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Original Article

Erythropoietin suppresses D-galactose-induced aging of rats via the PI3K/Akt/Nrf2-ARE pathway

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Abstract: EPO (erythropoietin) is a hormone-like substance with a putative role in hematopoietic regulation. Current research suggests that it exerts a neuroprotective effect by enhancing the activity of antioxidant enzymes. Our previous studies in vitro have confirmed that EPO can delay senescence of cultured neurons by activation of nuclear factor-erythroid 2-related factor 2 (Nrf2) and the phosphoinositide-3-kinase (PI3K)/AKT pathway. Thus we set out to further substantiate the mechanism in vivo. A rat model of aging was induced by continuous subcutaneous injection of 5% D-galactose for 6 weeks. Starting at the 7th week, physiological saline or EPO was administered twice daily. LY294002, an inhibitor of the PI3K/AKT pathway, was also given to one of the groups. Improvement of learning and memory abilities were observed in the EPO intervention group. Raised levels of Cu-Zn SOD protein were detected by immunohistochemical staining and Western blot after using EPO, together with increased expression of PI3K/AKT pathway proteins. Concomitantly, there was an increase in expression of Nrf2 mRNA and a decrease in expression of Keap1 mRNA by qRT-PCR. All these effects were not found in the group injected with LY294002. We conclude that EPO can suppress aging by reducing oxidative stress. The proposed mechanism is an upregulation of the PI3K/Akt/Nrf2-ARE pathway and thus maintenance of expression and activation of antioxidant enzymes in aging rats.

Keywords: EPO, PI3K/Akt, p-Akt, Nrf2, Cu-Zn SOD, anti-aging effects

Introduction

Aging is an inexorable law of life, which is usually thought of as the result of an accumulation of molecular and cellular damage under the action of various factors, leading to structural damage as well as functional decline and, ultimately, increasing the risk of disease and death. Causes of aging include but are not limited to oxidative stress, glycation, telomere shortening, side reactions, mutations, aggregation of proteins and so on [1]. The theory of free radical damage is a widely accepted mechanism of aging [2, 3] stating that free radicals or reactive oxygen species (ROS) excessively generated under oxidative stress can lead to cell and tissue damage paralleled by alterations in the function of genetic apparatus, resulting in untimely cell death and aging.

D-galactose is a naturally-occurring reducing sugar in animal body, which can be completely metabolized at physiological concentrations. However, the D-galactose in a supraphysiological concentration may convert to aldose, hydrogen peroxide, and galactose oxidase, serving as a catalyst, resulting in the generation of superoxide anion and oxygen-derived free radicals [4]. Studies reported that injected with d-galactose for 6 to 10 weeks, rodents showed progressive increases in oxidative stress, which led to the same result as natural aging [5]. Therefore, continuous subcutaneous injection of D-galactose is generally used to induce the experimental model of aging.

EPO is a hematopoietic cytokine synthesized in the kidney, involving in hematopoietic regulation. In recent years, EPO has been proven to have broad neuroprotective effects [6-8]. Researchers have found the use of exogenous EPO could enhance the learning and memory ability of rats [9]. Our previous study also showed that EPO played a role in anti-aging activities by increasing the activity of antioxidant enzymes and scavenging free radicals

[10]. The Nrf2/ARE/Cu-Zn SOD pathway is one of the important signal pathways in intracellular oxidative stress regulation [11-13], while PI3K and Akt are important upstream kinases that regulate the expression of Nrf2 [14]. Nrf2 is an important transcription factor in mammals, which can bind to the specific recognition site GCTGAGTCA on the ARE, thereby regulating the expression of several antioxidant enzymes and phase II detoxification enzymes at the gene level [15]. Keap1 is a negative regulator of Nrf2. Under physiological conditions, Nrf2 and Keap1 interact with each other to form a complex. Keap1 can also recognize and attach to the ubiquitin-protein ligase, facilitating the degradation of Nrf2 via ubiquitination [16]. When an organism is confronted with oxidative stress, the combining capacity of Keap1 declines, thus Nrf2 can dissociate from Keap1, and translocate into the nucleus to active ARE. This process finally promotes the transcription of downstream genes that encode the antioxidant enzymes [11]. LY294002 (2-(4-morpholinyl)-8-phenyl-chromone) is a commonly used pharmacological inhibitor that selectively inhibits the PI3K-Akt pathway, downregulating the expression of the downstream molecules.

Our previous studies have confirmed that EPO plays a part in anti-aging by activating the Nrf2-ARE pathway. In this study, we further explored the anti-aging mechanistic effects of the PI3K/Akt/Nrf2-ARE signalling pathway and how EPO affects this pathway by in vivo experiments.

Materials and methods

Materials and reagents

D-galactose was purchased from Sigma. rhEPO for injection was obtained from Shenyang Sansheng Pharmaceutical Company (Shenyang, China). LY294002 was obtained from American Cell Signaling Technology. Immunohistochemical antibodies were obtained from Abcam, Santa Cruz, ABgent and Wuhan Boster (Wuhan, China). PVDF membranes (0.45 μm) were purchased from Millipore. The SDS-PAGE gel configuration kit was purchased from Xi'an Woersen Company (Xi'an, China).

Animals

Ninety 2-month-old Sprague Dawley rats (280±10 g weight) were obtained from the Experimental Animal Center of the Medical College of Xi'an Jiaotong University (SCXK (shan) 2013-002). The rats were housed in a standard environment (22±2°C with 55±5% controlled humidity and a 12-h dark/light cycle) with free access to food and water. The animal experimental protocols used in this study were performed in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health and approved by the Institutional Animal Care and Use Committee (IACUC) of Xi'an Jiaotong University.

Establishment of aging model

The rats were randomly divided into 5 groups (n=18), including young control group (Control), D-galactose induced mimetic ageing model group (D-gal), EPO intervention aging group (EPO), EPO and LY294002 intervention aging group (EPO+LY294002), LY294002 intervention aging group (LY294002). The rats of D-gal group, EPO group, EPO+LY294002 group and LY294002 group were subcutaneously injected with 150 mg/kg of 5% D-galactose daily for 6 weeks to establish the aging model. The rats of the young control were similarly administered with saline (2.5 ml/kg) for 6 weeks.

Intracerebroventricular injections

The aging and control rats were anesthetized by intraperitoneal injection of 10% chloral hydrate (350 mg/kg). The fontanelle of each rat was exposed, and a cannula was implanted into the lateral ventricle. Stereotaxic coordinates were -1.1 mm antero-posterior, -1.5 mm medio-lateral, -4.5 mm dorso-ventral from bregma. After the operation, the rats were fed separately and injected with 80,000 IU penicillin daily for 3 days. The animals were given 3 days to recover after surgery, before the injection protocol.

Then via the cannula, EPO or saline were injected into the lateral ventricle by a 10 μl microsyringe fitted with a 33-gauge needle and an automated syringe pump at 0.5 μl/min. For the control group and D-gal group, 10 μl physiological saline was administrated. 10 μl EPO (10 IU/ml) was injected to the rats of EPO group. 5 μl LY294002 (5 μM) was injected to the rats of LY294002 group. For the EPO+LY294002 group, 5 μl LY294002 was administered, and 10 μl EPO was injected 30 min after. The medicines were delivered twice daily for a total of 7 days.

Table 1. Escape latent period and cross platform times of rats

Group	Escape latent	Number of platform crossings
Control	12.61±4.33	1.83±0.75
D-gal	20.82±8.57*,▲	0.33±0.52*,▲
EPO	9.14±4.89	1.50±1.05
EPO+LY294002	26.41±12.47*,▲	0.20±0.45*,▲
LY294002	28.11±8.12*,▲	0.30±0.13*,▲

Note: *P<0.05 *vs* Control group, ▲P<0.05 *vs* EPO group.

Learning and memory ability test

The Morris water maze was used to assess spatial learning and memory capabilities of rats. The maze consisted of a large stainless circular pool (100 cm in diameter and 40 cm in height) which was filled with water (25±1°C) to a depth of 30 cm. The pool was divided virtually into four equal quadrants, and a 9-cm diameter transparent platform (escape platform) was hidden 2 cm below the surface of the water in a fixed location of the first quadrant [17]. Spatial learning-memory tests included place a navigation test and a probe test. In the test, the performance of each rat was tracked and analysed by a video-computerized tracking system (MT 200 type, Chengdu, China). In the place navigation test, the rats experienced learning trials for 5 consecutive days, twice daily. At the beginning of each trial, rats were held facing the pool wall and placed into the water from one of the four fixed entry points randomly. Each trial ended when the rat climbed on the platform within 60 s and remained on it for 10 s. If the rats failed to find the platform within 60 s, they would be guided to the platform by the experimenters and allowed to remain on the platform for 10 s [18]. The time to reach the platform (escape latency, EL) was recorded. The probe test was carried out 24 h after the last learning trail. The platform was removed, and the rats were placed in the water as described. The frequencies of passing through the former position of the platform were tested for 60 s, and were recorded as the number of platform crossings.

Hematoxylin/eosin (HE) staining

After behavioural tests, 6 rats were selected randomly from each group and anesthetized by 10% chloral hydrate. A cannula was inserted into the ascending aorta via the left ventricle after thoracotomy and phosphate-buffered saline (250 mL, room temperature) and 4% paraformaldehyde (0.01 mol/L, 250 ml, room temperature) were infused sequentially through the cannula. The auricula dextra was opened to let the liquid out and the cerebrums were quickly separated and placed in 4% paraformaldehyde for 24 hours. The specimens were dehydrated, permeabilized, and embedded in wax. Finally the specimens were sectioned serially into coronal slices as the thickness of 4 μm on a rotary microtome (Leica/CM1900, Germany). The sections were then stained with haematoxylin-eosin using standard methods, and were observed using a light microscope (Nikon/E100, Japan).

Immunohistochemistry staining

The sections were hydrated after being deparaffinized and were incubated with serum for 20 min at 37°C to block non-specific antigens. The sections were then incubated with primary rabbit anti-rat p-Akt (phosphorylated Akt) antibody (1:1000, Abcam) and rabbit anti-rat Cu-Zn SOD antibody (1:200, Abcam) respectively at 4°C overnight. Next, the sections were washed by PBS and then incubated with biotinylated sheep anti-rabbit antibody (1:200) for 40 min at room temperature. Subsequently, the sections were incubated in streptavidin marked by horseradish peroxidase for 30 min at room temperature, followed with diaminobenzidine (DAB) and haematoxylin staining solution until brown precipitates appeared under the light microscope. Images were collected and analyzed from the hippocampus CA1 region. Five different visual fields were randomly selected from each section for quantification under a fluorescence microscope (400× magnification). The number of positive cells in each section was represented by a mean number. The expression of p-Akt and Cu-Zn SOD was measured by the average integrated optical density (IOD) of positive cells using Image-ProPlus 6.0 software.

Western blot analysis

For Western blots, six rats from each group were sacrificed after anesthesia, and the hippocampus was collected. The tissues were homogenised in ice-cold RIPA lysis buffer and protease inhibitor mixture. The lysate was cen-

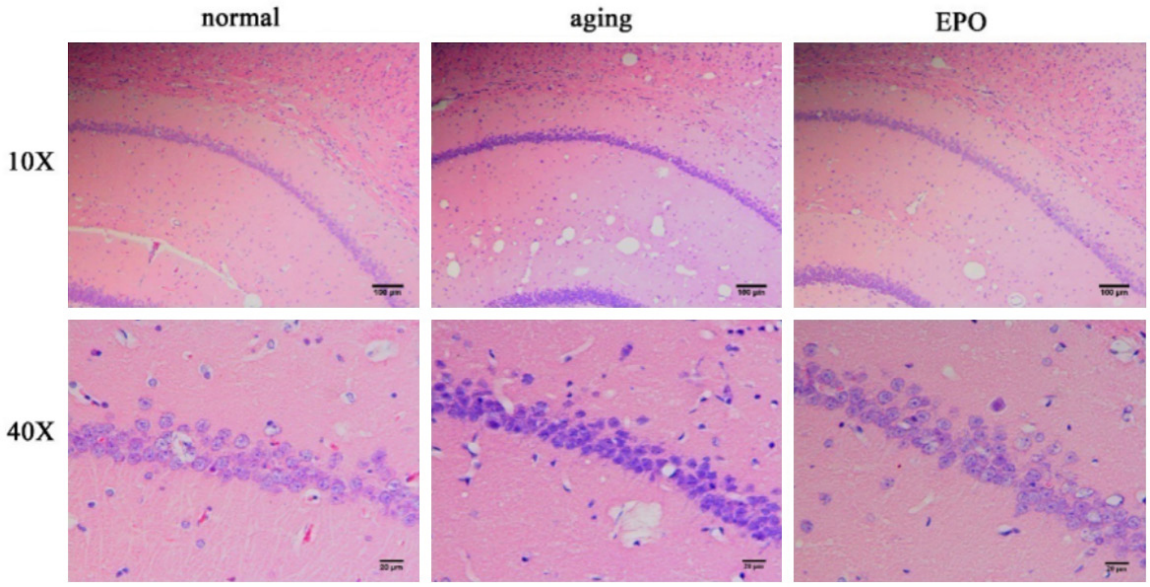

Figure 1. HE staining of the hippocampal CA1 area.

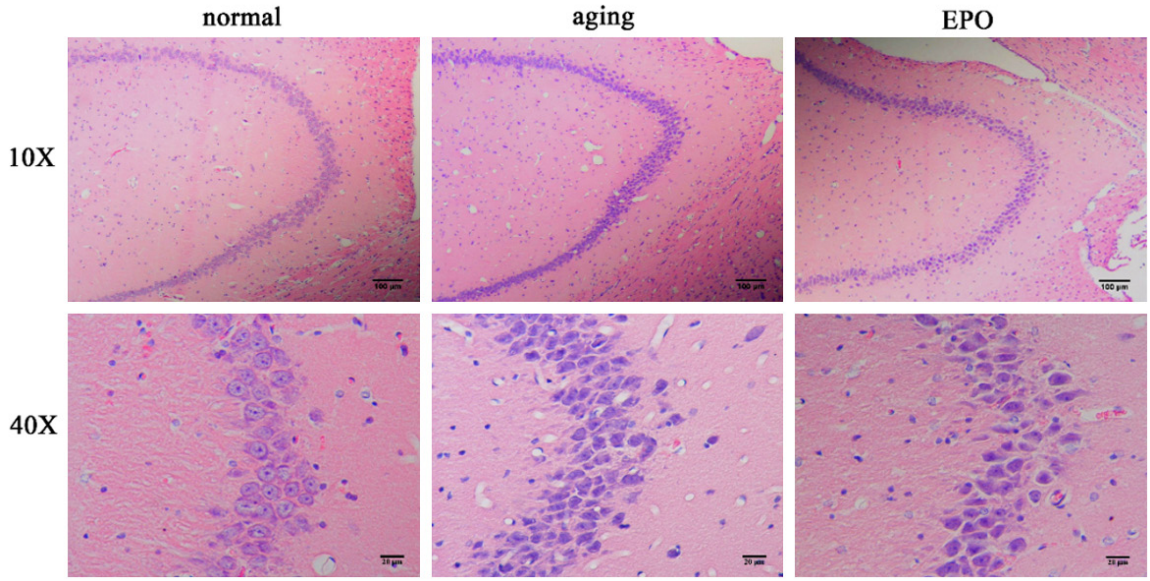

Figure 2. HE staining of the hippocampal CA3 area.

trifuged at 15,000 rpm for 10 min, and the supernatant was collected. Nuclear proteins were separated using the Nuclear and Cytoplasmic Protein Extraction Kit (Beyotime Biotechnology, Shanghai, China). Protein concentrations in the supernatants were determined by a spectrophotometer (Molecular Imager ChemiDoc™ XRS, ND-1000, NanoDrop, USA). Equal amounts of protein (80 μg) were heat denatured at 100°C for 5 min, electrophoretically separated on 5-10% gradient SDS-PAGE gels and then transferred to a PVDF membrane by electrophoretic transfer. The membrane was then blocked with 5% skim milk in Tris Buffered Saline with Tween (TBST) for 2 h and incubated with the primary rabbit anti-Akt antibody (1:2,000, Abcam), rabbit anti-p-Akt antibody (1:500, Abcam), rabbit anti-Cu-Zn SOD antibody (1:2,000, Abcam), rabbit anti-Nrf2 (1:1,000, Abcam) and the mouse anti-β-actin antibody (1:2,000; Santa Cruz Biotechnology) respectively at 4°C overnight. The

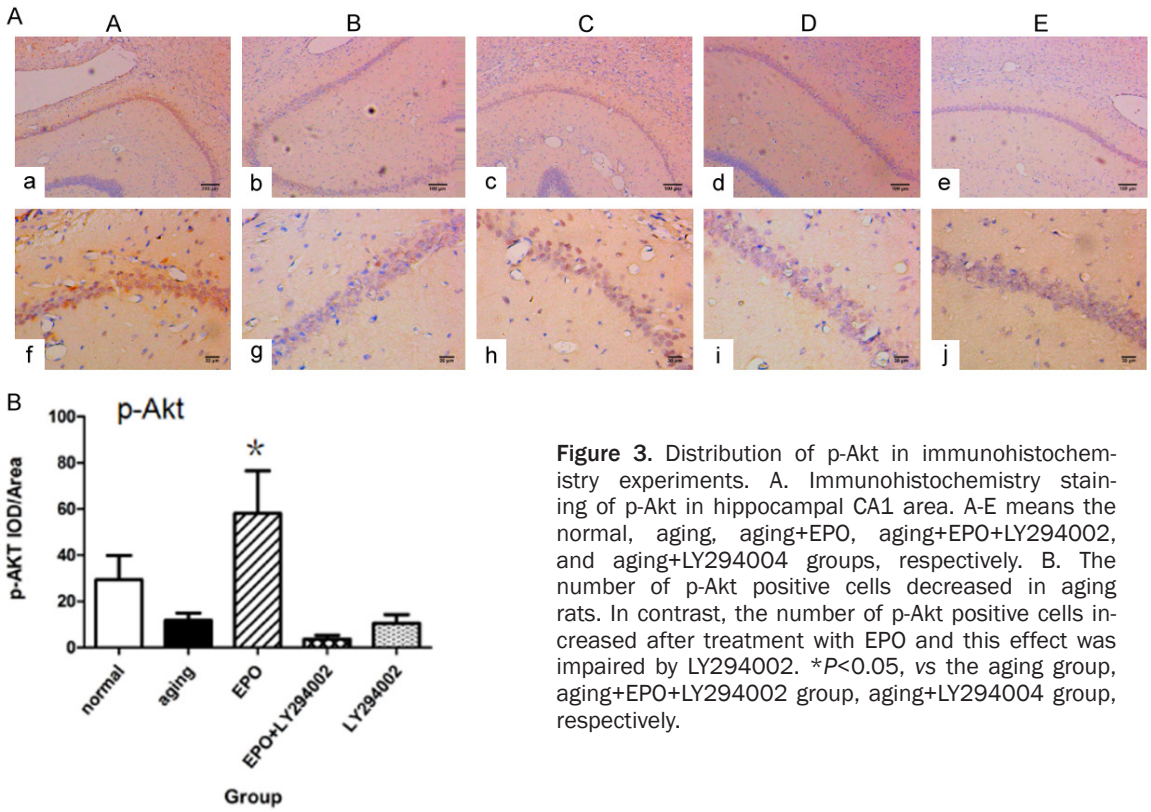

Figure 3. Distribution of p-Akt in immunohistochemistry experiments. A. Immunohistochemistry staining of p-Akt in hippocampal CA1 area. A-E means the normal, aging, aging+EPO, aging+EPO+LY294002, and aging+LY294004 groups, respectively. B. The number of p-Akt positive cells decreased in aging rats. In contrast, the number of p-Akt positive cells increased after treatment with EPO and this effect was impaired by LY294002. *P<0.05, *vs* the aging group, aging+EPO+LY294002 group, aging+LY294004 group, respectively.

blot was then incubated with horseradish peroxidase-conjugated respective secondary antibodies (goat anti-rabbit IgG, 1:10,000, Abcam; goat anti-mouse IgG, 1:5,000, ABgent) for 2 h. The optical density of bands was quantified using GboxChemi-HR16 BioImaging System (Bio-Rad, USA). The data were normalized using β-actin as an internal control and standardized with the control as 1.0.

RNA extraction and quantitative real-time PCR

Total mRNA was extracted from the hippocampus of rats using RNA Trizol reagent (Life Technologies) and then reverse transcribed to cDNA using a reverse transcription kit (Takara Biotechnology, China) according to the manufacturer's instructions. Quantitative real-time PCR was performed using SYBR Green Master Mix (Takara Biotechnology, China). The primer sequences were as follows: (forward) 5'-CACCAGGGCAGGATCTAC-3', (reverse) 5'-TTGCTTCCGACAGGGTTC-3' for Keap1; (forward) 5'-GAACGAGCTTCGCTGAG-3', (reverse) 5'-ATGACCTTGGGGTGGATG-3' for Nrf2; and (forward) 5'-TACCCACGGCAAGTTCAACG-3', (reverse) 5'-CACCAgCATCACCCCATTTg-3' for GAPDH. The relative expression of Nrf2 and Keap1 were calculated using the comparative threshold method ($2^{-\Delta\Delta Ct}$), and the data were normalized using GAPDH as an internal control. The specificity of amplification was assessed via melting curve analysis and gel electrophoresis.

Statistical analysis

Each test was repeated more than 3 times. All data were analysed using SPSS (ver. 19.0) software. Measurement data were expressed as the mean ± SD, and a one-way analysis of variance (ANOVA), and T-test were performed to assess the statistical significance. Enumeration data were compared between groups using the chi-squared test. $P \leq 0.05$ was considered statistically significant.

Results

EPO improved spatial learning-memory ability of D-galactose-induced rats

Results from the Morris water maze test demonstrated that the escape latency (EL) of D-gal group rats was significantly longer than that of the control group (P<0.05). The EL of the EPO

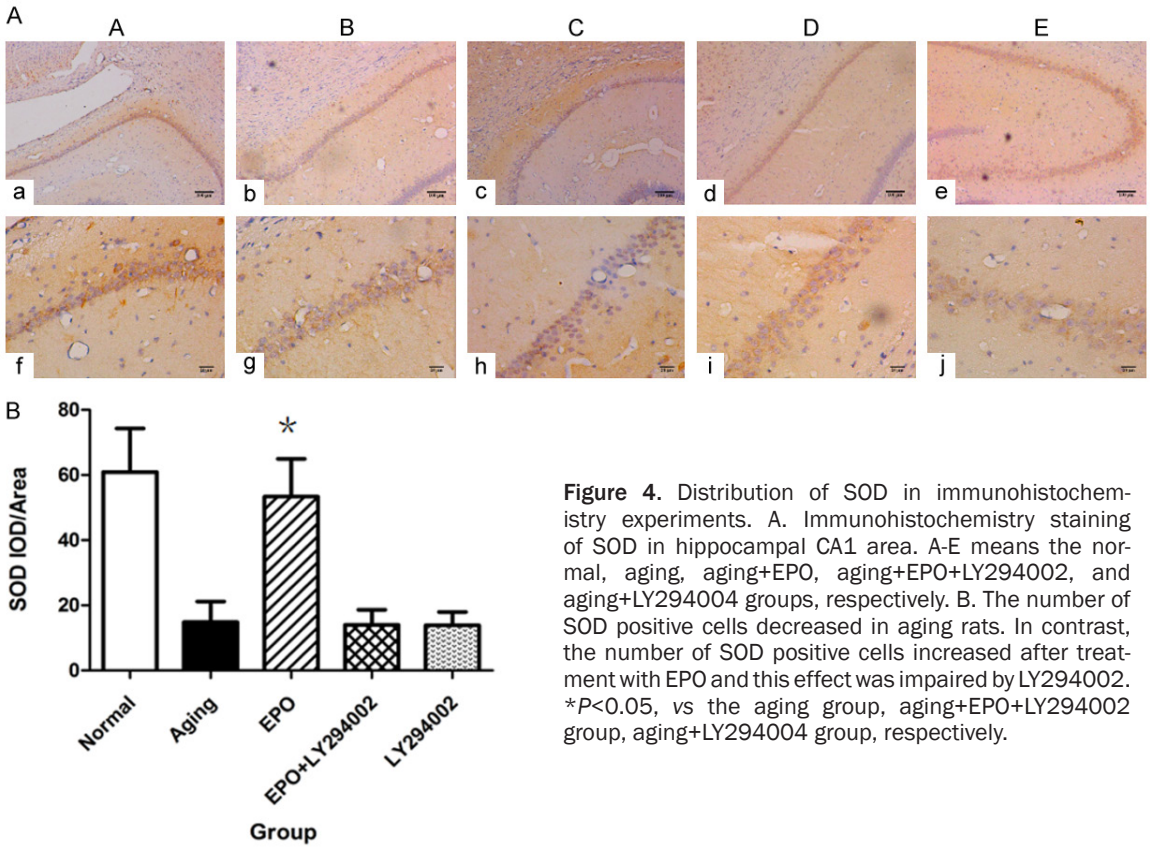

Figure 4. Distribution of SOD in immunohistochemistry experiments. A. Immunohistochemistry staining of SOD in hippocampal CA1 area. A-E means the normal, aging, aging+EPO, aging+EPO+LY294002, and aging+LY294004 groups, respectively. B. The number of SOD positive cells decreased in aging rats. In contrast, the number of SOD positive cells increased after treatment with EPO and this effect was impaired by LY294002. **P*<0.05, *vs* the aging group, aging+EPO+LY294002 group, aging+LY294004 group, respectively.

Table 2. IOD/Area values for p-Akt in the hippocampal CA1 area of rats for each group

Group	p-Akt IOD/Area
Control	29.40±10.50
D-gal	11.73±3.20*,#
EPO	58.04±18.49
EPO+LY294002	3.60±1.66*
LY294002	10.5±3.75*

Notes: Data are the means ± SD of three independent experiments. **P*<0.05 vs EPO group. #*P*<0.05 vs Control group.

group rats was significantly shorter than that of the D-gal group (*P*<0.05) but showed no significant differences with that of the control group (*P*>0.05). In the LY294002 group and EPO+LY294002 group, the EL of rats were significantly longer than that of EPO group (*P*<0.05), but had no distinction with that of D-gal group (*P*>0.05). The probe test revealed that D-gal group rats spent a significantly shorter retention time in the target quadrant that previously contained the platform than the control group (*P*<0.05). In contrast, the cross-platform times of rats in EPO group were significantly higher as compared with rats in D-gal group (*P*<0.05) and similarly, the cross-platform times of rats in the LY294002 group and EPO+LY294002 group showed no significant difference with that of D-gal group (*P*>0.05), but were significantly less than that of EPO group (*P*<0.05) (**Table 1**). These results suggest that administration of EPO can significantly improve the spatial learning-memory ability of D-galactose-induced rats, and LY294002, an inhibitor of the PI3K/AKT pathway may reduce this effect.

Effect of EPO on hippocampal morphology

The hippocampus CA1 and CA3 areas were observed using a light microscope. Compared with the control group, the number of hippocampal neurons of rats in the D-gal group was significantly decreased and the neurons showed abnormal morphological features including cell shrinkage and deformation. The cells were sparsely arranged and the cellular structures were unclear. In contrast, hippocampal neurons of rats in the EPO group were plump round and arranged tightly and neatly, which are similar to the neurons in the control group (**Figures 1** and **2**).

Table 3. Comparison of the Cu-Zn SOD IOD/Area values in the hippocampal CA1 area

Group	SOD IOD/Area
Control	60.87±13.45
D-gal	14.80±6.34*,#
EPO	53.35±11.62
EPO+LY294002	13.95±4.66*
LY294002	13.83±4.14*

Note: *P<0.05 vs EPO group. #P<0.05 vs Control group.

Table 4. Expression of Akt and p-Akt in the rat hippocampus

Group	Akt/Beta-Actin	p-Akt/Akt
Control	1.02±0.03	1.09±0.04
D-gal	1.35±0.06*	0.47±0.04*
EPO	1.25±0.08▲	0.68±0.06▲
EPO+LY294002	0.92±0.07	0.29±0.04
LY294002	1.36±0.12	0.26±0.03

Note: *P<0.05 vs control group; ▲P<0.05 vs D-gal group, EPO+LY294002 group and LY294002 group.

Table 5. Expression of Nrf2 protein in the whole cell and nucleus of the rat hippocampus

Group	Whole-cell Nrf2/Beta-Actin	Nuclear Nrf2/Beta-Actin
Control	0.79±0.05	0.86±0.03
D-gal	0.52±0.04*	0.44±0.04*
EPO	0.71±0.05▲	0.66±0.07▲
EPO+LY294002	0.33±0.03	0.24±0.03
LY294002	0.32±0.03	0.20±0.03

Notes: *P<0.05 vs control group; ▲P<0.05 vs D-gal group, EPO+LY294002 group and LY294002 group.

Table 6. The expression of Cu-Zn SOD of rat's hippocampus

Group	Cu-Zn SOD/Beta-Actin
Control	0.86±0.03
D-gal	0.49±0.05*
EPO	0.76±0.06▲
EPO+LY294002	0.44±0.09
LY294002	0.37±0.07

Notes: *P<0.05 vs control group; ▲P<0.05 vs D-gal group, EPO+LY294002 group and LY294002 group.

EPO activates the PI3K/Akt pathway in D-galactose-induced rats

The results of immunohistochemical staining showed increased expression of phosphorylated (activated) Akt (p-Akt) and Cu-Zn SOD after the administration of EPO. The staining showed that p-Akt and SOD were mainly located in the cytoplasm. The expression of p-Akt and Cu-Zn SOD decreased in the D-gal group compared with the control group (P<0.05). While in the EPO group, expression of p-Akt and Cu-Zn SOD increased significantly compared to the D-gal group, and showed no obvious difference with the control group. In the EPO+LY294002 group, expression of p-Akt and Cu-Zn SOD had no distinction with the D-gal group (**Figures 3** and **4**; **Tables 2** and **3**). This result indicated that EPO may upregulate the expression of anti-oxidants such as Cu-Zn SOD by activation of the PI3K/Akt pathway.

Western blot was performed to evaluate the protein expression of Akt, p-Akt, Nrf2, and Cu-Zn SOD in neurons of the hippocampal CA1 region. The results showed that the expression of Akt changed little in each group, while the protein content of p-Akt (activated) in the D-gal group was found to be significant decreased compared with the control group as well as the EPO group (P<0.05). In the EPO+LY294002 and LY294002 group, expression of p-Akt was not affected by EPO (P>0.05). From this we conclude that EPO can stimulate the PI3K/Akt/Nrf2 pathway via up-regulation of the phosphorylation level of Akt in the hippocampus. The expression trends of Nrf2 and Cu-Zn SOD were in accordance with the results of immunohistochemical. (**Tables 4**-**6**; **Figures 5**-**7**).

A quantitative analysis of the expression of Nrf2 and Keap1 genes was performed using quantitative real-time PCR. We found that the quantity of Nrf2-mRNA in the cells of EPO group was obviously higher than that of other groups. Meanwhile, the quantity of Keap1-mRNA in the cells of EPO group was much lower. The results demonstrate that EPO may activate the PI3K/Akt pathway at the gene level by upregulating expression of Nrf2 and decreasing expression of the negative regulatory factor Keap1 (**Figures 8** and **9**).

Discussion

In this study we demonstrate that EPO is an important protective factor in an anti-aging progress. In addition, we clarified that Akt kinase is activated by EPO depending on functional PI3K activity. It relied on the functional activity of the PI3K/Akt pathway and Akt kinase

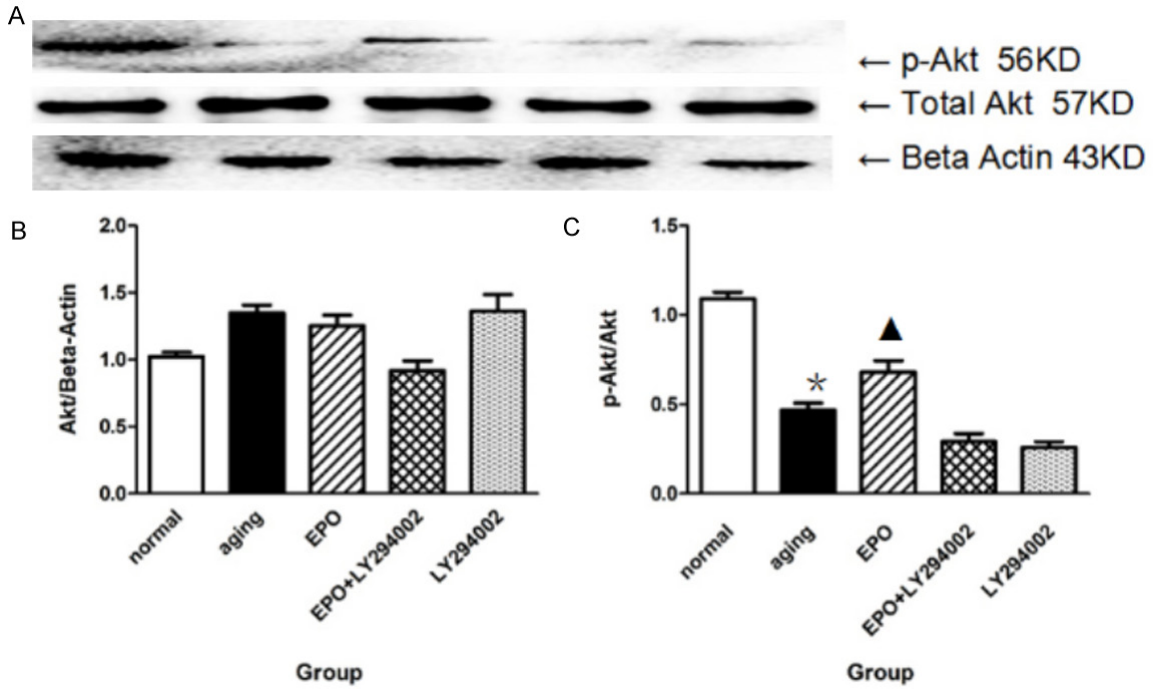

Figure 5. Expression of Akt and p-Akt in the rat hippocampus. The protein expression level of T-Akt (total Akt) and p-Akt was detected by Western blot analyses. A. Electrophoresis results for proteins. B. The relative expression level of T-Akt protein showed that the expression of T-Akt was stabilized, and EPO or LY294002 had little effect. C. The relative expression level of p-Akt protein showed that EPO enhanced expression of p-Akt, and LY294002 suppressed expression. Data are expressed as the mean ± SEM of three independent experiments (*P<0.05 *vs* normal group; ▲P<0.05 *vs* the aging group, aging+EPO+LY294002 group, aging+LY294004 group, respectively).

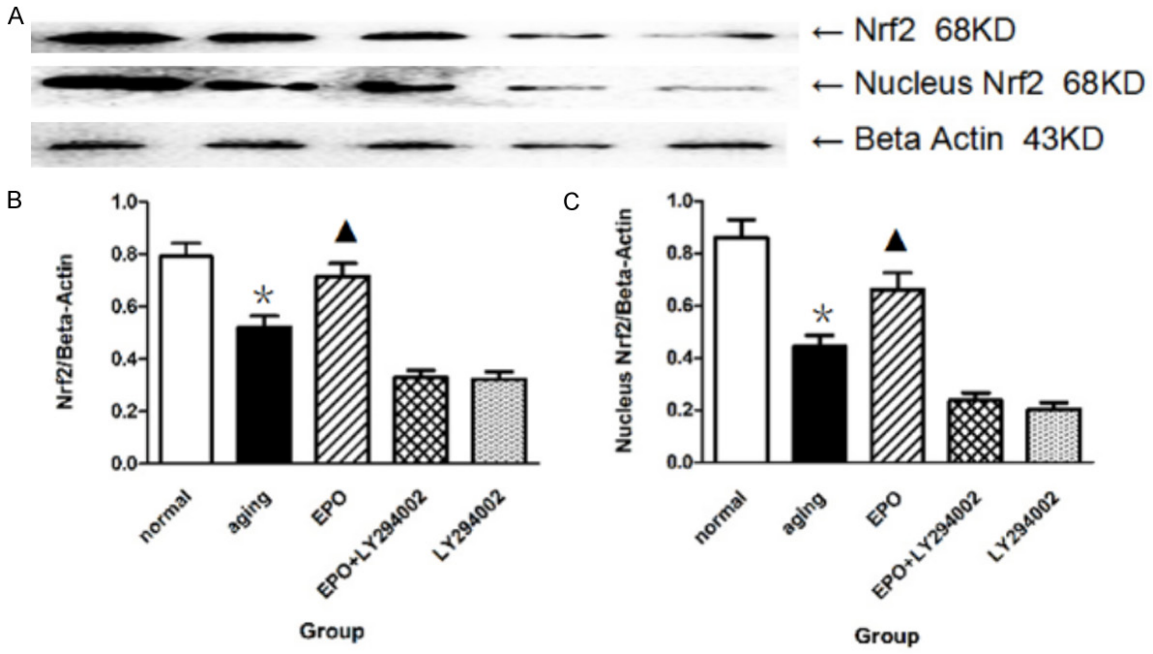

Figure 6. Expression of Nrf2 protein in the whole cell and nucleus. Western blot analyses were performed to evaluate the expression of Nrf2 protein in the neurons of the hippocampal CA1 region. A. Electrophoresis results for proteins. B, C. The relative expression levels of total Nrf2 protein and nuclear Nrf2 protein were both enhanced by EPO and suppressed by LY294002. Data are expressed as the mean ± SEM of three independent experiments (*P<0.05 *vs* normal group; ▲P<0.05 *vs* the aging group, aging+EPO+LY294002 group, aging+LY294004 group, respectively).

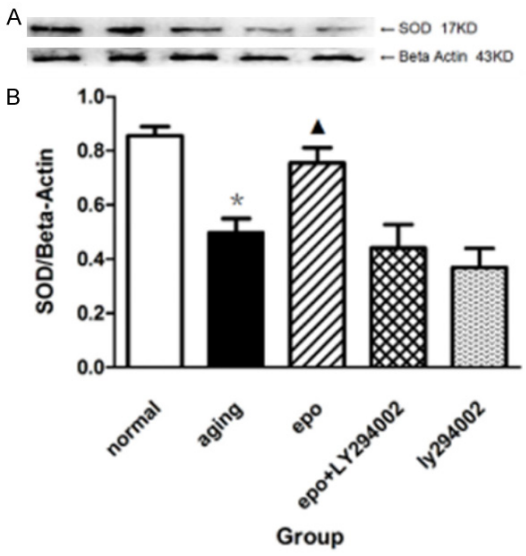

Figure 7. Expression of Cu-Zn SOD in the hippocampus. Western blot analyses were performed to evaluate the expression of Cu-Zn SOD. A. Electrophoresis results for proteins. B. The relative expression level of Cu-Zn SOD shows that EPO enhanced the expression of Cu-Zn SOD, and LY294002 suppressed the expression. Data are expressed as the mean ± SEM of three independent experiments. (**P*<0.05 *vs* the normal group; ▲*P*<0.05 *vs* the aging group, aging+EPO+LY294002 group, aging+LY294004 group, respectively).

downstream of EPO to increase expression of antioxidant enzymes in the hippocampus, and finally reduce neuron damage during aging.

Erythropoietin (EPO) is a glycoprotein hormone that controls erythropoiesis by acting as a cytokine, and was first used to treat patients with end-stage renal disease and anemia [19]. Identification of EPOR expression on different cell types kicked off a search for non-erythropoietic effects of EPO. It is now clarified that there are several non-erythropoietic functions of EPO, as far ranging as promoting cardiac and CNS development, blocking cell death in stroke models, and improving learning and memory [20]. Studies report that the use of exogenous EPO can reduce cell apoptosis in a cerebral ischemia mouse model, suggesting that EPO exerts a neuroprotective effect [21, 22]. Further applications of PI3K and MAPK inhibitors showed that the function of EPO was closely related to PI3K and MAPK [23]. Furthermore, several studies have found that the KEAP1-Nrf2 pathway is a key pathway underlying the EPO neuroprotective effect in an ischemic hypoxic cell model [23, 24]. Subsequently, studies found that EPO could enhance the ability of antioxidant enzymes to remove excess free radicals, although the specific mechanism has not yet been clarified [25, 26]. In our previous studies, we also confirmed that EPO enhanced cellular anti-oxidant capacity of long-term cultured primary nerve cells [27]. Recently, studies have reported that supplementation of EPO effectively restored impaired memory in the vascular dementia rat model [28].

Here, we clarified the neuroprotective effect of EPO in the D-galactose-induced-aging rat model. First, we found EPO rescued the impairment of spatial learning-memory ability in aging rats. Spatial memory (i.e. memory for spatial configurations and ability to navigate in an environment) is a critical component of episodic memory that has been generally shown to decline with age across species [29, 30]. Studies have reported that EPO could attenuate memory deficits in aging rats [31], and in present study, we found that EPO injection to aging rats for 1 week additionally reduced the decline of spatial learning and memory. In our study, after injection with D-galactose for 6 weeks, the rats gradually showed signs which were similar to natural aging, such as reduced physical activities, delayed movements, lessened responses to stimuli, and decreased weight growth rate. Compared with the normal control group, the aging rats behaved more clumsily in the Morris water maze test, indicating the decline of spatial memory. While the aging rats behaved more intelligently in the test after treatment with EPO, including escape latency time shortening and the increased cross-platform times which indicate improvement of the rat's spatial memory.

In addition, EPO treatment reversed the loss of neurons in the hippocampus and promoted plerosis of neuronal injury in the aging process. Extensive research has confirmed an essential role of the hippocampus in both storing and retrieving new memories as well as in spatial navigation [32-34]. In HE staining, we have observed impairment of hippocampal neurons as described in aging rats. After treatment with EPO, the density of the hippocampal neurons was restored and the morphological characteristics of nerve cells turned to be more similar to the normal neurons. This demonstrates the ultimate effect of EPO is in resisting cellular damage during aging.

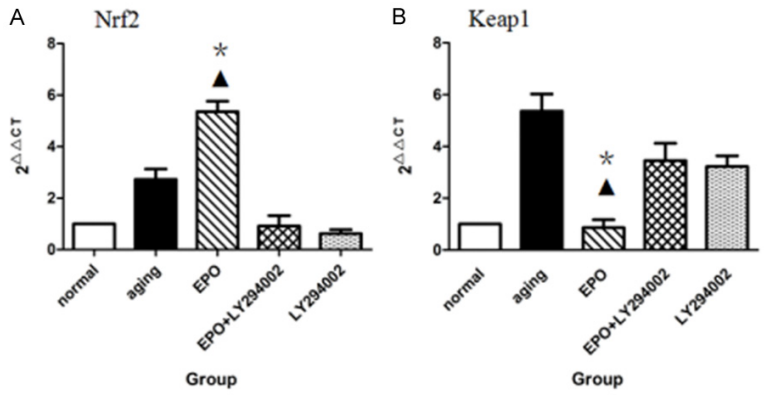

Figure 8. Expression of the Nrf2 and Keap1 genes. The abundance of Keap1 mRNA and Nrf2 mRNA was determined by quantitative real-time PCR. A. The abundance of Nrf2 mRNA was significantly increased after EPO injection, and this effect disappeared following LY294002 treatment. B. Expression of Keap1 mRNA exhibited an opposite trend to that of Nrf2. The relative mRNA expression was calculated using the $2^{-\Delta\Delta Ct}$ method and shown as the change in percentage from controls. Data are expressed as the mean ± SEM of three independent experiments (*P<0.05 *vs* the normal group; ▲P<0.05 *vs* the aging group, aging+EPO+LY294002 group, aging+LY294004 group, respectively).

In the present study, we demonstrated that the protective process of EPO on aging rats was associated with activation of the PI3K/Akt/Nrf2 pathway. We also clarified that Akt kinase is activated by EPO depending on functional PI3K activity. One of the earliest detectable signalling events initiated by EPO-R activation is phosphorylation at tyrosine residues of several intracellular proteins, and the Jak2 protein tyrosine kinase was first identified to serve as the principal kinase involved in mediating Epo-responsive signal transduction [28, 35]. The PI3K/Akt pathway is now known as an important signal transduction pathway of cell growth and proliferation, apoptosis, as well as anti-oxidative stress in many different cell types. Akt, the serine/threonine kinase protein kinase B (PKB), was identified as a downstream component in survival signaling through PI3K. Studies reported that EPO induced activation of Akt in the murine EPO-responsive cell line HCD57 [23]. In the present study, the protein abundance of AKT and p-AKT (activated) was detected by Western blot. The result showed that expression of Akt changed little in each group, while the protein content of p-Akt (activated) in the D-gal group was significantly decreased compared with the control group as well as the EPO group. This result indicates that the decrease of Akt phosphorylation in hippocampal neurons is a vital molecular biology change in the aging process and EPO treatment could facilitate phosphorylation of Akt in hippocampal neurons of aging rats. Furthermore, a PI3K-specific inhibitor LY294002 was also applied to the rats treated with EPO under the same conditions and the protein content of p-Akt of rats additionally treated with LY294002 remained very low compared with non-LY294002 rats. We conclude that phosphorylation of Akt induced by EPO is dependent on functional PI3K activity and our preliminary studies using, PD98059, a specific inhibitor of the MAPK/extracellular signal-regulated kinase 1 (MEK1), weakened the neuroprotective effect of EPO [36]. Thus, according to the literature and our research, we conclude that the anti-aging effect of EPO should be controlled via complicated networks including Jak2-Stat, MAPK-Erk, and PI3K-Akt activation pathways.

Importantly, we clarified EPO enhanced the expression of antioxidant enzymes in the hippocampus by stimulating the Nrf2-ARE pathway. The free radical damage hypothesis states that free radicals are one of the mechanisms inducing aging, via reactive oxygen species (ROS) excessively generated under oxidative stress that can lead to cell and tissue damage paralleled by alterations in the function of genetic apparatus, resulting in untimely cell death and aging [37]. In this study, D-galactose was injected subcutaneously to rats for 6 weeks to simulate chronic oxidative stress in natural aging. Expression of SOD, one of the most essential antioxidant enzymes was found significantly decreased in the D-gal group by Western blot. The results also revealed that EPO treatment attenuated the loss of SOD in aging rats. We conclude that that a lack of anti-oxidant enzymes is involved in the process of aging induced by D-gal. By increasing expression of SOD, EPO gives assistance to nerve cells to defence the damage of free radicals or

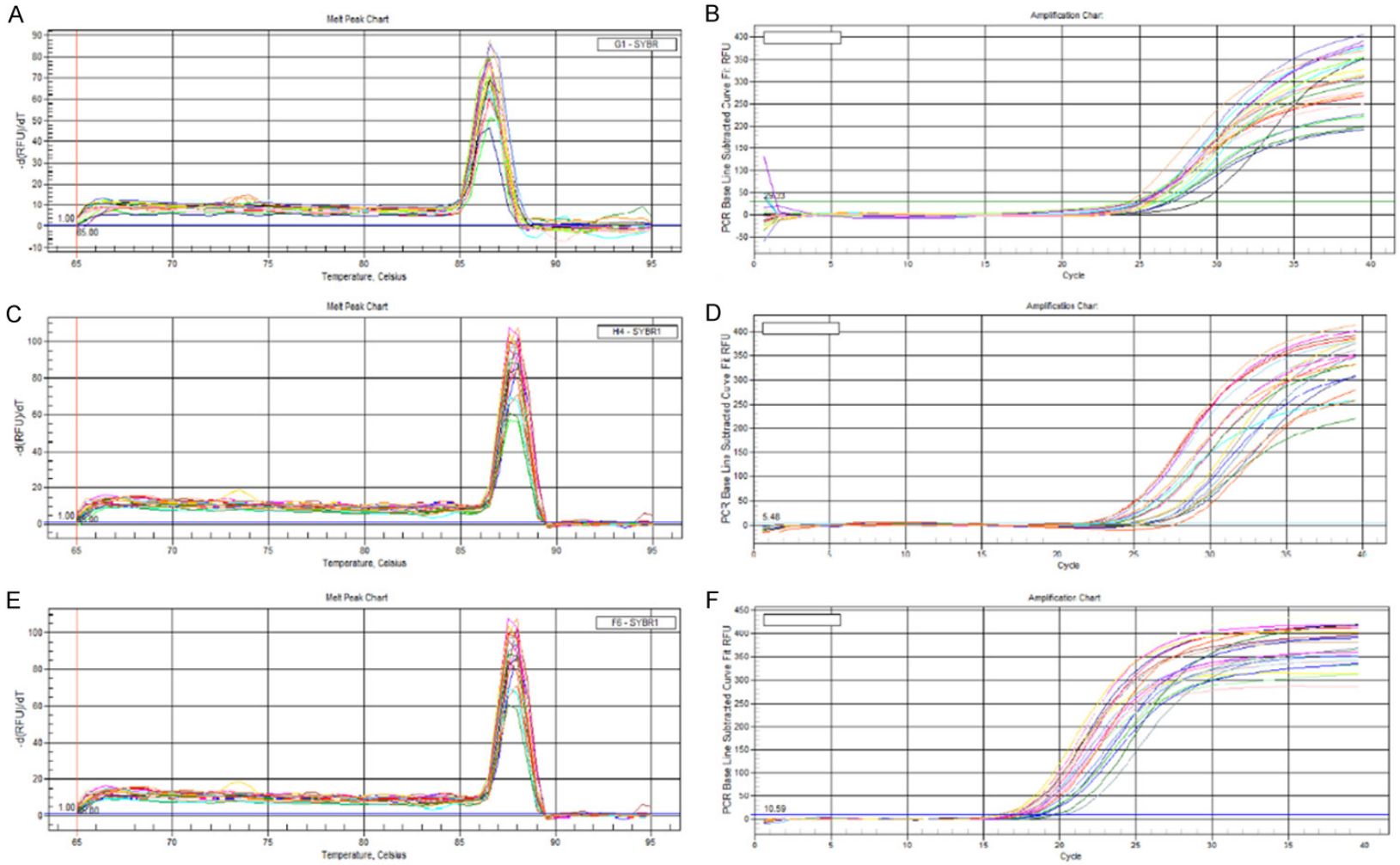

Figure 9. A, B. Melting curve and amplification curve for KEAP1. C, D. Melting curve and amplification curve for Nrf2. E, F. Melting curve and amplification curve for the reference gene GAPDH.

reactive oxygen species generated during aging.

In the antioxidant defense system, nuclear factor erythroid 2-related factor 2 (Nrf2) is the most important transcription factor in regulating multiple antioxidants, which binds to the antioxidant response elements (AREs). Furthermore, previous research has demonstrated that the PI3K/AKT pathway plays a critical role in modulating Nrf2/HO-1 protein expression as an upstream signaling molecule, which leads to Nrf2 activation and induces Nrf2-mediated downstream genes [38]. Under physiological conditions, Keap1 binds to Nrf2, hindering the activation and nuclear translocation of Nrf2. When exposed to oxidants, Keap1 can dissociate from Nrf2, which allows Nrf2 to translocate into the nucleus, and then bind to ARE, thereby upregulating expression of antioxidant stress protein genes. In this study, our data revealed that the abundance of Keap1 mRNA significantly decreased and Nrf2 mRNA significantly increased after EPO treatment, indicating a positive effect of EPO on activation of Nrf2/ARE pathway. As the expression of Keap1 decreased, Nrf2 could be easier to translocate into nucleus and activate ARE, ultimately initiating expression of antioxidant enzymes.

In conclusion, we have demonstrated that EPO plays an effective role in preventing D-gal-induced aging in rats. The specific mechanism may be associated with activating the endogenous antioxidant defense system via the PI3K/Akt/Nrf2 pathway. Moreover, our discovery gives us a clue that enhancing the endogenous antioxidant defense capacity is a promising strategy to delay senescence.

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Disclosure of conflict of interest

None.

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