Original Article
Antioxidant capacities of long-range cultured neurons enhanced by erythropoietin (EPO)-regulated Nrf2 pathway

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Abstract: This study aims to investigate erythropoietin (EPO) regulate the nuclear factor-erythroid 2-related factor 2 (Nrf2) pathway on the cellular level, thus enhancing the antioxidant capacities of neurons to exert their anti-aging effects. The long-range cultured neurons were set as the natural aging cell model, the cellular morphologies, antioxidant capacities and Nrf2 expression changes of naturally aged cells, and the cells that were administrated recombinant human erythropoietin (rh-EPO) and Nrf2 pathway blockers for intervention were observed. EPO intervention could increase Nrf2 expression and Nrf2 nuclear translocation, promote the expression of CuZn superoxide dismutase (SOD), enhance cellular antioxidant capacities, reduce malondialdehyde (MDA) retention and accumulation, thus significantly improving the morphology and neuronal networks of long-range cultured neurons; while LY294002 continuously blocked Nrf2 pathway, rhEPO lost its roles of promoting the proliferation of cytoplasmic Nrf2 protein levels and nuclear translocation of Nrf2. rhEPO could promote CuZn SOD expression, enhance the antioxidant capacities of long-range cultured neurons, increase cell survival by upregulating Nrf2 pathway.

Keywords: Erythropoietin, neurons, Nrf2, long-range culture, oxidative stress

Introduction

It was currently considered that the body's free radical scavenging abilities would be weakened with aging, a series of biochemical reactions resulted from the excessive accumulation of free radicals was the main reason towards the aging of nervous system [1]. Recent studies had found that erythropoietin (EPO) could enhance the body's free radical scavenging abilities, thus exhibiting protective effects towards a variety of brain injuries, it was even considered that "EPO had proposed a new concept for neuroprotection" [2-6]. Some studies found that EPO was involved in the pathogenesis of brain development and such age-related diseases as Alzheimer's disease [7, 8]. Our previous research on aging rats found that the exogenous EPO could enhance the body's antioxidative capacities, remove the excessive free radicals, thus playing the anti-aging role towards the nervous system, but the exact mechanism was still unclear [9]. As one of the key neuroprotective pathways, Nrf2 pathway regulated the expressions of a variety of antioxidant enzymes [10, 11]. Thus, we hypothesized that: EPO might also regulate Nrf2 pathway, thus upregulating the expressions of antioxidant enzymes, enhancing body's antioxidant capacities, and playing the anti-aging effect towards the nervous system. This study intended to establish the long-range cultured neurons as the natural aging model [12], thus observed the changes of Nrf2 pathway and cellular antioxidant capacities after recombinant human erythropoietin (rh-EPO) intervention, and verified this hypothesis from the cellular level.

Materials and methods

Animals

Sprague-Dawley (SD) neonatal rats (1 d), male or female, were purchased from Laboratory Animal Center, School of Medicine, Xi'an Jiaotong University, all experiments were completed in the Experimental Center of School of Medicine, Xi'an Jiaotong University, and all

operations were in compliance with the ethics regulations of animal experiments. This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The animal use protocol has been reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) of Xi'an Jiaotong University.

Primary long-range culture of neurons

The bilateral cerebral cortexes were removed from newborn SD neonatal rats (1 d) under sterile conditions. with the assistance of stereo microscope (OLYMPUS, Japan), the vascular membranes and the cerebral pia maters were removed, followed by washing with DMEM-high glucose medium, cutting into pieces and digestion with 0.125% trypsin, after filtrated, the single cell suspension was prepared (DMEM-high glucose 80 ml, FBS 20 ml, HEPES 10 mM, L-glutamine 1 mM, glucose 8 mM, Sodium pyruvate 1 mM, Penicillin-Streptomycin 100 mg/L), thus diluted (1 × 10^5 cells/ml) and inoculated 0.5 ml into 24-well polylysine-coated plates [12]. The inoculated 24-well plates were placed into an incubator (37°C, 5% CO_2), 12 hours later, the whole amount was changed to neuron culture medium (DMEM/F12 98 ml, B27-OA 2 mL, L-glutamine 1 mM, Penicillin-Streptomycin 100 mg/L), which was then semi-amount changed every 3 d.
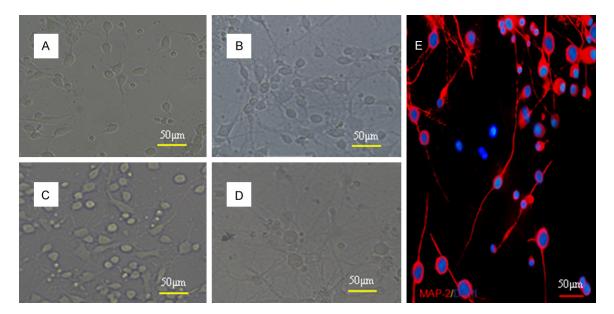
Grouping

After 7-day culture (DIV7), the cells entered the young mature phase, and entered the aging period after 20-day culture (DIV20) [13, 14]. DIV7 was set as the standard indicators of young phase, while DIV20 was set as the indicators of early aging phase, and DIV30 was set as the terminal indicators of aging. Natural aging group (N): did not give the drug intervention. EPO group (E): added rhEPO (100 U/ml) within DIV7 medium [10] (Shenyang Pharmaceutical Co.). LY294002 + EPO group (L + E): added 5 μM LY294002 within DIV7 [15] (Cell Signaling Technology Inc., USA), then rhEPO (100 U/ml) 1 hour later. LY294002 group (L): added 5 μM LY294002 within DIV7.

Staining of neurons

The cells were administrated in order: 4% paraformaldehyde (30°C, 10 min) for fixation, 0.3%

Triton X-100 (30°C, 10 min) for permeability and 5% BSA (30°C, 30 min) for closure. After that, the rabbit anti-rat Microtubule Associated Protein 2 (MAP2) antibody (1:100, Abcam, UK) was incubated at 4°C overnight, and added goat anti-rabbit IgG-CY3 antibody (1:200, Abcam, UK) 24 hours later and incubated at room temperature for 1 h. The neuronal nuclei were re-stained with 2-(4-Amidinophenyl)-6-indolecarbamidine dihydrochloride (DAPI) (1:1000, Shanghai Beyotime biotechnology Co., Shanghai, China) for 5 min, which was then observed with inverted fluorescence microscope (Ti-E, Nikon, Japan). The blue fluorescence-stained nuclei inside the specimens were observed under UV excitation light (Ex 358 nm, Em 461 nm), and other excitation light wavelengths were converted towards the same field of view to observe the red fluorescence-stained neurons (Ex 540 nm, Em 568 nm); five view fields of each specimen were selected, observed and photographed; and each photo was randomly selected 3 view fields for the cell counting; the proportion of red fluorescent cells was calculated by (the positive cells/total cells × 100%).

Western blot detection

The Radio Immunoprecipitation Assay (RIPA) lysis buffer (Wolfson, China, Xi'an) was used to split cells and extract the proteins, the trace NanoDrop spectrophotometer (NanoDrop, USA) was then used to measure the protein concentrations. The target proteins of sample and 5 × SDS-PAGE protein loading buffer (Wolfson, China, Xi'an) were then mixed and heated in 100°C water bath for 5 min to fully denature the proteins, then cooled to room temperature and centrifuged, loaded and performed electrophoresis (80 V, constant voltage), which was terminated when the bromophenol blue indicator migrate to the appropriate location of gel. After transfected onto the film, the protein blot hybridization was performed: the rabbit anti-rat CuZn superoxide dismutase (SOD) antibody (1:2000, Abcam, UK), mouse anti-rat β-actin antibody (1:5000, Santa Cruz, USA) and rabbit anti-Nrf2 rat antibody (1:1000, Abcam, UK) were added and incubated at 4°C overnight. After rinsing, the second antibody: HRP goat anti-rabbit (1:10000, Abcam, UK) and HRP goat anti-mouse (1:5000, Santa Cruz, USA) were added for the detection of protein bands by ECL chemiluminescence, and used the gel imager

Figure 1. Primary long-range culture of rat cortical neurons (scale = 50 μm, 20 ×). Under an inverted phase contrast microscope, it could be seen that 24-hour after inoculation, the single cell suspension became wall-adherent, partial cells exhibited small processes (A); in 3rd day, the cell bodies increased, the processes became longer and thicker (B); in the 7th day, the neuronal bodies were further increased, and the processes were more coarse, wove into a network (C); in the 20th day, the neurons began degeneration, the cytoplasm exhibited tiny vacuoles, the processes became tiny, followed by nuclear condensation and cleavage, the cell grid became sparse (D); percentage of MAP-2-positive cells after 7-day culture was greater than 90% by immunocytochemistry (E). (A-D) neurons seen under an inverted phase contrast microscope. Scale = 50 μm; (E) under the fluorescence microscope, among which the red fluorescence was MAP-2 positive, and was the neurons; the blue fluorescence was DAPI-labeled nuclei.

to record the images. The chemiluminescence gel imaging system (Syngene, UK) was used for the analysis.

Statistical analysis

The results were expressed as mean ± standard deviation ($\overline{x} \pm s$), the intergroup comparison used ANOVA and Tukey test, all the data were processed by SPSS13.0, with $P < 0.05$ considered as statistical significance.

Results

Establishment of natural aging model

After separated from the brain tissues of newborn rat (1 d), the single cell suspension was inoculated and observed by an inverted phase contrast microscope, it could be seen that the inoculated single cells were uniformly round, mainly suspended and drifted inside medium, began wall-adherent 3-4 hours later, and almost completely wall-adherent 12 hours later, 24 hours later, partial cells exhibited small processes, and the cell bodies were increased than previous (**Figure 1A**). In the 3rd

day, the cell bodies increased, the processes became longer and thicker, and the cells clumped together, with processes intertwined, thus forming a sparse grid (**Figure 1B**). In the 7th day, the neuronal bodies were further increased, and the processes were coarser, wove into a network, with obvious halos around the cells (**Figure 1C**). With the incubation time extension, in the 20th day, the neurons began degeneration, the cytoplasm exhibited tiny vacuoles, the processes became tiny, followed by nuclear condensation and cleavage, and the cell grid became sparse under the inverted phase contrast microscope, and could not connect to form a network (**Figure 1D**). During this period, the neuronal morphology was clear, the cell bodies were plump, the processes were thick, and the growth was in good condition.

The cells in the 7th day were performed the immunocytochemistry with neuron-specific marker MAP-2, and the fluorescence microscopy was performed to calculate the percentage of positive cells. The results showed that: the cell bodies and the processes of MAP-2-positive cells were red, while the nuclei were blue
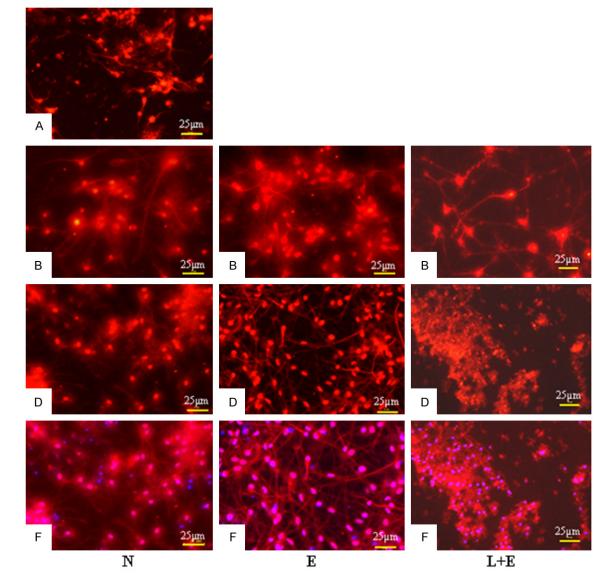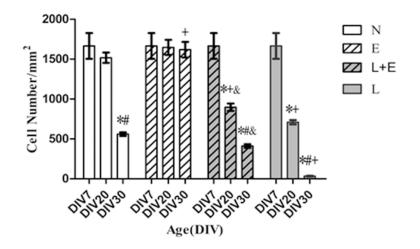
Figure 2. Morphologies of cortical neurons in different groups N. fluorensence microscope, scale = 25 μm (20 ×). A: DIV7 (20 ×); B: DIV20 (20 ×); D: DIV30 (20 ×); F: DIV30 (20 ×) (Merged image of MAP-2 and DAPI). N Group: DIV7: the neuronal bodies appeared cone-shape, coud be seen processes around, and connections could be seen among processes and between process-neurons. DIV20: the cell bodies were further increased, the processes were increased, formed the extensive contacts among processes and neurons, the neural network formed. DIV30: the cell structures existed, but the cell bodies shrank; the processes were intermittent, the structures were fuzzy, partial neuronal processes disappeared; the neural network degraded; the number of neurons was reduced. The partial neuronal nuclei appeared such manifestations of apoptosis as dense bright fluorescein gathering and scattered fluorescence debris. E Group: DIV20: compared with N group, the neuronal bodies were more full, with more rich processes, the neural network was intensively and extensively developed. DIV30: the neuronal morphology was no significant change, intact neurons could be seen embedding into the large amounts of processes-constructed neural network; the neuronal nuclei were round, with regular shapes, the blue fluorescence uniformly dispersed and distributed, while no such apoptosis-related changes as fluorescence debris and dense particles appeared. L + E Group: the neuronal morphologies were normal in DIV20, but the processes were less and partial processes were intermittent. In DIV30, the cell bodies dramatically shrank, the processes' structures were fuzzy, partial processes disappeared, and the neural network degraded; the partial nuclei had dense and bright fluorescence aggregation, and associated with multiple fragmented fluorescence.

(**Figure 1E**). Five fields of view were randomly selected to calculate the percentage of MAP-2-positive cells, the result was greater than 90%, suggesting that the neuronal purity was greater

Figure 3. Changes of neuronal numbers in group N, E, L + E and L MAP2 (+) counted the neuronal numbers ($\bar{x} \pm s_{\bar{x}}$/mm², n = 12). *P < 0.05, vs. the same group in DIV7; #P < 0.05, vs. the same group in DIV20; +P < 0.05, vs. group N at the same time point; &P < 0.05, vs. group E at the same time point. N Group: Before DIV20, the number of neurons remained relatively stable (P > 0.05). Then with the number of neurons decreased, only about 30% MAP2 (+) cells existed till DIV30 (*P < 0.05; #P < 0.05). E Group: the number of neurons remained relatively stable before DIV30 (P > 0.05). In DIV20, there was no significant difference in neuronal cells between the N and E groups (P > 0.05), when in DIV30, the number of neurons in E group was significantly higher than N group (+P < 0.05). L + E group: the number of neurons decreased with the extension of incubation time, when in DIV30, about 30% cells survived (*P < 0.05; #P < 0.05). It significantly decreased than those at the DIV20 and DIV30 in group E (&P < 0.05).

than 90%, and the neurons cultured by this method could be used for the subsequent tests.
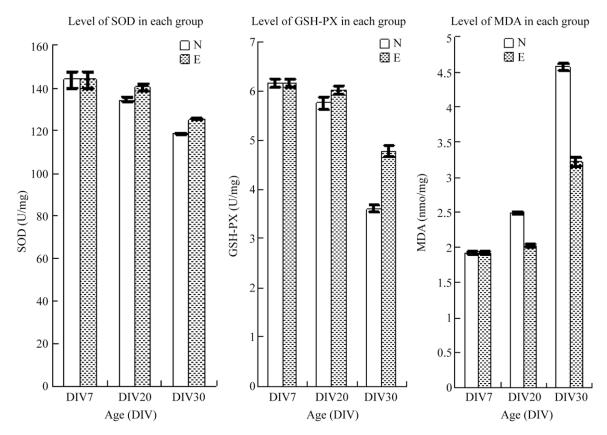
Morphological changes

Morphological observation: Natural aging group: DIV7: the neuronal bodies appeared cone-shape, could be seen processes around, and connections could be seen among processes and between process-neurons. DIV20: the cell bodies were further increased, the processes were increased, formed the extensive contacts among processes and neurons, the neural network formed. DIV30: the cell structures existed, but the cell bodies shrank; the processes were intermittent, the structures were fuzzy, partial neuronal processes disappeared; the neural network degraded; the number of neurons was reduced; after DAPI restaining, the neuronal nuclei appeared such manifestations of apoptosis as dense bright fluorescein gathering and scattered fluorescence debris (**Figure 2**). EPO group: DIV20: compared with N group, the neuronal bodies

were fuller, with more rich processes; the neural network was intensively and extensively developed. DIV30: the neuronal morphology of E group showed no significant change than the situations of DIV20 of the same group, intact neurons could be seen embedding into the large amounts of processes-constructed neural network; the DAPI re-staining revealed that the neuronal nuclei were round, with regular shapes, the blue fluorescence uniformly dispersed and distributed, while no such apoptosis-related changes as fluorescence debris and dense particles appeared (**Figure 2**).

The MAP2 (+) cells were used to count the number of neurons. Natural aging group: Before DIV20, the number of neurons remained relatively stable (P > 0.05). Then with the number of neurons decreased, only about 30% MAP2 (+) cells existed till DIV30 (P < 0.05). EPO group: the number of neurons remained relatively stable before DIV30 (P > 0.05). Comparison of neuronal numbers in Natural aging group and EPO group revealed that in DIV20, there was no significant difference in neuronal cells between the 2 groups (P > 0.05), when in DIV30, the number of neurons in EPO group was significantly higher than Natural aging group (P < 0.05), indicating rhEPO significantly inhibited the aging-associated neuronal morphological abnormalities, apoptosis and neuronal loss (**Figure 3**).

Enhanced antioxidant capacities

The neurons in Natural aging group and EPO group were selected to culture at the same time points in DIV7, DIV20 and DIV30, and then the total proteins were extracted. According to the instructions of SOD, MDA and GSH-PX assay kit (Nanjing Jiancheng Co.), the enzyme activity of SOD and GSH-PX, the content MDA within the cell proteins of each group were

Figure 4. Impacts of EPO towards SOD, MDA and GSH-PX in long-range cultured neurons. Group N: the levels of SOD and GSH-PX in DIV20 and DIV30 were decreased than DIV7 ($P < 0.05$), while the MDA levels were significantly increased ($P < 0.05$). Group E: the levels of SOD and GSH-PX in DIV20 and DIV30 of group E were significantly increased than group N ($P < 0.05$), while the MDA levels were significantly decreased ($P < 0.05$).

measured by spectrophotometry. The results showed: Natural aging group: the levels of SOD and GSH-PX in DIV20 and DIV30 were decreased than DIV7 ($P < 0.05$), while the MDA levels were significantly increased ($P < 0.05$). EPO group: the levels of SOD and GSH-PX in DIV20 and DIV30 of EPO group were significantly increased than group N ($P < 0.05$), while the MDA levels were significantly decreased ($P < 0.05$), indicating rhEPO could improve the antioxidant capacities of long-range cultured neurons and reduce the retention and accumulation of toxicants inside the aged neurons (**Figure 4**).
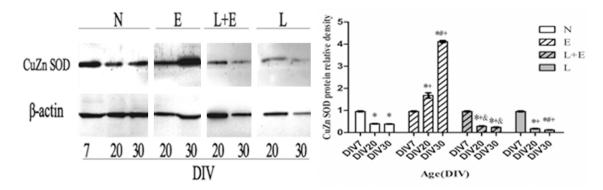
Upregulation of neuronal CuZn SOD expression

The CuZn SOD expression was detected by Western blot. The results showed: Natural aging group: the CuZn SOD expression in DIV20 was decreased by about 50% than in DIV7 ($P < 0.05$), then maintained a low level till DIV30 ($P > 0.05$). After continuous EPO treatment, the
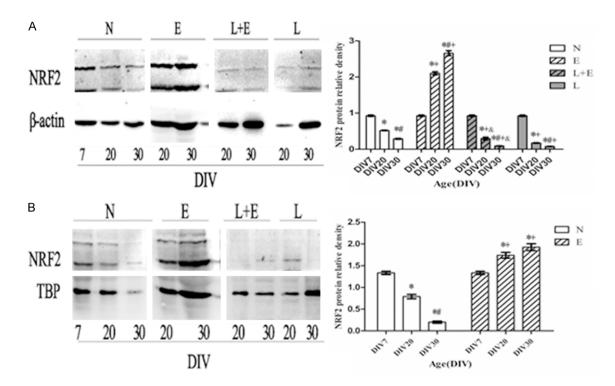
CuZn SOD expression level in EPO group was gradually increased with time going, which were increased to about 1.5 and 4 times in DIV20 and DIV30 than in DIV7 ($P < 0.05$), and significantly higher than those at the same time point in Natural aging group ($P < 0.05$) (**Figure 5**).

Upregulated Nrf2 pathway

Western blot was performed to detect the Nrf2 protein levels in cytoplasm and nucleus, the results showed: Natural aging group: the cytoplasmic Nrf2 protein level decreased with the extension of incubation time, which dropped to about 50% of DIV7 when in DIV20, and about 30% in DIV30 ($P < 0.05$); Nrf2 had a similar downward trend in nucleus, which dropped to approximately 50% and 15% of DIV7 when in DIV20 and in DIV30 ($P < 0.05$). EPO group: the cytoplasmic Nrf2 protein level was gradually increased, which reached more than 2-fold and 2.5-fold of DIV7 when in DIV20 and in DIV30, respectively ($P < 0.05$); higher than those at the same time points in Natural aging group ($P <

Figure 5. Changes of CuZn SOD in long-range cultured neurons detected by Western blot. *P < 0.05, vs the same group in DIV7; #P < 0.05, vs the same group in DIV20; +P < 0.05, vs group N at the same time point; &P < 0.05, vs group E at the same time point. Group N: the CuZn SOD expression in DIV20 was decreased by about 50% than in DIV7 (*P < 0.05), then maintained a low level till DIV30 (P > 0.05). Group E: the CuZn SOD expression level was gradually increased with time going, which were increased to about 1.5 and 4 times in DIV20 and DIV30 than in DIV7 (*P < 0.05), and significantly higher than those at the same time point in group N (+P < 0.05). Group L + E: the CuZn SOD expression in fell to 30% of DIV7, which subsequently remained the low expression till DIV30 (*P < 0.05; #P < 0.05); compared with those at the same time points of group E, its expressions were significantly decreased (&P < 0.05).

Figure 6. Impacts of rhEPO towards Nrf2 protein levels in long-range cultured neurons detected by Western blot. *P < 0.05, vs. the same group in DIV7; #P < 0.05, vs. the same group in DIV20; +P < 0.05, vs. group N at the same time point; &P < 0.05, vs. group E at the same time point. A: The cytoplasmic Nrf2 protein level: Group N: the cytoplasmic Nrf2 protein level decreased with the extension of incubation time, which dropped to about 50% of DIV7 when in DIV20, and about 30% in DIV30 (*P < 0.05); Group E: the cytoplasmic Nrf2 protein level was gradually increased, which reached more than 2-fold and 2.5-fold of DIV7 when in DIV20 and in DIV30, respectively (*P < 0.05); higher than those at the same time points in group N (+P < 0.05); L + E Group: the cytoplasmic Nrf2 level declined with aging (*P < 0.05; #P < 0.05); about 13% and 4% of those in group E in DIV20 and DIV30 (&P < 0.05). B: Nuclear Nrf2 protein level: Group N: dropped to approximately 50% and 15% of DIV7 when in DIV20 and in DIV30 (*P < 0.05); Group E: the nuclear Nrf2 level was increased to about 1.5 times of DIV7 when in DIV20 (*P < 0.05), then maintained that level till DIV30 (P > 0.05); compared with those at the same time points in group N, the nuclear protein levels were significantly increased (+P < 0.05). L + E group: only showed faint protein bands.

0.05); the nuclear Nrf2 level was increased to about 1.5 times of DIV7 when in DIV20 ($P <$ 0.05), then maintained that level till DIV30 ($P >$ 0.05); compared with those at the same time points in Natural aging group, the nuclear protein levels were significantly increased ($P <$ 0.05) (**Figure 6**).

LY294002 significantly inhibited the protective effects of rhEPO

Cellular immunofluorescence morphological observation: LY294002 + EPO group: the neuronal morphologies were normal in DIV20, but the processes were less and partial processes were intermittent. In DIV30, the cell bodies dramatically shrank, the processes' structures were fuzzy, partial processes disappeared, and the neural network degraded; DAPI re-stained nuclei exhibited that partial nuclei had dense and bright fluorescence aggregation, and associated with multiple fragmented fluorescence (**Figure 2**).

MAP2 (+) cell counting: LY294002 + EPO group, the number of neurons decreased with the extension of incubation time, when in DIV30, about 30% cells survived (L + E Group: DIV7 vs. DIV20, $P <$ 0.05; DIV30 vs. DIV20, $P <$ 0.05). The EPO group showed no significant changes in neuronal morphology and number in DIV30 when compared with those in DIV7, but significantly increased than those at the same time point in LY294002 + EPO group (DIV20: E vs. LE, $P <$ 0.05; DIV30: E vs. LE, $P <$ 0.05) (**Figure 3**).

Detection of intracellular CuZn SOD expression by Western blot analysis showed in DIV20, the CuZn SOD expression in LY294002 + EPO group fell to 30% of DIV7, which subsequently remained the low expression till DIV30 (L + E: DIV7 vs. DIV20, $P <$ 0.05; DIV20 vs. DIV30, $P <$ 0.05); compared with those at the same time points of EPO group, its expressions were significantly decreased (DIV20: E vs. L + E, $P <$ 0.05; DIV30: E vs. L + E, $P <$ 0.05) (**Figure 5**).

Western blot analysis revealed that: LY294002 + EPO group: the cytoplasmic Nrf2 level declined with aging (L + E: DIV7 vs. DIV20, $P <$ 0.05; DIV20 vs. DIV30, $P <$ 0.05); about 13% and 4% of those in EPO group in DIV20 and DIV30 (DIV20: E vs. L + E, $P <$ 0.05; DIV30: E vs. L + E, $P <$ 0.05) (**Figure 6**). The test results of

nuclear Nrf2 showed: LY294002 + EPO group only showed faint protein bands (**Figure 6**). These results suggested that after LY294002 continuously blocked Nrf2 pathway, rhEPO lost its roles of promoting the proliferation of cytoplasmic Nrf2 protein levels and nuclear translocation of Nrf2.

Discussion

EPO was an important glycoprotein hormone in vivo, involved in the development of nervous system, and played the neuroprotective roles in a variety of pathological lesions [2-4, 6]. Detection of EPO expression in different brain regions of older animals and EPO activities in cerebrospinal fluid of elderly human suggested it occurred the age-related declining [16, 17]. Exogenous EPO supplements could delay the development of such neurodegenerative diseases as Alzheimer's disease (AD), Parkinson's disease (PD), amyotrophic lateral sclerosis (ALS), Huntington's disease (HD) and others [4, 7], reduce the oxidative stress levels in D-galactose-induced aged rats, and improve their learning and memory functions [9], but it still lacked the evidence from the cellular level.

The numbers and morphology of long-range cultured neurons could maintain relatively stable in their development and maturity periods, while the number of survived cells might decline after entering aging period, and the declining met the Gompertz survival model [18]. With the extension of culture time, the mitochondrial membrane potential was decreased, reactive oxygen species (ROS) was increased, while the glutathione (GSH) expression was reduced, the protein carbonyl and Aβ became accumulated, and the expressions of apoptosis-related proteins were increased [19, 20]. These changes were similar to those of central nervous system in aging. This study found that long-range cultured neurons exhibited age-related changes in morphology and number when cultured to DIV20, suggesting that the long-range cultured neurons were the good model to simulate the natural aging of nervous system. After given rhEPO for the continuous intervention, the neuronal morphologies, quantities and the neural network could maintained at a relatively stable state at least till DIV30. Thus, rhEPO had protective effects towards natural aging neurons on the cellular level.

The radical theory considered that aging was the cellular damages caused by oxidative stress-resulted MDA retention. Increased ROS production and decreased functions of such antioxidant defense repairing system as SOD, enzyme catalase (CAT), Gpx and glutathione peroxidase (GSH-PX) would lead to the occurrence of oxidative stress. The results of this study showed: the SOD and GSH-PX levels in naturally aged neurons in DIV20 and DIV30 were significantly decreased than DIV7, while the MDA levels were significantly increased, indicating that with the extension of incubation time, the levels of radical scavengers, SOD and GSH-PX, inside neurons were significantly decreased, thus their antioxidant capacities were decreased, resulting in a significant content increase of malondialdehyde, thus causing the aging damages of neurons, and this was in line with the physiological natural aging process of neurons. It was also found that after the rhEPO intervention, the SOD and GSH-PX levels in DIV20 and DIV30 were significantly higher than the naturally aged cells, while the MDA levels were significantly decreased, proving that rhEPO could improve free-radical scavenging abilities in long-range cultured neurons, enhance the antioxidant capacities in long-range cultured neurons, and reduce the accumulation of cytotoxic substances in neurons.

Mammals had three kinds of SOD: CuZn SOD was in cytoplasm and cell membrane, Mn SOD was in mitochondria, and EC SOD was in extracellular matrix. CuZn SOD was the important SOD in cells, and played an important role in aging and aging-related diseases, EPO could increase the expressions and activities of CuZn SOD, reduce the oxidative stress-induced vascular injuries [21-24]). This study found that with the extension of incubation time, the expression of intracellular CuZn SOD protein was decline, down to 50% of DIV7 in DIV20, and then maintained low expression until DIV30; after continuous treatment of rhEPO, the CuZn SOD expression levels did not appear the aging-related decreasing, while increased to about 1.5 times of DIV7 when in DIV20, even further increased to four times of DIV7 in DIV30. Thus, increasing the expression of CuZn SOD in neurons was an important way for rhEPO to enhance the antioxidant capacities of long-range cultured neurons.

Dysfunction of antioxidant defense repairing system was an important feature of aging. the

basic and inducible expressions of various antioxidant enzymes (AOEs), such as CAT, CuZn SOD and Human heme oxygenase 1 (HO-1) and others, were subject to regulation of transcription factor Nrf2. study towards Nrf2 in aging process found: Nrf2 transcription, intracellular protein level, nuclear translocation and binding with Antioxidant response element (ARE) were all declined with aging process, the above results were confirmed in myocardial tissues, liver tissues and nerve tissues [25]. The observation towards in vitro long-range cultured astrocytes found that in DIV60, the total intracellular Nrf2 protein levels were decreased to 70% of DIV14 and 50% of DIV30 [26]; and the declining of Nrf2 protein levels had a similar trend with those of AOEs expressions; the Nrf2 gene-knockout could significantly shorten the lifetime of transgenic animals, and significantly inhibit the effects of lifespan extension by caloric restriction [27]. Our study towards in vitro long-range cultured neurons obtained the similar results: the Nrf2 expressions in cytoplasm and nuclei of naturally aged neurons showed decreasing with aging: in DIV20, the cytoplasmic and nuclear Nrf2 were decreased to 50% of DIV7; and in DIV30, only 30% cytoplasmic Nrf2 and 15% nuclear Nrf2 existed.

Genc et al. [10] found the continuous action of EPO for 24 hours could increase the Nrf2 nuclear translocation of SH-SY5Y under non-stress conditions, but would not increase the Nrf2 transcription. While it was found in TBI, SAH or ischemic stroke models that the neuroprotective effects of EPO were related with the increased Nrf2 transcription, maintaining its protein stability and promoting its nuclear translocation [11, 28, 29]. This study found that rhEPO almost completely inhibited the decreasing of Nrf2 in long-range cultured neurons; on the contrary, in DIV20 and DIV30, the cytoplasmic and nuclear Nrf2 proteins were significantly higher than in DIV7. The results suggested that rhEPO could not only promote the Nrf2 nuclear translocation in long-range cultured neurons, but also increase the Nrf2 expression in whole cells. It might be related with reducing the Nrf2 degradation and increasing its stability. But also it was possible that EPO promoted Nrf2 transcription. We would confirm in our late studies through inhibiting proteasome-dependent degradation or inhibiting protein synthesis by MG132 and cycloheximide, etc.

Although studies had suggested that the Nrf2 activities decreased in senescent cells, the administration of cytoprotective agents could still induce the Nrf2 nuclear translocation and the expression of downstream genes. Bergström used sulforaphane to maintain the sustained activation of Nrf2 in astrocytes, and found the accumulative expression increasing of downstream NAD(P)H: quinoneoxidoreductase (NQO1)-mRNA and its protein [30]. This study found that after the rhEPO intervention, as the downstream gene regulated by Nrf2 pathway, the expression of CuZn SOD was increased significantly, and accumulated. It was thus reasoned that rhEPO might increase intracellular overall Nrf2 protein levels and increase its nuclear translocation in long-range cultured neurons, thus maintaining the high intracellular expression of CuZn SOD, and prolonging the occurrence of aging.

LY294002 could block Phosphoinositide-3-kinase/Protein Kinase B (PI3K/Akt) signaling pathway, thus blocking the regulatory roles of Nrf2 [3, 15, 26, 29, 31]. In this study, 5 μM LY294002 was given for the continuous intervention in DIV7 during the culture process of long-range cultured neurons [15], and achieved the sustained suppression of Nrf2 upregulation in long-range cultured neurons while most neurons survived. To ensure the blocking effects of LY294002, when the medium was changed every time, LY294002 pretreatment was given 1 hour before the rhEPO treatment. The results showed that compared to group E, in DIV20 and DIV30, L + E group exhibited significant decreasing of cytoplasmic and nuclear Nrf2, the downstream CuZn SOD also showed the similar expression changes. Observation towards neuronal morphologies and quantities found that L + E group exhibited process reduction, neural network degradation and other aging-related changes in DIV20, the number of neurons also decreased; in DIV30, most processes shrank, accompanied by the cell body shrinkage, as well as the formation apoptotic bodies and chromatin debris, the neuronal numbers also dropped to about 30% of DIV7. While the group E exhibited relatively stable neuronal morphologies and numbers before DIV30. The results confirmed that rhEPO could indeed upregulate Nrf2 pathway, thus promoting the CuZn SOD expression, enhancing the antioxidant capacities of long-range cultured neurons, and delaying cellular senescence.

Disclosure of conflict of interest

None.

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