Genetics and Molecular Research

Effects of intermittent hypobaric hypoxia preconditioning on the expression of neuroglobin and Bcl-2 in the rat hippocampal CA1 area following ischemia-reperfusion

Q. Wu, K.X. Yu, Q.S. Ma and Y.N. Liu

Pathophysiology Department of Qinghai University Medical College, Xining, Qinghai, China

Corresponding author: Y.N. Liu
E-mail: liuyongnin_yn@163.com

Genet. Mol. Res. 14 (3): 10799-10807 (2015)
Received February 11, 2015
Accepted May 14, 2015
Published September 9, 2015
DOI http://dx.doi.org/10.4238/2015.September.9.18

ABSTRACT. This study was aimed at understanding the effect of intermittent hypobaric hypoxia preconditioning (IHHP) on neuroglobin (NGB) and Bcl-2 expression in the hippocampal CA1 region of rats following global cerebral ischemia-reperfusion. Wistar rats were randomly divided into sham, IHHP control, global cerebral ischemia-reperfusion (IR group), and IHHP+IR groups. The four-vessel occlusion rat model of Pulsinelli was used for the IR groups, in which the common carotid artery was occluded for 8 min before reperfusion. Thionin and immunohistochemical staining were used to observe NGB and Bcl-2 expression in the hippocampal CA1 region. Data was analyzed using the SPSS software. There was a significant increase in the number of surviving cells in the hippocampal CA1 region of the IHHP+IR group (119.5 ± 14) compared to the IR group (41.7 ± 3.8) ($P < 0.05$). There was a significant increase in the expression of NGB and Bcl-2 in the hippocampal CA1 region of the IHHP+IR group compared to the IR group. By upregulating hippocampal NGB and Bcl-2 expression, IHHP

may play a role in neural protection by reducing hippocampal neuronal apoptosis following IR.

Key words: Immunohistochemistry; Intermittent hypobaric hypoxia; Global cerebral ischemia-reperfusion;

INTRODUCTION

A previous study (Wu et al., 2012) suggested that intermittent hypobaric hypoxia preconditioning (IHHP) could induce delayed preconditioning of rat hippocampal neurons and reduce the degree of brain injury following ischemia and reperfusion (IR). The protective mechanisms of IHHP on neurons are unclear. Advanced delayed preconditioning was induced in neurons using a common method, in which moderate pretreatment is applied followed by a latent period at intervals of 1 to 2 days with neuroprotective effects continuing for several days. This phenomenon indicated that advanced delayed preconditioning of neurons might occur because of changes in gene expression and synthesis of new proteins (Racay et al., 2009; Brown et al., 2010). In this study, immunohistochemistry was used to detect the effects of IHHP on the expression of neuroglobin (NGB) and B cell lymphoma/leukemia-2 (Bcl-2) in the rat hippocampal CA1 area following IR and to investigate the possible protective mechanisms of IHHP.

MATERIAL AND METHODS

Materials

Animals

Healthy male Wistar rats (N = 48) weighing 280 to 300 g were provided by Center for Laboratory Animal Care of Hebei Medical University.

Instruments and reagents

Olympus optical microscope, Motic6.0 medical image analysis system (Motic Med 6.0), SP9001 kit (Beijing Zhongshan Jinqiao Biotechnology Co., Ltd., Beijing, China), thionin (Sigma, St. Louis, MO, USA), NGB rabbit anti-rat polyclonal antibody (China Academy of Military Medical Science), Bcl-2 rabbit anti-rat polyclonal antibody (Wuhan Boster Biological Technology, Ltd.).

Methods

Groups

Rats were randomly divided into four groups with 12 rats in each group (six rats for thionin staining and six for immunohistochemistry): 1) sham group (coagulation of the bilateral vertebral arteries for 2 days without blockage of the bilateral common carotid arteries); 2) IHHP group (exposed to hypothermia and hypoxia for 4 days, the bilateral vertebral arter-

ies were coagulated once a day, and 4 days later the carotid arteries of conscious rats were occluded for 2 days followed by removal of the blockage of the bilateral common carotid arteries); 3) I/R group (coagulation of the bilateral vertebral arteries for 2 days and 2 days later the carotid arteries of conscious rats were occluded for 8 min followed by reperfusion); 4) IHHP+I/R (exposed to hypothermia and hypoxia for 4 days once a day, then coagulated on day 5. Two days later the carotid arteries of conscious rats were occluded for 8 min followed by reperfusion). Six rats from each group were sacrificed on day 7 and their hippocampal CA1 area obtained. The survival neuronal density (ND) of the hippocampal CA1 area was obtained by thionin staining. Another six rats were sacrificed after reperfusion for one more day. An immunohistochemistry assay was used to detect the expression of NGB and Bcl-2 in the hippocampal CA1 area.

Establishment of IHHP model and rat global cerebral ischemia model

The IHHP model (Wu et al., 2012) was established by optimization of an acute repeated hypoxia model reported by Lu et al. (1992). The rat global cerebral ischemia model was achieved by occluding the four arteries supplying the brain, as reported by Pulsinelli and Brierley (1979).

Thionin staining and survival neuronal density count

Six rats from each group were sacrificed on day 7 and their brains obtained. Brain tissues were collected from 1 to 4 mm behind the optic chiasm in the coronal plane, fixed in 4% paraformaldehyde for 48 h, embedded and sectioned according to conventional methods, and stained with thionin. The ND was obtained from these sections.

Immunohistochemistry assay and analysis of NGB and Bcl-2

Rat brains were collected after an extra day of reperfusion, then fixed, embedded and sectioned, and stained according to the Figure 1. The negative control was 0.01 M phosphate-buffered saline (PBS). Results were observed under a microscope. The positive staining area and average optical density were analyzed by an image analysis system.

Statistical Analysis

Data were analyzed using the SPSS 13.0 software and reported as mean ± standard deviation (SD). The results were analyzed with a One-Way ANOVA. If there was statistical significance, the intra-group comparison was conducted by the least significant difference (LSD) method. A P value of 0.05 was considered to be statistically significant.

RESULTS

Effects of IHHP on the ND in the rat hippocampal CA1 area following IR

Survival ND was high in the sham group (Figure 1A, Table 1). The morphologic changes in the rat hippocampal CA1 area were not significant in the IHHP group compared with sham group ($P > 0.05$, Figure 1B, Table 1). Pyramidal cells disappeared completely in the

hippocampal CA1 area of the I/R group, with the survival ND value significantly decreased compared with the sham group ($P < 0.01$, Figure 1C, Table1). The arrangement and morphology of pyramidal cells in the hippocampal CA1 area significantly changed in the IHHP+I/R group compared with the I/R group, and the survival ND value significantly increased ($P < 0.01$, Figure 1D, Table 1).

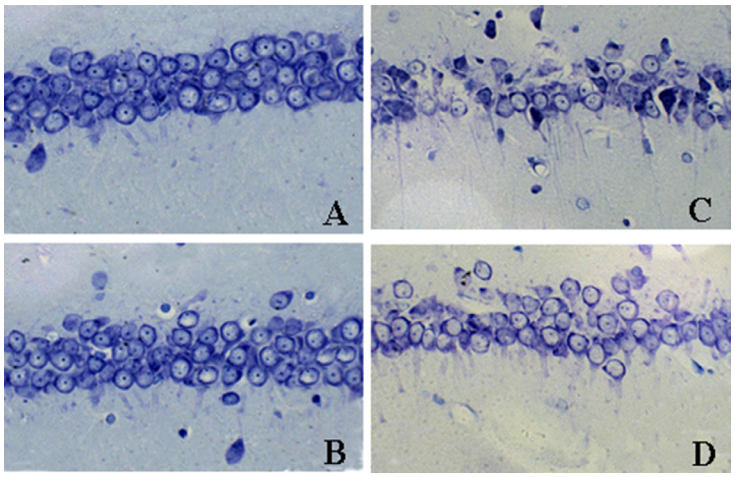

Figure 1. Thionin staining in the hippocampal CA1 area (400X). **A.** Sham; **B.** Control; **C.** IR; **D.** IHHP+IR.

Table 1. Neuronal density determined by thionin staining in the hippocampal CA1 region (means ± SD, N = 6).

Group	Neuronal density (Thionin staining, number/mm^2)
Sham	149.3 ± 2.5
HHP Control	141.0 ± 12.8
IR	41.7 ± 3.8*$^{\Delta}$
HHP+IR	119.5 ± 14*$^{\Delta\blacktriangle}$

*$P < 0.05$ *vs* Sham group; $^{\Delta}P < 0.05$ *vs* Control group; $^{\blacktriangle}P < 0.05$ *vs* IR group. IHHP: intermittent hypobaric hypoxia preconditioning.

Effects of IHHP on the expression of NGB in the rat hippocampal CA1 area with IR

There were a few brownish-yellow NGB-positive cells within the hippocampal CA1 area in the sham group (Figures 2A, 3, and 4). Compared with the sham group, the NGB-positive cell area increased in the IHHP group ($P < 0.01$), and the average optical density also increased ($P < 0.01$, Figures 2B, 3, and 4). In the I/R group, NGB-positive cells in the pyramidal layer were few, their color significantly decreased ($P < 0.01$), and the average optical density increased compared with the sham group ($P < 0.05$, Figures 2C, 3, and 4). Compared with the I/R group, the NGB-positive cell area and average optical density of positive cells were significantly increased in the IHHP+I/R group ($P < 0.01$), but decreased compared with the IHHP group ($P < 0.01$, Figures 2D, 3, and 4).

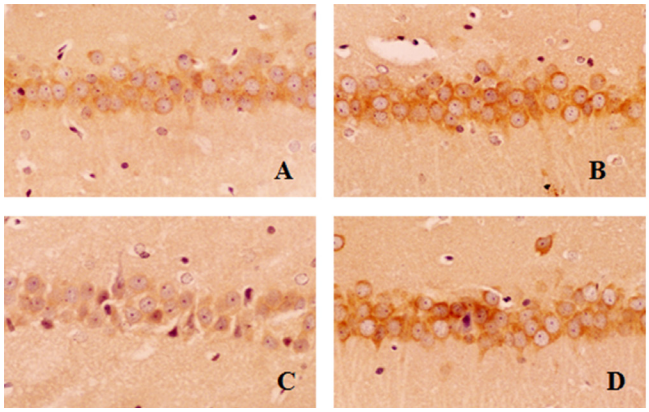

Figure 2. Effect of IHHP on NGB expression in the hippocampal CA1 subfield of rats with global cerebral IR by immunohistochemistry (400X). **A.** Sham group; **B.** IHHP group; **C.** IR group; **D.** IHHP+IR group.

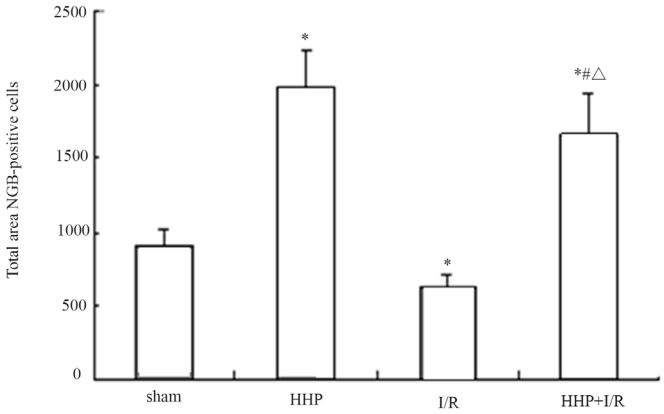

Figure 3. Effect of IHHP on the total area of NGB positive cells in the hippocampal CA1 subfield of rats with global cerebral IR (400X). Means ± SD, N = 6. *P < 0.01 *vs* Sham group; $^{\#}$P < 0. 01 *vs* IR group; $^{\Delta}$P < 0.01 *vs* IHHP group.

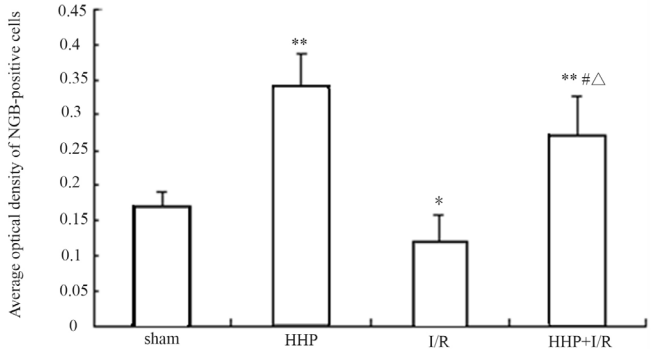

Figure 4. Effect of IHHP on the average optical density of NGB positive cells in the hippocampal CA1 subfield of rats with global cerebral IR (400X). Means ± SD, N = 6 *P < 0.05, **P < 0.01 *vs* Sham group; $^{\#}$P < 0.01 *vs* IR group; $^{\Delta}$P < 0.01 *vs* IHHP group.

Effects of IHHP on the expression of Bcl-2 in the rat hippocampal CA1 area with IR

Microscope observations showed that the Bcl-2-positive cell area and average optical density of positive cells were significantly increased ($P < 0.01$) in the IHHP group compared with sham group (Figures 5B, 6, and 7). The Bcl-2-positive cell area and average optical density of positive cells were significantly increased in the I/R group ($P < 0.01$, Figures 5C, 6, and 7). The Bcl-2-positive cell area and average optical density of positive cells were significantly increased in the IHHP+I/R group ($P < 0.01$) compared with I/R group, and significantly higher than the IHHP group ($P < 0.01$, Figures 5D, 6, and 7).

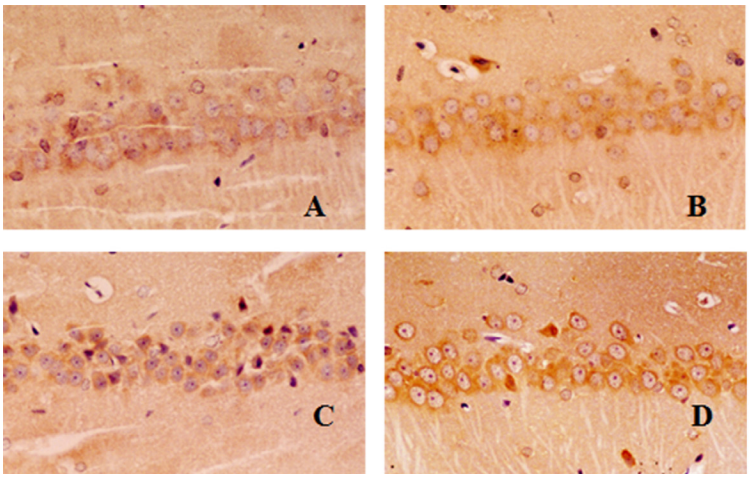

Figure 5. Effect of IHHP on Bcl-2 protein expression in the hippocampal CA1 subfield of rats with global cerebral IR by immunohistochemistry (400X). **A.** Sham group; **B.** IHHP group; **C.** IR group; **D.** HHP+IR group.

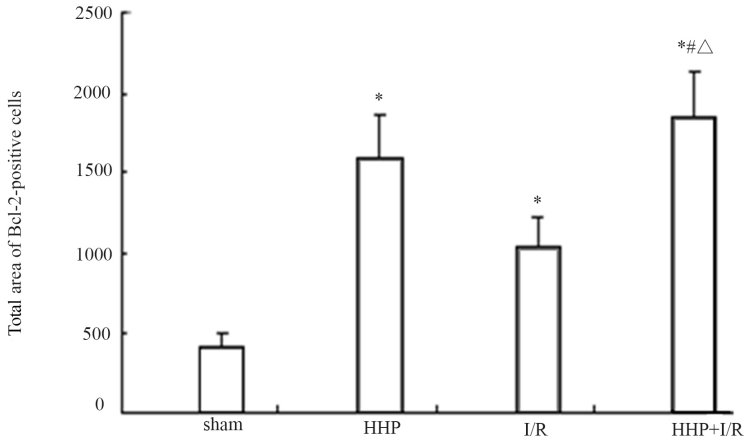

Figure 6. Effect of IHHP on the total area of Bcl-2 positive cells in the hippocampal CA1 subfield of rats with global cerebral IR (400X). Means ± SD, N = 6, *P < 0.01 *vs* Sham group; $^{\#}P < 0.01$ *vs* I/R group; $^{\Delta}P < 0.01$ *vs* IHHP group.

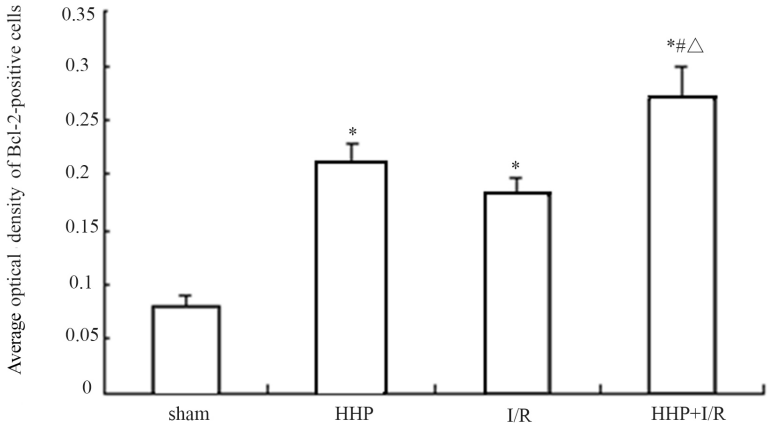

Figure 7. Effect of IHHP on the average optical density of Bcl-2 positive cells in the hippocampal CA1 subfield of rats with global cerebral IR (400X). Means ± SD, N = 6, *P < 0.01 *vs* Sham group; #P < 0.01 *vs* I/R group; △P < 0.01 *vs* HHP group.

DISCUSSION

NGB is the third kind of oxygen-carrying protein discovered by Burmester et al (2000). It can enhance the ability of oxygen coming into mitochondrion and improve the oxygen utilization rate, so as to provide enough oxygen for neuronal cellular metabolism. *In vitro* and *in vivo* experiments confirmed that anoxic environments could upregulate the expression of NGB (Jin et al., 2010), and that increasing NGB brain expression had significant neuroprotective effects. In this study, NGB-positive cells in the hippocampal CA1 area and the average optical density were significantly higher in the IHHP group than the sham group and the expression of NGB in the IHHP+I/R group was significantly higher than that in the I/R group. This suggests intermittent hypobaric hypoxia could increase the expression of NGB, in agreement with the view of Chen et al (2008). They found that the expression level of NGB mRNA and protein were increased in re-perfused mice after a short time (6-48 h), indicating NGB might have stress protective effects on brain tissues. Zhang et al. (2013) suggested that *Potentilla anserina* L. alcohol extract might enhance the tolerance of neurons to hypoxia, relieve neuronal damage, and protect neurons by promoting NGB expression, indicating that NGB was beneficial in protecting neurons from another side.

Burmester et al. (2000) showed there was close relationship between the distribution degree of NGB in different brain regions and the tolerant ability of brain regions to hypoxia. In this study, NGB expression in the rat hippocampal CA1 area was lower in the I/R group than the sham group. There were also many areas where NGB was completely not expressed, which might be caused by neuron loss resulting from brain tissue injury due to hypoxic ischemia. The expression of NGB in the hippocampal CA1 area was increased in the IHHP and IHHP+I/R groups, which suggests hypobaric hypoxia could upregulate NGB expression in the hippocampal CA1 area. It also indicates that NGB had important protective effects against cerebral ischemic injury, and that its expression level might be a factor in the differential tolerant ability of different brain regions to cerebral ischemic injury. Another study (Reuss et al., 2002) revealed that the content of NGB and tolerance of nerve tissues to hypoxia was low, indicating that NGB plays important roles in adaptively protective processes during cerebral anoxia, and

also suggesting that NGB might increase the oxygen supply to nerve cells to improve their survival rate and function. NGB is an oxygen-carrying protein and has a close relationship with oxygen supply in the brain. It has a significant effect in glial cells and could help to understand and treat cerebral hypoxia and neurodegenerative diseases (such as senile dementia). It might be possible to decrease the incidence and development of hypoxia at very early stages using NGB, making cerebral hypoxia possible to control (Yin et al., 2005).

Bcl-2 is a kind of endogenous defender against apoptotic death. Over-expression of Bcl-2 in neurons can inhibit neuron apoptosis induced by IR injury through maintaining the integrity of mitochondrion (Xing et al., 2008; Zhang et al., 2008). Bcl-2 content is extremely low in normal brain tissues and almost undetectable using common immunohistochemistry assays (Hong et al., 2002). In our study, there was scattered and low expression of Bcl-2 in the sham group, which might be due to operation injury. Bcl-2 expression was higher in the I/R group compared with the sham group. We hypothesize that Bcl-2 could be highly expressed in surviving cells early after I/R to inhibit apoptosis. The Bcl-2-positive cell area was increased in the IHHP group compared with the sham group, and Bcl-2 expression was significantly higher in the IHHP+I/R group than the I/R group, suggesting IHHP reduced neuronal apoptosis after cerebral ischemic by upregulating Bcl-2 expression in the hippocampal CA1 area.

Li (2009) found that the expression of Bcl-2 mRNA was significantly increased after NGB expression upregulation in the hippocampal CA1 area by limb ischemic preconditioning. The expression of Bcl-2 mRNA was significantly decreased after downregulating the expression of NGB by injecting NGB antisense-oligodeoxynucleotides (AS-ODNs) into the rat lateral ventricle before limb ischemic preconditioning. An *in vitro* experiment found NGB was over-expressed in PC12 cells using the single nucleotide polymorphisms (SNP), nitric oxide (NO) donor, which could significantly relieve cell injury induced by SNP. A subsequent study found that Bcl-2 expression was significantly improved after overexpression of NGB (Chen, 2008). Thus, we hypothesized that upregulation of NGB was induced by IHHP preconditioning, and its mechanism against injury might be on the mitochondrial apoptosis pathway, in which it decreased neuron apoptosis from cerebral ischemic by increasing the expression of Bcl-2. While the anti-apoptotic ability of Bcl-2 is limited, it cannot be the only pro- or anti-apoptotic factor after cerebral ischemia. There should be other apoptosis regulation genes participating and further study is needed to investigate the regulation effects of various genes.

REFERENCES

Brown JE, Zeiger SL, Hettinger JC, Brooks JD, et al. (2010). Essential role of the redox-sensitive kinase p66shc in determining energetic and oxidative status and cell fate in neuronal preconditioning. *J. Neurosci*. 30: 5242-5252.

Burmester T, Weich B, Reinhardt S and Hankeln T. (2000). A vertebrate globin expressed in the brain. *Nature* 407: 520-526.

Chen XL, Chen R, Gao CJ, Xu SM, et al. (2008). Kinetic change in the expression of neuroglobin in mouse hippocampus after transient forebrain ischemia. *Chin. J. Zool.* 43: 147-152.

Chen TF (2008). Preliminary study of myoglobin neuroprotective function and mechanism of the brain. Doctoral thesis, Academy of Military Medical Sciences, Beijing.

Hong KW, Kim KY, Lee JH, Shin HK, et al. (2002). Neuroprotective effect of (2S,3S,4R)-N-cyano-N-(6-amino-3,4-dihydro-3-hydroxy-2-methyl-2-dimethoxymethyl-2H-benzopyran-4-yl)-N'-benzylguanidine (KR-31378), a benzopyran analog, against focal ischemic brain damage in rats. *J. Pharmacol. Exp. Ther.* 301: 210-216.

Jin K, Mao Y, Mao X, Xie L, et a1. (2010). Neuroglobin expression in ischemic stroke. *Stroke* 41: 557-559.

Li SQ (2009). Neuroglobin effect and mechanism of limb ischemic preconditioning in brain protection. Doctoral thesis, Department of Physiology, Hebei Medical University, Hebei.

Lu GW, Shi MT, Li L and An YY (1992). A preliminary study of repeated acute hypoxic hypoxia tolerance in mice and its mechanism. *Zhongguobinglishenglizazhi* 8: 425-430.

Pulsinelli WA and Brierley JB (1979). A new model of bilateral hemispheric ischemia in the unanesthetized rat. *Stroke* 10: 267-272.

Racay P, Chomova M, Tatarkova Z, Kaplan P, et a1. (2009). Ischemia induced mitochondrial apoptosis is significantly attenuated by ischemic preconditioning. *Cell Mol. Neurobio1*. 29: 901-908.

Reuss S, Saaler-Reinhardt S, Weich B, Wystub S, et al. (2002). Expression analysis of neuroglobin mRNA in rodent tissues. *Neuroscience* 115: 645- 656.

Wu Q, Qi J, Liu WL, Li QJ (2012). The protective effect of intermittent hypobaric hypoxia preconditioning on the hippocampal neuron of rats with cerebral ischemia/reperfusion. *J. Qinghai Medical College*. 33: 16-21

Xing B, Chen H, Zhang M, Zhao D, et al. (2008). Ischemic preconditioning inhibits apoptosis after focal cerebral ischemia/reperfusion injury in the rat. *Stroke* 39: 2362-2369.

Yin SH, Gong SS, Yan KS, Li S, et al. (2005). Construction of recombinant plasmid pEGFP-NGB mice and experimental study of the expression of glial cells in culture. *Zhongguobinglishenglizazhi* 21: 1388- 1392.

Zhang H, Li Q and Li Z (2008). The protection of Bcl-2 overexpression on rat cortical neuronal injury caused by analogous ischemia/reperfusion *in vitro*. *Neurosci. Res.* 62: 140-146.

Zhang Y, Li LZ, Yang H, Gong HY, et al. (2013). Juema affect brain tissue protein in red on hypobaric hypoxia in rat brain. *Tianjinzhongyiyao* 30: 224-227.