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Full Length Research Paper

Effect of erythropoietin (EPO) on plasticity of nervous synapse in CA1 region of hippocampal of vascular dementia (VaD) rats

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The effects of erythropoietin (EPO) on plasticity of nervous synapse in CA1 region of hippocampal of vascular dementia (VaD) rats were studied. The Wistar rats reaching standard were randomly divided into three groups: Sham control group, VaD group, VaD + EPO injection treatment (E) group. The bilateral common carotid arteries of rats were permanently ligated to establish VaD model. Special study and memory were observed by Y-maze test. The expressions relative rate of MAP-2 and SYN in CA1 region of hippocampus at different time were detected by reverse transcription-polymerase chain reaction (RT-PCR) and Immunohistochemistry (ICH). 4, 8 and 12 weeks after operation, there were obvious decrease in MAP-2 and SYN expression in both VaD and E groups, while it was much more for VaD group. EPO could comparatively increase the MAP-2 and SYN protein and mRNA expression in the hippocampal CA1 region of VaD rats. These results suggested that EPO might develop its improvement by regulating the plasticity of nervous synapse, which were in terms of increasing MAP-2 and SYN protein and mRNA expression in the CA1 region of hippocampal of VaD rats.

Key words: Erythropoietin, vascular dementia, nervous synapse, MAP-2, SYN.

INTRODUCTION

Erythropoietin (EPO), an endogenous cytokine, plays an adjusting and regulating role in the genesis and development of brain through paracrine action (Csete et al., 2004; Wang et al., 2004), and has also good protective effects on cells cultured *in vitro* under ischemic/hypoxic state (Kawata et al., 2006). Clinical reports demonstrated that EPO could improve cognitive function while treating renal anemia (Grimm et al., 1990). Meanwhile, in our previous study (Huang and Shao, 2006), we also found that EPO could improve the cognitive function of vascular dementia (VaD) rats. However, the mechanisms of its improvement are still unknown.

Synapse is a structure that permits a neuron to pass an electrical or chemical signal to another cell (neural or otherwise). Obviously, the synapse plays important roles in transmission, processing and storing of information among neurons. Earlier studies have showed that cognitive functions were closely related to the structures and plasticity of synapse (Waterman et al., 1999; Sanchez et al., 2000). The loss of synapse is one of the important mechanisms of cognitive function impairments of VaD (Sloboda et al., 1976). Microtubule-associated protein-2 (MAP-2) is a highly conservative protein and belongs to structural microtubule-associated protein family regulates the reversible polymerization and stability of microtubules (McIlwain and Hoke, 2005). MAP-2 is especially vulnerable to acute central nervous system (CNS) injury and secondary events, which result from increased extracellular levels of excitatory amino acids and excessive calcium entry into cells (González et al., 2009; Young, 1992; Cao et al., 2011). Meanwhile, the protein expression of MAP-2 is closely related to the loss of synapse (Li et al., 1998). Synaptophysin (SYN) is correlated with the release of neurotransmitters, as one symbolic protein for the synaptic vesicle (Sudhof and Janh, 1991). The protein expression of SYN is the important indexes of evaluating the degree of cognitive function impairment of VaD rats (Masliah et al., 1991; Brock and

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O'Callaghan, 1987). Therefore, we assumed that EPO might improve the cognitive function impairments of VaD rats by regulating the MAP-2 and SYN expressions.

In this current work, firstly, we established rat models of VaD by permanent ligation of bilateral common carotid artery and investigated the spatialorientated learning and memory ability of rats by lateral cerebral ventricle injection of EPO so as to demonstrate its improvement on VaD rats. The MAP-2 and SYN mRNA expression in CA1 region of hippocampal of VaD rats were determined by reverse transcription-polymerase chain reaction (RT-PCR). The MAP-2 and SYN protein expression in CA1 region of hippocampal of VaD rats were detected by immunohistochemistry (ICH).

MATERIALS AND METHODS

Reagents and apparatus

Recombinant human EPO (rhEPO) was purchased from Sunshine Pharmaceutical (Shenyang, China). Trizol, MAP-2 (rabbit polyaclonal) and SYN (rabbit polyaclonal) were obtained from Wuhan Biostor (Wuhan, China). Y-maze (type MG -3) was obtained from Zhangjiagang Biomedical Instrument Factory (Zhangjiagang, China);

Laboratory animals

Fifty-four Wistar rats, aged >16 months, weighing (300 ± 41) g, of either gender, were provided by the experimental animal center of the Second Military Medical University of Chinese PLA between July 2008 and December 2010. The rats were housed under controlled conditions (room temperature, 22 ± 2°C).

Lateral ventricular cannula embedding

A hole was drilled with minimized electric trephine with the help of PF5-48 stereotaxic apparatus according to atlas of brain of Wistar rats (Bao and Shu, 1991). A stainless steel tracheal tube with 0.5 mm external diameter was inserted into left cerebral ventricle (AP-1.0 mm, L-1.5 mm, and H-3.0 mm) and fixed on the crinial bone with adhesive and dental base acrylic resin powder. A stainless steel internal tube with 0.3 mm external diameter was inserted into tracheal tube. The top end of internal tube was 1 mm longer than tracheal tube, and the other end was connected with microinjector. Antibiotics were intramuscularly injected after operation for 1 week for anti-infection.

Establishment of VaD rats models

Following the rats were intraperitoneally anesthetized by chloral hydrate, a median incision was made at the cervical part. Muscle was carefully bluntly dissected to expose bilateral common carotid artery. A No.7 surgicalsuture was embedded under common carotid artery. Vagus nerve was avoided to be stimulated and bilateral common carotid artery was ligated carefully and incision was sutured.

Grouping and intervention

Fifty-four Wistar rats were randomized into three groups: sham operation group (n = 18); Bilateral common carotid artery was dissected, but ligation was not conducted following surgical suture embedding, then surgical suture was drawn out and incision was sutured. VaD group (n = 18): rat models of VaD were developed. EPO treatment group (n = 18): following a cannula was buried in lateral cerebral ventricle for one week, rat models of VaD were developed and injected with 200 U/kg of rhEPO via lateral cerebral ventricle, three times a week. The experimental temperature was kept at 25℃. Rats in the Sham operation group and VaD group were intraperitoneally injected with the same amount of normal saline.

Behavioral evaluation

The changes in action and behavior of rats were observed with MG-3 Y-maze type before and 4, 8 and 12 weeks after operation. The parameters of Y-maze were set as voltage 70 V and time delay 3 s. Following random rest method (Yin et al., 2000; Xu et al., 2000), safety area was changed in irregular order. The arm which rats stood on was used as the starting point of test. After rats escaped to safety area, signal lamp still lighted on for 15 s. Test began following another 1 s. Test was conducted repeatedly to train rats to form the conditioned reflex of light-dark discrimination, and there were 30 s time intervals between two tests. The rats were daily trained 20 times for 3 days successively. Error number (EN), which referred to the times of errors in everyday training, in the last day ≤ 2 and total reaction time (TRT) ≤ 120 s indicated the rats had formed the conditional reflex (Wang et al., 1997). EN ≥ 8 was used as the criterion of cognitive function impairments.

Criteria of correct and wrong reaction

Rats escaped to safety area after pelma was electrified for 10 s, which indicated correct reaction, otherwise indicated wrong reaction. Rats escaped to starting area that was also considered as wrong reaction. Generally, rats were daily trained 20 times, which reflected the degree of correct reaction of rats. Reaction time (latency) referred to time period from signal lamp was lighted to rats firstly escaped to light area. TRT refer to the time needed by all the reactions (including correct reaction and wrong reaction) in a whole experimental day. TRT reflected the reaction time of rats. Both EN and TRT could be used together to evaluate the learning and memory ability of rats. Before being sacrificed, all the rats were daily retrained 20 times for 3 days successively with Y-maze. EN and TRT in the last day were recorded.

Reverse transcription-polymerase chain reaction (RT-PCR)

Rats were anaesthetized with 10% of urethane and then sacrificed by transcardiac perfusion with PBS followed by separate their hippocampus immediately, cryoprotected by immersion in 30% sucrose for 24 h at 4 to 8°C and frozen in a tissue-freezing medium. The total RNA was extracted with Trizol reagent according to the manufacturer's protocol. A_{260} readings were taken on the extracted samples to quantify the amount of RNA present, and A_{260}/A_{280} ratios were calculated to determine the purity. The reverse transcription reaction was carried out with 1 mM of dNTP, 40 U/μl of Rnase innhibitor, 5 U/μl of AMV-RT and 5 U/μl of AMV- Optimized Taq in the provided reaction buffer at 50°C for 30 min. The primers were designed and synthesized based on the published gene sequence as shown in Table 1. The PCR reaction was done in a thermal cycler (Model 7500, Beckman) with an initial denaturation step at 94°C for 5 min, followed by a variable number of cycles of denaturation 94°C for 30 s, annealing for 30 s, elongation 72°C for 1 min and a final elongation step at 72°C for 7 min. The number of

Table 1. Primers for the various genes investigated in this study.

Gene	Direction	Primer sequence (5'- 3')	Product size (bp)
β-actin	Forward	CCTCTATGCCAACACAGTGC	211
	Reverse	GTACTCCTGCTTGCTGATCC	
MAP-2	Forward	AGCACACGAAGCAGGGTACA	259
	Reverse	GCAATAGAATCAAGGCAAGAC	
SYN	Forward	CACGGACCCAGAGAACATTA	447
	Reverse	GGACTTCACTGACCAGATTAC	

Table 2. Error number (EN) of rats at different time points in different groups.

Group	Error number（EN）			
	Before operation	4 weeks after operation	8 weeks after operation	12 weeks after operation
Sham	1.12 ± 0.51 (n = 18)	0.97 ± 0.57 (n = 18)	0.98 ± 0.62 (n = 12)	0.19 ± 0.69 (n = 6)
VaD	1.14 ± 0.52 (n = 18)	2.78 ± 0.83 (n = 18)$^{\triangle}$	8.15 ± 1.72 (n = 12)$^{\triangle\triangle}$	13.64 ± 1.92 (n = 6)$^{\triangle\triangle}$
E	1.19 ± 0.52 (n = 18)	2.04 ± 0.73 (n = 18)$^{\triangle}$*	5.27 ± 1.05 (n = 12)$^{\triangle\triangle}$**	9.78 ± 1.52 (n = 6)$^{\triangle\triangle}$*

(VS Sham: $^{\triangle}$, $p < 0.05$, $^{\triangle\triangle}$, $p < 0.01$; vs VaD: *$p < 0.05$, **, $p < 0.01$).

cycles was 32 for MAP-2 and 28 for SYN. The cycle numbers were defined after titration between 20 and 45 cycles and were within the logarithmic phase of amplification. The amplified products were run on a 1.5% agarose gel with ethidium bromide. Images were documented with a digital CCD camera in a BX60, and the intensity of the product bands was measured with Flous-SmutiImage Spectrum software (Bio-Rad, USA).

Immunohistochemistry (ICH)

Rats were anaesthetized with 10% of urethane and then sacrificed by transcardiac perfusion with PBS followed by separate their hippocampus immediately, cryoprotected by immersion in 30% sucrose for 24 h at 4 to 8℃ and frozen in a tissue-freezing medium. The brains were cut on a freezing microtome at the level of the anterior hippocampus, into six adjacent series of 4-μm-thick coronal sections. The sections were dehydrated through an alcohol series. Prior to immunohistochemical processing, sections were rinsed in 2% PBS-Triton X-100 and mounted onto gelatine-coated slides. Immunohistochemistry was performed on slide-mounted sections utilizing the following antibodies: MAP-2 (dilution 1:100); SYN (dilution 1:100). The sections were incubated overnight at room temperature with the primary antibody diluted in PBS-bovine serum albumin (BSA). After rinsing, sections were incubated for 1 h at room temperature in biotinylated goat antimouse serum (1:500), sections were incubated for 1 h in avidin–biotin–horseradish peroxidase complex (1:200). Following rinses, sections were placed for 30 min in chromagen solution consisting of 0.05% diaminobenzidine and 0.01% H_2O_2. The reaction was monitored visually and stopped by rinses of 0.1 M PBS. In order to minimize variability, sections from all animals were stained simultaneously. Cell counts were performed blindly in all sections using a Nikon Eclipse E800 microscope. Counts were made in six randomly selected optical fields under 400× magnification by individuals who were blinded to diagnosis. MAP-2 and SYN immunoreactivity were assessed semi-quantitatively using image pro plus software version 4.5.129 (Media Cybernetics). The percentage area covered by immunoreactivity was measured and the mean value taken.

Statistical analysis

The database was set up with the SPSS 16.0 software package for analysis. Data were represented as mean ± S.D. The means of multiple groups were compared with One-way ANOVA, after the equal check of variance, and the two–two comparisons among the means were performed by Student's t-test. $p < 0.05$ was considered as statistically significant.

RESULTS

Evaluation of learning and memory ability of rats

There were no significant differences in EN and TRT before operation among groups ($p > 0.05$). At post operative 4, 8 and 12 weeks, EN and TRT in the VaD group and EPO treatment group were significantly more than those in the sham-operation group ($p < 0.05$ or 0.01), while EN and TRT in EPO treatment group was significantly less than those in the VaD group ($p < 0.05$ to 0.01) (Tables 2 and 3). These results suggested that EPO could improve the learning and memory ability of Vad rats, which were induced by permanent ligation of bilateral common carotid artery.

Effects of EPO on the expression of MAP-2 and SYN mRNA in CA1 region of hippocampal of rats

At post operative 4, 8 and 12 weeks, MAP-2 and SYN mRNA in the VaD group and EPO treatment group were significantly much less than those in the sham-operation group ($p < 0.05$ or 0.01), while MAP-2 and SYN mRNA in

Table 3. Total reaction time (TRT) of rats at different time points in different groups.

Group	Total reaction time (TRT)			
	Before operation	4 weeks after operation	8 weeks after operation	12 weeks after operation
Sham	100.65 ± 2.52 (n = 18)	100.43 ± 1.93 (n = 18)	101.34 ± 3.05 (n = 12)	107.25 ± 1.98 (n = 6)
VaD	100.86 ± 2.55 (n = 18)	173.54 ± 5.62 (n = 18)$^{\Delta\Delta}$	212.38 ± 8.24 (n = 12)$^{\Delta\Delta}$	275.25 ± 4.94 (n = 6)$^{\Delta\Delta}$
E	100.47 ± 2.51 (n = 18)	128.39 ± 6.19 (n = 18)$^{\Delta *}$	169.84 ± 4.72 (n = 12)$^{\Delta\Delta **}$	216.80 ± 2.98 (n = 6)$^{\Delta\Delta **}$

(VS Sham: Δ, $p < 0.05$, $^{\Delta\Delta}$, $P < 0.01$; VS VaD: *, $p < 0.05$, ** p, < 0.01).

Table 4. Expression relative rate of MAP-2 mRNA in the hippocampal CA1 region.

Group	4 weeks after operation	8 weeks after operation	12 weeks after operation
Sham	0.810 ± 0.042	0.803 ± 0.036	0.751 ± 0.051
VaD	0.424 ± 0.058$^{\Delta\Delta}$	0.380 ± 0.029$^{\Delta\Delta}$	0.326 ± 0.060$^{\Delta\Delta}$

(VS Sham: Δ, $p < 0.05$, $^{\Delta\Delta}$, $p < 0.01$; VS VaD: *$p < 0.05$; **, $p < 0.01$).

Table 5. Expression relative rate of SYN mRNA in the hippocampal CA1 region.

Group	4 weeks after operation	8 weeks after operation	12 weeks after operation
Sham	0.796 ± 0.065	0.793 ± 0.053	0.783 ± 0.069
VaD	0.395 ± 0.043$^{\Delta\Delta}$	O.385 ± 0.033$^{\Delta\Delta}$	0.323 ± 0.036$^{\Delta\Delta}$
E	0.601 ± 0.034$^{\Delta\Delta **}$	0.535 ± 0.045$^{\Delta\Delta **}$	0.410 ± 0.031$^{\Delta\Delta **}$

(VS Sham: $^{\Delta}$, $p < 0.05$, $^{\Delta\Delta}$, $p < 0.01$; VS VaD: *, $p < 0.05$, **, $p < 0.01$).

EPO treatment group was significantly more than those in the VaD group ($p < 0.05$ to 0.01) (Table 4 and 5; Figure 1 and 2). These results suggested that EPO could up-regulating the decreased expressions of MAP-2 and SYN protein of Vad rats, which were induced by permanent ligation of bilateral common carotid artery.

Effects of EPO on the expressions of MAP-2 and SYN protein in CA1 region of hippocampal of rats

At post operative 4, 8 and 12 weeks, MAP-2 and SYN protein in the VaD group and EPO treatment group were significantly much less than those in the sham-operation group ($p < 0.05$ or 0.01), while MAP-2 and SYN protein in EPO treatment group was significantly more than those in the VaD group ($p < 0.05$ to 0.01) (Tables 6 and 7; Figure 3 and 4). These results suggested that EPO could up-regulating the decreased expressions of MAP-2 and SYN protein of Vad rats, which were induced by permanent ligation of bilateral common carotid artery.

DISCUSSION

In the western world, VaD is the second most common form of adult-onset dementia after Alzheimer's disease (AD). It has an overall prevalence of 1.2 to 4.2% in people aged 65 years or older, and accounts for 10 to 50% of dementia cases, depending on the diagnostic criteria and study population (Knopman et al., 2003). In terms of symptomatology, VaD is characterized by progressive cognition decline, functional ability impairment and behavioral problems. VaD results mainly from ischemic injury or oligaemia to brain areas involved in cognition, memory, and behaviour (Román, 2003). Therefore, we established the VaD model by permanent ligation of bilateral common carotid artery, which could result ischemic injury *in vivo*. Moreover, we evaluated the effect of EPO on learning and memory ability of VaD rats by Y-maze test. According to the theories relevant to plasticity of nervous, the MAP-2 and SYN were choosing to investigate the mechanisms of the improvements.

Based on model of 2-vascular occlusion, we used Y-maze to test the learning and memory ability of VaD rats before and 4, 8 and 12 weeks after operation. Results found that as compared with sham-operation group, rats of VaD group presented increased EN and TRT of Y-maze task (at post operative 4, 8 and 12 week, $p < 0.05$), and these symptoms are more and more obvious with the elongation of time to blockage. As compared with VaD group, EN decreased and TRT shortened in the EPO treatment group ($p < 0.05$). These results demonstrated that EPO could improve the learning and memory ability

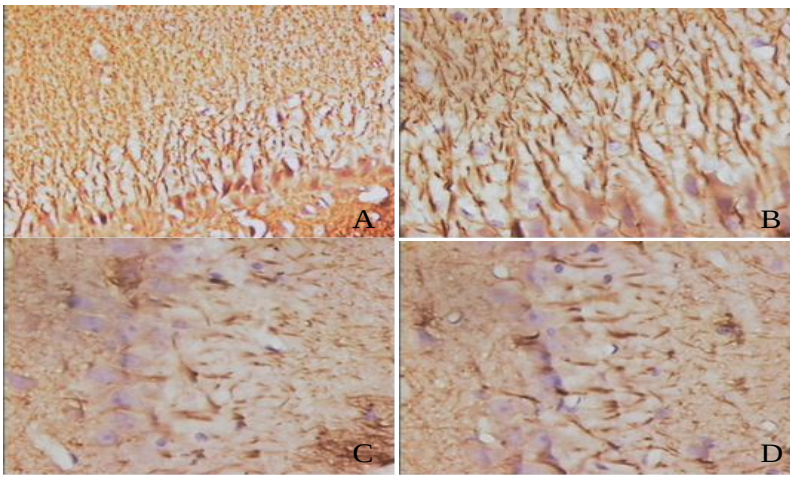

Figure 1. MAP-2 positive cells of CA1 region of hippocampus among groups at different time points (×400).A and B: MAP-2 immunoreactivity in CA1 region of hippocampus in sham group; C: MAP-2 immunoreactivity in CA1 region of hippocampus in VaD group at postoperative 12 weeks; D: MAP-2 immunoreactivity in CA1 region of hippocampus in E group at postoperative 12 weeks.

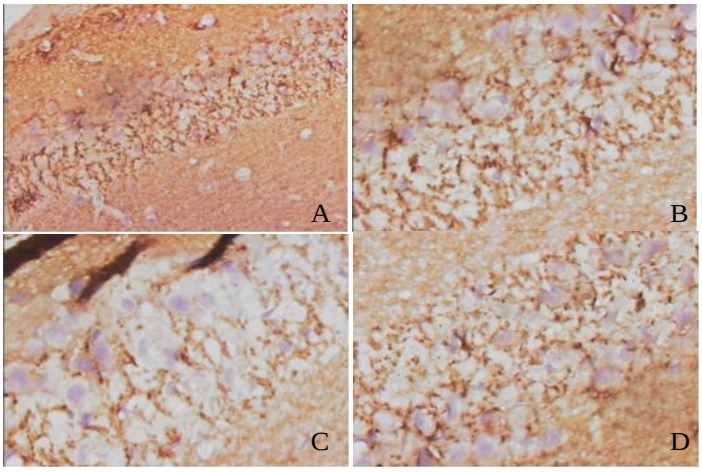

Figure 2. SYN positive cells of CA1 region of hippocampus among groups at different time points (×400). A and B: SYN immunoreactivity in CA1 region of hippocampus in sham group; C: SYN immunoreactivity in CA1 region of hippocampus in VaD group at postoperative 12 weeks; D: SYN immunoreactivity in CA1 region of hippocampus in E group at postoperative 12 weeks.

Table 6. The IOD of MAP-2 immunoreactivity in the hippocampal CA1 region.

Group	4 weeks after operation	8 weeks after operation	12 weeks after operation
Sham	140.25 ± 6.55	149.67 ± 5.88	159.17± 6.73
VaD	98.25 ± 4.31$^{\triangle\triangle}$	98.58 ± 3.37$^{\triangle\triangle}$	98.67± 3.47$^{\triangle\triangle}$
E	120.08 ± 9.65$^{\triangle\triangle **}$	129.50 ± 7.51$^{\triangle\triangle **}$	138.33 ± 7.17$^{\triangle\triangle **}$

(VS Sham: $^{\triangle}$, $p < 0.05$, $^{\triangle\triangle}$, $p < 0.01$ vs VaD: *$p < 0.05$, ** $p < 0.01$).

Table 7. The IOD of SYN immunoreactivity in the hippocampal CA1 region.

Group	4 weeks after operation	8 weeks after operation	12 weeks after operation
Sham	135.25 ± 6.12	140.08 ± 6.80	148.33 ± 5.79
VaD	95.17 ± 5.61$^{\triangle\triangle}$	96.58 ± 6.61$^{\triangle\triangle}$	97.17 ± 4.15$^{\triangle\triangle}$
E	13.67 ± 7.74$^{\triangle\triangle **}$	120.42 ± 7.17$^{\triangle\triangle **}$	126.5 ± 5.21$^{\triangle\triangle **}$

(VS Sham: $^{\triangle}$, $p < 0.05$, $^{\triangle\triangle}$, $p < 0.01$; VS VaD: *, $p < 0.05$, **, $p < 0.01$).

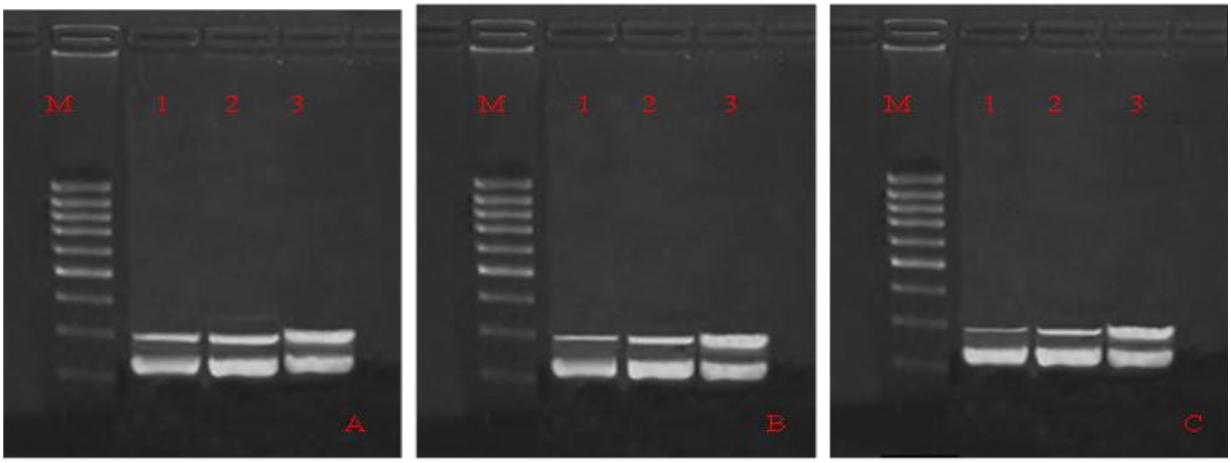

Figure 3. The expressions of MAP-2 mRNA among groups at different time points. A: The expressions of MAP-2 mRNA among groups at postoperative 4 weeks; B: The expressions of MAP-2 mRNA among groups at postoperative 8 weeks; C: The expressions of MAP-2 mRNA among groups at postoperative 12 weeks. M, Mark; 1, VaD group, 2 , E group; 3, Sham group.

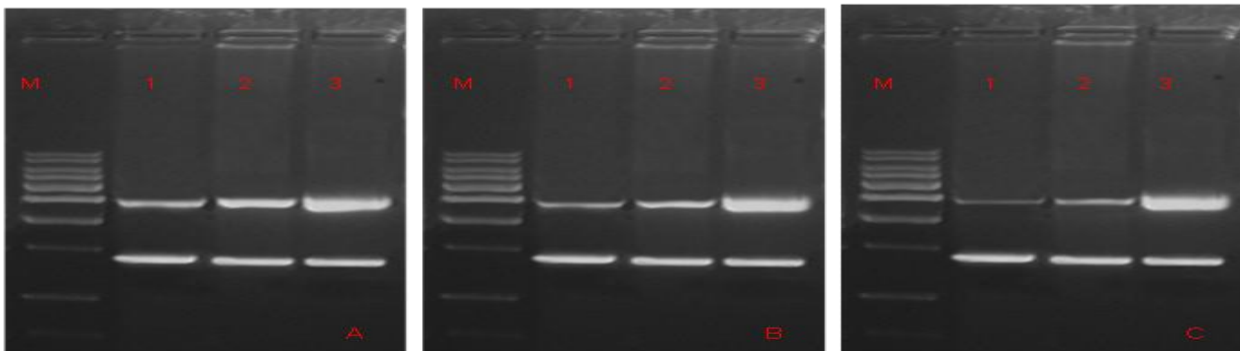

Figure 4. The expressions of SYN mRNA among groups at different time points. 4A: The expressions of SYN mRNA among groups at postoperative 4 weeks; B: The expressions of SYN mRNA among groups at postoperative 8 weeks; 4C: The expressions of SYN mRNA among groups at postoperative 12 weeks. M , Mark; 1, VaD group; 2, E group; 3, Sham group.

of VaD rats. The mechanisms of its improvement were investigated by RT-PCR and ICH. The RT-PCR and ICH results showed that as compared with Sham operation group, the MAP-2 and SYN protein and mRNA expressions decreased in VaD rats (at post operative 4, 8 and 12 week, $p < 0.05$). As compared with VaD group, the MAP-2 and SYN protein and mRNA expressions increased in the EPO treatment group ($p < 0.05$). These findings indicated that EPO might improve the learning and memory ability of VaD rats by increasing the MAP-2 and SYN protein and mRNA expressions.

In the current work, we found that the learning and memory ability of rats with VaD obviously were improved following EPO treatment, suggesting that EPO not only has protective effect on nerve cells cultured *in vitro* but also improve the learning and memory ability of VaD rats

in vivo. Meanwhile, EPO might develop its improvement by regulating the plasticity of nervous synaps, which were in terms of increasing MAP-2 and SYN protein and mRNA expression in the CA1 region of hippocampal of VaD rats.

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