

Review Article

Microenvironmental change after synthetic E-selectins interfere in ischemia-reperfusion in rats and its contribution to endogenous/exogenous neural stem cells

Wei-Hua Lin^{1*}, Feng Xu¹, Long Bao¹, Shi-Ying Zheng², Wen-Ming Shen^{2,3*}

Departments of ¹Emergency Surgery, ²Cardio-Thoracic Surgery, The First Affiliated Hospital of Soochow University, Suzhou 215006, Jiangsu Province, China; ³The Affiliated Wujin People's Hospital of Jiangsu University, Changzhou 213000, Jiangsu Province, China. *Co-first authors.

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Abstract: Objective: To explore the special significances in advantages of using anti-inflammatory drugs, such as amelioration of growing conditions and the promotion of cell growth. Methods: Utilizing anti-adhesive effects of synthetic E-selectins, we observed the changes of inflammatory cytokines (TNF- α , IL-1 β) contented in brain tissues and rat serums in rats hind cerebral ischemia-reperfusion models. Both growth and expression of endogenous/exogenous neurological stem cells were detected after ameliorated local microenvironment. Results: The contents of TNF- α and IL-1 β were decreased in brain tissues and rat serums after applying synthetic E-selectins. Expression of exogenous neurological stem cells was enhanced. Animal neurological functions improved. Conclusion: Anti-inflammatory therapy in early stage could enhance proliferation of stem cells so that it has vital significations in treating cerebrovascular diseases.

Keywords: Ischemia-reperfusion, inflammatory cytokines, synthetic e-selectins, endogenous/exogenous neural stem cells

Introduction

In the mechanism of ischemia-reperfusion injury, the inflammatory response plays a very important role. After the ischemia-reperfusion injury, the endogenous stem cells in the state of body rest are activated and begin to migrate, proliferate as well as differentiate towards the ischemic injury area, for the purpose of compensating injured neurological function. However, the self-repair capacity of central nervous system is limited, which is related to the reason that ischemic microenvironment with local inflammatory is not conducive to the proliferation and survival of newborn cells. Selectins play a role of promoting activation, aggregation and leakage of leukocyte in inflammatory response after ischemia reperfusion as well as infiltration in the ischemic area. Synthetic E-selectin is a kind of selectins antagonist, which can inhibit selectins-mediated leukocyte and endothelial adhesion and have a good anti-

inflammatory property. In this study, by observing the changes of the 2 inflammatory cytokines-TNF- α and IL-1 β in rats' serum with ischemia-reperfusion injury after synthetic E-selectins, we mainly research the impact of changes of local microenvironment with ischemia reperfusion injury after being applied the synthetic anti-inflammatory E selectins on the endogenous and exogenous stem cells and neurological function in experimental animals. Moreover, we also make a preliminary study on the significance of using inhibitors inflammation to improve environment of stem cells as well as promote their growth condition.

Materials and methods

Reagents: Synthetic E-selectins (Shanghai Biochemical Research Institute); rat IL-1 β , TNF- α ELISA kit (Shanghai Langke Bioengineering Co., Ltd.); FITC labeled rabbit anti-mouse IgG (Southern Biotechnology Company); mouse

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Table 1. Change of plasma IL-1 β content in different groups ($x \pm s$, ng/ml)

	Sham control group	IR group	SeS group
IR 2 h	2.706 \pm 0.022	2.732 \pm 0.026	2.704 \pm 0.015
IR 6 h	2.694 \pm 0.011	3.406 \pm 0.076*	2.794 \pm 0.031▲
IR 12 h	2.678 \pm 0.023	4.144 \pm 0.053*	2.960 \pm 0.043*,▲
IR 24 h	2.676 \pm 0.024	4.648 \pm 0.089*	3.050 \pm 0.047*,▲
IR 48 h	2.684 \pm 0.018	4.804 \pm 0.072*	3.204 \pm 0.070*,▲
IR 72 h	2.666 \pm 0.056	3.234 \pm 0.072*	2.926 \pm 0.046*,▲

Note: SeS: synthetic E-selectins; IR: ischemia-reperfusion. *compared with the sham control group, $P < 0.05$; ▲ Compared with the ischemia-reperfusion group, $P < 0.05$.

Table 2. Changes of plasma TNF- α content in different groups ($x \pm s$, ng/ml)

C	Sham control group	IR group	SeS group
IR 2 h	1.586 \pm 0.059	1.832 \pm 0.055*	1.606 \pm 0.045▲
IR 6 h	1.588 \pm 0.034	2.250 \pm 0.086*	1.750 \pm 0.047▲
IR 12 h	1.602 \pm 0.052	2.362 \pm 0.036*	1.846 \pm 0.066▲
IR 24 h	1.610 \pm 0.043	2.146 \pm 0.042*	1.728 \pm 0.044▲
IR 72 h	1.600 \pm 0.037	1.766 \pm 0.084*	1.580 \pm 0.026▲

Note: IR: ischemia-reperfusion; SeS: synthetic E-selectins; *compared with the sham control group, $P < 0.05$; ▲ Compared with the ischemia-reperfusion group, $P < 0.05$.

anti-BrdU monoclonal antibody (Becton Dickinson Company).

Experimental animal grouping: 170 healthy adult SD rats, male or female, weighing 280-320 g. They are randomly divided into 5 groups, namely (1) sham operation group 46; (2) Ischemia-reperfusion group 46; (3) Treatment group of synthetic E-selectins 46; (4) simple hAMSCs transplantation group 16; (5) hAMSCs synthetic graft + E-selectins treatment group 16. In the 5 groups of experimental animals, we should randomly select 5 rats for blood sampling in (1)-(3) group at the ischemic time point of 2 h, 6 h, 12 h, 24 h, 48 h and 72 h, in which the 48 h-group can be used to make the brain homogenate plasma specimen. The remaining 16 rats in each group as well as the (4), (5) groups will be used for neurological function scoring. (1)-(3) groups mainly detect the growth condition of endogenous stem cells and BrdU intraperitoneal injection time in each group: after ischemia for about 1 w, continuously injuring for 6 d and 1 d before being killed, a dose of 50 mg/kg for each time; synthetic E-selectins should be injected in the tail vein after the ischemia-reperfusion model being made and

before the rats regaining consciousness, with a dose of 10 mg/kg. The (4), (5) groups detect the growth condition of exogenous stem cells: hAMSCs has been labeled BrdU before transplantation, so there is no need to conduct intraperitoneal injection of BrdU in order to avoid confusion with endogenous stem cells, which may impact the results of experimental observation. hAMSCs migration path should choose the tail vein transplantation and the time is 24 h after ischemia-reperfusion. This study was conducted in accordance with the declaration of Helsinki.

Establishment of an animal model

This experiment uses the Longa's suture method to make the artery ischemia-reperfusion model in rats' brain. For those rats participating in neurological function score in each experimental animals function score group, we will change to use the modified neurological severity score (mNSS) respectively at the time points

of 1 d, 3 d, 1 w, 2 w and 4 w after ischemia (**Table 1**) to conduct neurological evaluation. The highest score is 18 point, for 1-6 is mild injury, 7-12 is the moderate injury and 13-18 is the severe injury.

Collection of plasma

Draw blood using cardiac puncture for 3-4 ml in rats in (1)-(3) group at different time points (2 h, 6 h, 12 h, 24 h, 48 h, 72 h) after ischemia-reperfusion, natural solidify at room temperature after 10-20 min, centrifuge 20 min with the rotate speed of 2000-3000 r/min. Then carefully collect the supernatant, package, and place it in the -70°C refrigerator for examination.

Preparation for the brain tissue homogenate

Rats (1)-(3) group with ischemic 48 h will be killed by the overdose anesthesia after the heart puncture blood. Then we should remove their brains quickly, separate the bilateral cortical on ice plate, clean it using ice saline rinse, dry it by filter paper, accurately weigh its brain tissue, and add the 10% homogenate with 9

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Table 3. Content of IL-1 β , TNF- α in brain tissue at the 48th h after Ischemia-reperfusion ($x \pm s$, ng/g)

	Sham control group	IR group	SeS group
IL-1 β content	0.357 \pm 0.019	1.266 \pm 0.044*	0.648 \pm 0.033*, \blacktriangle
TNF- α content	0.693 \pm 0.012	1.265 \pm 0.042*	0.856 \pm 0.025*, \blacktriangle

Note: IR: ischemia-reperfusion; SeS: synthetic E-selectins; *compared with the sham control group, $P < 0.05$; \blacktriangle Compared with the ischemia-reperfusion group, $P < 0.05$.

Table 4. Neurologic function score of rats at different time points

	ICG	SeSG	hAMSCsT + SeSG	hAMSCsT
IR 1 d	11.36 \pm 0.74	11.75 \pm 1.04	11.29 \pm 1.11	11.25 \pm 1.04
IR 3 d	9.57 \pm 0.98	9.88 \pm 0.99	9.38 \pm 1.41	9.43 \pm 1.27
IR 1 w	7.88 \pm 1.26	7.25 \pm 0.71	5.13 \pm 0.84**	6.13 \pm 0.99
IR 2 w	6.88 \pm 0.90	4.63 \pm 0.92*	2.63 \pm 0.92**	4.00 \pm 0.76
IR 4 w	6.13 \pm 0.64	3.25 \pm 0.87*	2.25 \pm 0.71**	3.25 \pm 0.70

ICG: ischemia control group; SeSG: synthetic E-selectins group; hAMSCsT + SeSG: hAMSCs transplantation + synthetic E-selectins group hAMSCsT: hAMSCs transplantation group; IR: ischemia-reperfusion. Note: *compared with the ischemia control group, $P < 0.05$; **compared with the pure amniotic stem cell transplantation group, $P < 0.05$.

times physiological saline according to its volume and weight ratio, 4°C, 12000 r/min, centrifuging for 5 min. Finally carefully collect the supernatant and place it in -70°C refrigerator for examination.

ELISA detects the content of IL-1 β , TNF- α in the supernatant and plasma of the brain tissue

All the operations should be strictly in accordance with the instructions steps in the rat IL-1 β , TNF- α ELISA kits and determinate the content of IL-1 β , TNF- α in rats' plasma and brain tissue supernatants.

Preparation for the pathological specimens: After experimental animals in each group finish the mNSS score respectively in 2 w, 4 w, we should randomly select 8 killed rats. First, embed them in paraffin, regard the infarct locus as the neutral infarction, slice it to coronal shape with the slice thickness of 12 μ m, and apply the immunofluorescence staining to detect the fate of implanted cells. Conduct the HE staining, BrdU immunohistochemistry, and immunofluorescence staining of endogenous stem cells on the sham surgery group, ischemia-reperfusion group, and the synthesized E-selectins group while treatment group.

Statistical processing

Use SPSS10.0 software to conduct the statistical analysis. Indicate the measurement data as $x \pm s$ and analyze the t test and ANOVA of lines between any two group. $P < 0.05$ is considered statistically significant.

Results

Changes of plasma IL-1 β content: plasma IL-1 β content change of rats in each group: at different time points (2 h, 6 h, 12 h, 24 h, 48 h, 72 h) after the ischemia-reperfusion is shown in **Table 1**, whose results show that the plasma IL-1 β content doesn't have a significant change during the 2 h after focal ischemia-reperfusion, and the it will rise at 6 h and peak at

24-48 h, then decreasing gradually. Compared with the sham control group, it has significant difference. The IL-1 β content of synthetic E-selectins group is significantly lower and has a significantly difference compares with ischemia-reperfusion model group ($P < 0.05$).

Plasma TNF- α content change: plasma TNF- α content change of rats in each group at different time points (2 h, 6 h, 12 h, 24 h, 48 h, 72 h) after the ischemia-reperfusion is shown in **Table 2**, whose results show that the plasma IL-1 β content will rise at 2 h after rats' focal ischemia-reperfusion and peak at 24-48 h, and then decrease at 24 h gradually. Compared with the sham control group, it had significant difference. The TNF- α content of synthetic E-selectins group is significantly lower and has a significantly difference compares with ischemia-reperfusion model group ($P < 0.05$).

IL-1 β , TNF- α content of brain tissue: at the time point of 48 h is as shown in **Table 3**. The results show that after 48 h of rats' focal ischemia-reperfusion, the IL-1 β , TNF- α content in brain tissue has been significantly increased, and the difference is significant ($P < 0.05$) compared with sham control group. After using the synthetic E-selectins, the IL-1 β , TNF- α content has been significantly decreased and the differ-

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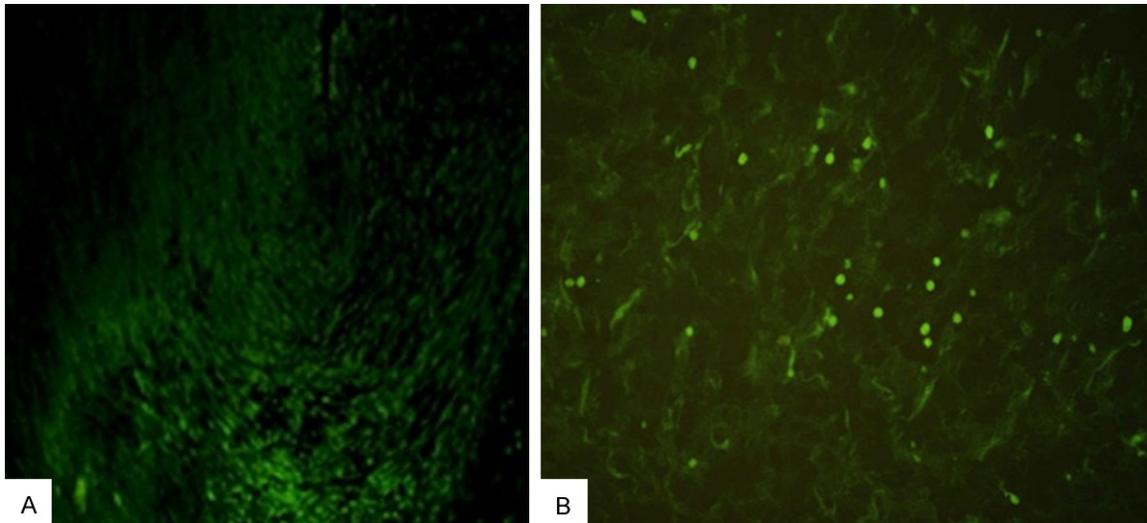


Figure 1. A: Tail vein transplantation group + synthetic E-selectins group (fluorescence microscopy 100×). There gathers large number of green fluorescence positive cells in the ischemia area. B: Tail vein transplantation group (fluorescence microscopy 100×). There scatters some green fluorescence positive cells in the ischemia area.

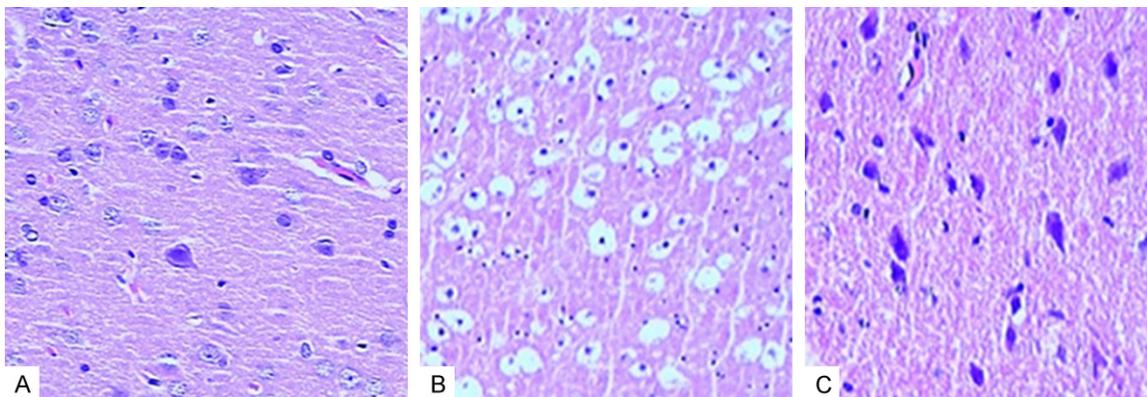


Figure 2. A: Sham group; B: IR group; C: Treatment group.

ence is significant ($P < 0.05$) compared with the ischemia-reperfusion group.

Functional score for animals: in each group is as shown in **Table 4**. In the 3rd d after ischemia-reperfusion, the neurologic dysfunction in all groups has been improved in varying degrees, but there is no significant difference among the groups ($P > 0.05$); at the 1st W after ischemia-reperfusion, the neurologic dysfunction in all groups has been further improved, but there is no significant difference between the synthetic E-selection group and the ischemia control group ($P > 0.05$), while the score in hAMSCs transplantation + synthetic E-selectins group has been significantly lowered compared with pure hAMSCs transplantation group, and the

difference is significant ($P < 0.05$). At the 2nd W after ischemia-reperfusion, the score in all groups expect the ischemia control group all suffers a further decrease, and the neurologic dysfunction of ischemic control group only has a little improvement compared with it at 1 week, while the score of synthesized E-selectins treatment group is significantly lower than the ischemia control group ($P < 0.05$), the score of hAMSCs transplantation + synthetic E-selectins group is significantly lower than pure hAMSCs transplantation group ($P < 0.05$).

Detection of immunofluorescence

(Figure 1A, 1B) The immunofluorescence result shows that in the 2nd w, 4th w after ischemia-

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reperfusion, there are no green fluorescence positive cells appearing in the synthetic E-selectins treatment group, sham operation group and ischemia-reperfusion group; while there are green fluorescence positive cells (transplanted hAMSCs) appearing in the hAMSCs transplantation + synthetic E-selectins treatment group and the pure hAMSCs transplantation group.

BrdU immunohistochemical staining: If the brown-yellow material appears, it will be positive and BrdU-positive material is in the nucleus. The experimental result shows that there are no BrdU-positive cells in sham operation group, ischemia-reperfusion group and synthetic E-selectins group.

HE staining result (Figure 2A-C)

The neuron in the brain of rats in sham operation group has a clear outline and complete structure, mostly shaped as conical, star as well as spindle. The tissue space surrounding cells is normal. The nucleolus is large and round, and its color is lighter and uniform, located in the central cell and rich in Nissl bodies, besides, its nucleolus is clear; the neuron death in central area of necrosis can be observed in ischemia-reperfusion groups, whose shape is not easily to distinguish, besides, its cell structure disappears, its cytoplasm empty bubble-like degenerates, its cells are sparse with disorder arrangement; part of the cytoplasmic autolyzes, part of the particles drop out, Nissl bodies significantly reduce, nucleus size shrinks, pycnosis stain, and the structure is unclear; the neuron ischemic change in the synthetic E-selectins treatment group is significantly reduced.

Discussion

Cerebral ischemia-reperfusion may induce a wide range of inflammatory reactions, on one hand, it may increase the impairment of brain function; on the other hand, and it may inhibit the regeneration of brain tissue. Once the ischemic brain injury occurs, the intrinsic repair mechanisms of the body starts and the injured tissue as well as the surrounding normal tissue will secrete some cell factors through the forms of paracrine and/or autocrine to promote the growth of survive neuron axon after ischemic injury and establish a new synaptic connection,

for the purpose of restoring part functions [1, 2]. It is regrettable that in the central nervous system, this self-repair capacity is very limited, which is mainly because ischemic local inflammatory microenvironment is not conducive for the proliferation and survival of new cells. A series of studies show that the local inflammatory reaction of brain tissue after the ischemia-reperfusion is one of the main reasons that cause the brain injury [3]. Adhesion molecules and cytokines generated by ischemia and reperfusion in the early period constitute the basis of ischemic injury changing to the inflammatory injury [4]. Many studies show that when the cerebral ischemia-reperfusion injures the body, the synthesis mechanism of TNF- α , IL-1 β is activated, resulting in the TNF- α , IL-1 β content rising. The co-expression of two factors--TNF- α and IL-1 β has played an important role in regulating the inflammatory response induced by cerebral ischemia [5, 6]. TNF- α and IL-1 β may be involved in and promote cerebral ischemia-reperfusion injury through the following ways: 1) excessive inflammatory response: TNF- α releases and activate excessive polymorph nuclear leukocyte, and increase the adhesion molecule expression of leukocyte as well as endothelial cell to promote leukocyte infiltrating to the brain tissue; it also activates the macrophages, endothelial cells and microglia cells to produce inflammatory metabolites, and further promote the activated leukocytes going into the ischemic area as well as the surrounding area. The IL-1 β expression also can induce the adhesion molecules expression, and promote neutrophil to go into the central nervous system. 2) The blood-brain barrier injury: TNF- α has a direct toxic effect on the capillaries, leading to small artery spasm, increasing vascular permeability, and opening the blood-brain barrier. The synergic action of TNF- α and IL-1 β can increase blood-brain barrier injury and increase peripheral leukocyte infiltration [7, 8]. 3) The apoptosis promotion: in the case of cerebral ischemia, TNF- α and IL-1 β can promote the injury and apoptosis of neurons through activating multiple apoptosis pathway such as Bcl-2, cathepsin B. 4) Stimulation of excitatory amino acids and the release of free radicals, inducing cerebral ischemia-reperfusion injury.

Our experiments will confirm that IL-1 β content begins to increase at the 6th after cerebral isch-

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emia, and reach peak during 24~48 h, while the TNF- α content begins to increase at the 2nd h after cerebral ischemia-reperfusion, reaches peak during 6~12 h, gradually decreasing at 24th h. At the 48th h, the content of IL-1 β and TNF- α in brain tissue is significantly higher than that in sham ischemic control group, which suggests that there appears secondary inflammatory response after cerebral ischemia-reperfusion, constituting a micro-inflammatory environment of local cerebral ischemia. It is not conducive to the proliferation and survival of new neurological cells and should be conducted anti-inflammatory treatment in early stage.

The synthetic E-selectins and P-selectins respectively have a inhibited effect on E-selectins as well as L-selectins [9], which can block the adhesion of leukocyte on endothelial cells and improve the blood supply of infarcted brain tissue, making the brain tissue get an effective protection when being conducting ischemia reperfusion [10, 11].

In this study, after applying synthetic E-selectins, the TNF- α and IL-1 β content in brain tissue and plasma has decreased significantly, and the difference has a statistical significance compared with the cerebral ischemia-reperfusion group, so we suggest that the synthetic E-selectins can inhibit the synthesis mechanism of IL-1 β and TNF- α , leading to decrease of IL-1 β and TNF- α content. On this basis, we examine the expression of endogenous stem cells at 2nd week after ischemia-reperfusion. Our experiments show that there are no green fluorescence-positive cells in synthetic E-selectins treatment group, ischemia-reperfusion group and sham group, and there are also no BrdU-positive cells when conducting BrdU immuno-histochemistry staining, which may be because there exists factors to inhibit neurons' differentiation and survival in micro-environment or there lacks of factors to promote the neuronal differentiation and survival [12]. While although the micro-environment can theoretically promote the proliferation and differentiation of endogenous stem cells after the synthetic E-selectins treatment, it still cannot overcome the negative factors, may be due to the limited endogenous stem cells and our limited detecting ability, etc. The function score of experimental animals in synthetic E-selectins treatment group decreases, and the difference has a statistical significance compared with the

ischemia-reperfusion group. HE staining shows that the ischemic change in brain tissue is significantly reduced after applying the synthetic E-selectins, which may be due to the increase of local blood flow in cerebral ischemia area and the improvement of blood supply in brain tissue after the anti-adhesion E-selectins, as well as anti-inflammatory of the synthetic E-selectins. Besides, we also detect the growth and survival condition of the transplanted hAMSCs in the use of synthetic E-selectins, and find that compared with pure transplanted hAMSCs, its growth and survival condition is much better after using the synthetic E-selectins, and there gathers large number of green fluorescence positive cells in the ischemic area, which shows that the animal neurological score is further reduced. Thus we believe that applying the synthetic E-selectins can improve the inflammatory micro-environment after ischemia-reperfusion and the blood supply for infarcted brain tissue, which is conducive to the growth of exogenous stem cells. Moreover, the transplanted hAMSCs can also secrete a number of nutritional factors which can improve the ischemic microenvironment and promote the survival of stem cells, so the combination of synthetic E-selectins and the transplantation of exogenous neural stem cells can enhance the proliferation, differentiation of neurological cells, protect the brain tissue function, and promote the recovery of experimental animals' neurological function. This is can provide clinical guidance for the application of the therapy using the stem cell transplantation to treat cerebral ischemia in clinic medicine in the future. However, how to improve the growth and survival condition of endogenous as well as exogenous stem cells after the improvement of inflammatory micro-environment still needs to be further studied.

Disclosure of conflict of interest

None.

Address correspondence to: Drs. Feng Xu and Shi-Ying Zheng, Departments of Emergency Surgery and Cardio-Thoracic Surgery, The First Affiliated Hospital of Soochow University, Suzhou 215006, Jiangsu Province, China. E-mail: fengxudoc@163.com (FX); shiyingzheng@163.com (SYZ)

References

- [1] Raineteau O, Schwab ME. Plasticity of motor systems after incomplete spinal cord injury. *Nat Rev Neurosci* 2001; 2: 263-273.

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- [2] Wieloch T, Nikolich K. Mechanisms of neural plasticity following brain injury. *J Curr Opin Neurobiol* 2006; 16: 258-264.
- [3] Matsuo Y, Onodera H, Shiga Y, Nakamura M, Ninomiya M, Kihara T, Kogure K. Correlation between myeloper-oxidasequantified neutrophil accumulation and ischemic brain injury in the rat: effects of neutrophil depletion. *Stroke* 2003; 25: 1469-1472.
- [4] Lan Haitao, Zhang Qingjun. The protective function of recombinant human erythropoietin on rats' cerebral ischemia-reperfusion inflammatory injury. *Neurosurgery* 2006; 1: 51-54.
- [5] Minami M, Kuraishi Y, Yabuuchi K, Yamazaki A, Satoh M. Induction of interleukin-1 beta mRNA in rat brain after transient forebrain ischemia. *J Neurochem* 1992; 58: 390-392.
- [6] Zhu Y, Saito K, Murakami Y, Asano M, Iwakura Y, Seishima M. Early increase in mRNA levels of proinflammatory cytokines and their interactions in the mouse hippocampus after transient global ischemia. *Neurosci Lett* 2006; 393: 122-126.
- [7] Hartz AM, Bauer B, Fricker G, Miller DS. Rapid modulation of P-glycoprotein-mediated transport at the blood brain barrier by tumor necrosis factor-alpha and lipopolysaccharide. *Mol Pharmacol* 2006; 69: 462-470.
- [8] Lee NY, Kang YS. The brain-to-blood efflux transport of taurine and changes in the blood-brain barrier transport system by tumor necrosis factor-alpha. *Brain Res* 2004; 1023: 141-147.
- [9] Zhang SM, Zhou D. On the application of E-selectins synthetic to treat rats' focal cerebral ischemia reperfusion injury. *Clin Neurosci* 2000; 8: 250-252.
- [10] Zhang SM, Zhou D. On the adhesion molecules and cerebral ischemia. *Jof Experimental Surgery* 1999; 16: 286-287.
- [11] Li JW, Zhang SM, Xie XS. The impact of E-selectins on the blood oxygen rate in cerebral ischemia-reperfusion, *Chinese Journal of Hemorheology* 2004; 14: 30-31.
- [12] Dempsey RJ, Sailor KA, Bowen KK, Türeyen K, Vemuganti R. Stroke-induced progenitor cell proliferation in adult spontaneously hypertensive rat brain: effect of exogenous IGF-1 and GDNF. *J Neurochem* 2003; 87: 586-297.