture system to increase in response. In the next step, we will establish a mixed culture model of senescent glial cells and neuronal cells and apply relevant interventions to explore and identify specific mechanisms.

The use of specific inhibitors has shown that the EPO transduction mechanism in nerve cells is similar to that in red blood cells, and both perform biological functions through combination with EPOR [27]. It has been found that the downstream signal transduction pathways of EPOR include mainly the PI3K/Akt pathway [28], STAT 5 pathway [29], NF-κB pathway [30], and MAPK/ERK pathway [31]. In previous studies, it was found that rhEPO has a protective effect on senescent long-term cultured primary neural cells, but its cell transduction pathways needed to be studied [1]. Changes in EPOR expression with age in brain

tissues have not yet been reported. In the present study, EPOR expression was studied in a long-term cultured primary neural cell model. Significantly higher levels of EPOR and p-Akt were observed in cells at DIV10 than at DIV4. However, as the culture time increased, the EPOR and p-Akt expression levels decreased successively at DIV16 and DIV22. Western blotting was used to compare and observe the changes in the expression of EPOR and p-Akt protein in nerve cells after rhEPO intervention. The p-Akt level in the EPO group was significantly higher than that in the control group, whereas the p-Akt level in the EPO + LY294002 group was significantly lower than that in the EPO group. The results showed that rhEPO affects the activation of the PI3K/Akt pathway in senescent, long-term cultured nerve cells. To determine whether rhEPO can protect senescent long-term cultured primary nerve cells by activating the PI3K/Akt pathway, we compared and observed whether the protective effect of rhEPO on senescent long-term cultured primary nerve cells was weakened after the addition of the PI3K/Akt pathway inhibitor LY294002. The results showed that the antioxidant capacity of EPO + LY294002 group was significantly lower than that of the EPO group, the intracellular MDA was significantly increased, the total number of cells and the number of nerve cells were significantly decreased, and the ratio of apoptotic cells and senescent cells was significantly increased. This confirmed that rhEPO can enhance the antioxidant capacity of cells by activating the PI3K/Akt pathway to protect senescent and long-term cultured primary nerve cells. In the next step, we will carry out in-vivo experiments on ageing animal models to verify the research conclusion.

In addition, we found that although the antioxidant capacity and cellular senescence-related indicators of EPO + LY294002 group were significantly different from those of the EPO group, they did not reach the level of the control group. Analysis of this situation may be related to the concentration of LY294002 used. Chromatographic analysis indicated that for purified PI3K, the IC50 of LY294002 was 1.4 μM [32]. However, in different cell models, its inhibitory concentration has been reported to range from 1 to 50 μM [33–35]. The concentration of LY294002 used in this study was 5 μM, and the experimental results also showed that p-Akt was still expressed in the LY294002 group, suggesting that this experiment only partially inhibited the PI3K/Akt pathway, and rhEPO exerted a protective effect through the remaining part of the PI3K/Akt pathway. In addition to the factor of LY294002 intervention concentration, this situation may be related to the diversity of EPO's pathways of action in nerve cells. This suggests that rhEPO can activate pathways besides the PI3K/Akt pathway to play a protective role in ageing long-term cultured primary nerve cells. It needs to be confirmed by further research in the future.

Conclusion

In summary, this study explored the relationship between the rhEPO and PI3K/Akt pathways in the protection of ageing nerve cells at the cellular level and found that rhEPO protects long-term cultured ageing primary nerve cells by upregulating the PI3K/Akt pathway. These findings provide a theoretical basis and experimental evidence for the antiageing mechanism of EPO in the nervous system.

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H.W. designed the study. X.Y. supervised this study. H.W. was the project manager and funding provider. M.C. participated in several steps of the experiment: long-term culture of primary nerve cells and observations of the antioxidant capacity by immunochemical methods. Z.G. and Y.G. participated in the SA-β-gal staining, immunofluorescence staining, and western blot analysis. H.W. collected and analysed data. H.W. also participated in the whole process of experimentation and wrote the manuscript. X.Y. and H.W. revised and edited the manuscript.

Conflicts of interest

There are no conflicts of interest.

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