Protective effect of Yulangsan polysaccharide on focal cerebral ischemia/reperfusion injury in rats and its underlying mechanism

Jian Chen, *MD, PhD,* ***Ren-Bin Huang,*** *MD, PhD.*

ABSTRACT

الأهداف: دراسة إصابة إعادة إشباع الخلية للإقفار (I/R) الدماغي البؤري المعالج بواسطة إطباق الشريان الدماغي الوسطي (MCAO) وتأثيرات عديد سكرايد يولنقسان (YLS) في هذه الإصابة.

الطريقة: أجريت هذه الدراسة في مختبر أبحاث علم الأدوية – جامعة قوانقشي الطبية – الصين – خلال الفترة مارس حتى مايو 2007. تم تقسيم 240 جرذ عشوائياً إلى مجموعة (I/R) و مجموعة أجريت لها عملية خادعة، و مجموعات تلقت جرعة عالية، و متوسطة، و قليلة من عديد سكرايد YLS، ومجموعة نيموديبين (Nim). تم إعطاء الحيوانات الأدوية لمدة 7 يوم. تم إجراء العملية لتحريض نموذج MCAO في الجرذان. تم البدء بإعادة إشباع الخلية بعد 2 ساعة من MCAO. تم فحص تأثيرات عديد سكرايد YLS في المؤشرات العصبية، محتوى ماء الدماغ، حجم الإحتشاء، نشاطات SOD و NOS، محتويات MDA و NO، كما تم فحص تعبيرات Bcl-2 و باكس في النسيج الدماغي. تمت ملاحظة تغيرات في الخلايا العصية القشرية الدماغية للجرذ.

النتائج: مقارنة مع مجموعة I/R، يقلل عديد سكرايد YLS بشكل ملحوظ المؤشرات العصبية، وكمية ماء الدماغ، و حجم الإحتشاء، ومحتويات MDA و NO، ونشاط NOS، و تعبيرات الباكس، ويزيد نشاط SOD و تعبير Bcl-2 في أنسجة الدماغ، كما يقلل من الوذمة العصبية.

خاتمة: أن لعديد سكرايد YLS أثر حماية في إصابة إعادة إشباع الخلية للإقفار الدماغي، كما أن آليته متعلقة بالتقليل من زيادة الجذر الحر Bcl-2 و أشعة باكس.

Objectives: To study the focal cerebral ischemia/reperfusion (I/R) injury induced by a middle cerebral artery occlusion (MCAO), and the effects of Yulangsan (YLS) polysaccharide on this injury.

Methods: This study took place in the Pharmacology Research Laboratory at Guangxi Medical University, China, between March and May 2007. Two hundred and forty rats were randomly divided into I/R group, sham-operated group, high-, medium-, and low-dose of YLS polysaccharide groups, and nimodipine (Nim) group. The animals were intragastrically administered with drugs for 7 days. An operation was performed to induce an MCAO model in the rats. Reperfusion was started after 2 hours of MCAO. The influences of YLS polysaccharide on the neurological score, the brain water content, the infarct volume, the activities of super oxide dismutase (SOD) and nitric oxide synthase (NOS), the contents of malondialdehyde (MDA) and nitric oxide (NO), the expressions of B-cell lymphoma/leukemia-2 (Bcl-2) and Bcl-2-associated X protein (Bax) in brain tissue were investigated; the morphological changes of rat cerebral cortical neurons were observed.

Results: Compared with the I/R group, YLS polysaccharide reduced the neurological score, the brain water content, the infract volume, MDA and NO contents, the NOS activity, and the expression of Bax, and increased SOD activity, and the expression of Bcl-2 in the brain tissue, and neuronal edema was reduced.

Conclusion: The YLS polysaccharide has a protective effect on cerebral ischemia/ reperfusion injury; the mechanism may be related to attenuating free radicals, and increasing the Bcl-2/Bax ratio.

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From the Department of Pharmacology (Chen, Huang), Guangxi Medical University, Nanning, and the Department of Physiology (Chen), Guilin Medial University, Guilin, China.

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Address correspondence and reprint request to: Dr. Ren-Bin Huang, Department of Pharmacology, Guangxi Medical University, Nanning 530021, China. Tel. +86 (0771) 5358014. Fax. +86 (0771) 5358014. E-mail: huangrenbin@163.com

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Over the past few years, research has focused on the damage to cerebral tissues after ischemia. The pathophysiological mechanism of cerebral ischemia is very complex. Previous studies have demonstrated that it is related to many factors, such as energy metabolism dysfunction[1] of brain tissue following cerebral ischemic injury, production of massive free radicals,[2] platelet activation, changes of hemorheology, and so forth. At present, the theory of cascade of damage has been raised, which includes energy failure, peri-infarct depolarizations, inflammation,[3] and programmed cell death.[4,5] Yulangsan (YLS) is the root of the *Millettia pulchra Kurz var-laxior (Dunn) Z. Wei*.[6] The extract of YLS has proved to be an effective antioxidant, and is used in the treatment of neurological and cardiovascular diseases.[7,8] Yulangsan polysaccharide is the major effective ingredient.[9,10] The YLS polysaccharide is capable of inhibiting peroxidation in vitro and suppressing production of free radicals in excess in vivo.[11] The aim of this study was to elucidate the beneficial effects of YLS polysaccharide on cerebral injury. Based on the results of experiments,[10,11] nimodipine (Nim) has a beneficial effect on active treatment, and so was used as a control measure in this study.

Methods. This study took place in the Pharmacology Research Laboratory at Guangxi Medical University, China, between March and May 2007. Two hundred and forty male Sprague-Dawley rats weighing 230-280g were obtained from the Experimental Animal Center. All the experimental procedures were performed in accordance with the guidelines of the Experimental Research Institute of Guangxi Medical University. Rats were divided into 6 groups: the sham-operated group; the vehicle-treated ischemia/reperfusion (I/R) group that received NS0.5ml·kg^{-1} for 7 days before ischemia; the YLS polysaccharide-treated I/R groups that received YLS polysaccharide in 0.6, 0.3, and 0.15 g·kg^{-1} doses for 7 days before ischemia; and the Nim-treated I/R group that received Nim 0.02 g·kg^{-1} for 7 days before ischemia. The YLS polysaccharide, was dissolved in normal saline (NS). One hour after administration, all the rats were anesthetized with 10% chloral hydrate (350 mg·kg^{-1}) intraperitoneally (ip), and brain I/R injury was induced by a middle cerebral artery occlusion (MCAO). By introducing a nylon suture as described previously,[12] the right common carotid artery, external carotid artery (ECA), and the internal carotid artery (ICA) was isolated via a ventral midline incision. A nylon monofilament, with its tip rounded by heating near a flame, was introduced into the ECA lumen and advanced into the ICA to block the origin of the middle cerebral artery (MCA). The rectal temperature was maintained at 37-38°C with a heating lamp and heating pad during the operation. The room temperature was controlled in the range of 25-27°C throughout the experimental procedure. After 2 hours of ischemia, the suture was removed and the animals were allowed to recover. Neurological deficits were evaluated after 24 hour reperfusion using a 5-point scoring system according to the method of Longa et al.[12] Then 120 rats were anesthetized with 10% chloral hydrate (mg·kg^{-1}) ip. The brains were carefully removed after the rats were sacrificed at different times. The area of cerebral infarction was quantified with 2,3,5-Triphenyltetrazolium chloride (TTC) staining. The brains were sectioned coronally with a brain slicer at 2 mm intervals from the frontal pole. All slices were incubated for 30 minutes in a 2% solution of TTC at 37°C, and fixed by immersion in 10% formaldehyde solution. With a computerized image analysis system (NIH Image, National Institutes of Health, Bethesda (MD), Version 1.63), the area of infarction of each section was determined. The total lesion volume was calculated by summing up the infarct area in each section and multiplying it by the distance between sections. The brain water content was assessed by comparing the wet weight against the dry weight, which was determined after being kept in an oven at 105°C until the weight remained unchanged. The water content of these samples was then measured by the wet and dry method as follow: water content (%) = (wet weight-dry weight)/wet weight × 100%. Sixty of the other rats were sacrificed and the brains were rapidly removed and separated into right and left hemispheres on ice. Tissue samples, obtained from the surrounding region of the MCA distribution in the ipsilateral hemisphere were isolated and stored at -80°C for further biochemical analysis. The spectrophotometer methods were used to assay super oxide dismutase (SOD), and nitric oxide synthase (NOS) activity, and malondialdehyde (MDA), and nitric oxide (NO) levels according to the description of the assay kits. The other 60 rats were anesthetized again and perfused with normal saline solution, followed by 4% paraformaldehyde. The brain ischemic penumbra area was removed, and post-fixed in the same fixative and cryoprotected in 25% sucrose solution. The brain tissues were embedded in paraffin blocks and sectioned at 8 µm thickness for hematoxylin-eosin (HE) staining or 20 µm for immunohistochemical study. The expression of B-cell lymphoma/leukemia-2 (Bcl-2) and Bcl-2-associated X protein (Bax) protein in the brain was visualized by immunohistochemical assay kit (Boster, Wuhan, China), as recommended by the manufacturer. Briefly, the endogenous peroxidase activity of the sections was blocked with H_2O_2. Then, the sections were incubated with the rabbit anti-rat Bcl-2 and Bax polyclonal antibody (Boster, China), biotinylated goat anti-rabbit IgG (Boster, China), and

avidin-biotin-peroxidase complex in turn. After staining with 3,3'-Diaminobenzidine (DAB), the sections were observed under light microscopy. Four fields were randomly selected from the cortex and striatum to count the positive cells under high-power microscope (×400).

The data were expressed as mean ± standard deviation. The Statistical Package for Social Sciences version 13.0 (SPSS Inc, Chicago, IL) was used for standard statistical analysis including one-way ANOVA and Student's t-test. A value of $p<0.05$ was considered statistically significant.

Results. As shown in **Table 1**, after treatment with YLS polysaccharide the infarct volume and the brain water content were significantly reduced. The neurological deficit was ameliorated. There was no significant difference in neurological score between the YLS 0.15 $g \cdot kg^{-1}$ group and the I/R group. As shown in **Table 2** and **Figure 1**, after treatment with YLS polysaccharide, the MDA, NO content, and NOS activity were significantly reduced and SOD activity was significantly increased as compared with the I/R group. As shown in **Figure 2**, in the sham-operated group, the morphology of neurons was oval or multi-angled in shape, in the ischemic areas the nuclei of the remaining neurons were pyknotic and the neural fibers became vacuolar, there were obvious gaps around the cells, with the absence of nucleolus, and there were many scattered cellular fragments. Neuron defects in the infracted center of YLS polysaccharide groups were significantly slighter than that of model group. As shown in **Figures 3 & 4**, and **Table 3**, less positive expressions of Bcl-2 and Bax were found in the cerebral cortex in the sham operation group, and in

Table 1 - Effect of Yulangsan (YLS) polysaccharide on brain function, water content, and infract volume in cerebral ischemia reperfusion rats (mean ± SD, n=10).

Group	Dose ($g \cdot kg^{-1}$)	Neurological score	Ratio of infarct area (%)	Water content (%)
Sham		0.0±0.00		78.56±0.91
Ischemia/reperfusion		3.2±0.79	25.65±2.97	83.23±1.44
YLS polysaccharide (high)	0.6	1.7±1.06 (p=0.0021)*	14.28±3.76 (p<0.0001)*	79.21±1.11 (p<0.0001)*
YLS polysaccharide (medium)	0.3	2.1±0.99 (p=0.0134)*	18.36±4.02 (p=0.0002)	81.22±1.48 (p=0.0064)
YLS polysaccharide (low)	0.15	2.7±1.16	21.44±4.99 (p=0.0341)	81.74±1.54 (p=0.0389)
Nimodipine	0.02	2.2±0.92 (p=0.0177)*	15.41±2.41 (p<0.0001)*	79.43±1.26 (p<0.0001)*

*versus ischemia/reperfusion group

Table 2 - Effect of Yulangsan (YLS) polysaccharide on the activities of SOD, NOS and the contents of MDA, NO in cerebral ischemia reperfusion rats (mean ± SD, n=10).

Group	Dose ($g \cdot kg^{-1}$)	Superoxide dismutase (U/mgprot)	Nitric oxide synthase (U/mgprot)	Malondialdehyde (nmol/mgprot)	Nitric oxide (umol/gprot)
Sham		134.52±17.04	0.82±0.15	1.32±0.33	7.62±0.57
Ischemia/reperfusion		70.15±20.43	2.46±0.28	5.27±0.90	14.91±0.62
YLS polysaccharide (high)	0.6	119.10±23.81 (p=0.0002)*	1.16±0.16 (p<0.0001)*	2.01±0.56 (p<0.0001)*	8.44±0.34 (p<0.0001)*
YLS polysaccharide (medium)	0.3	97.97±20.06 (p=0.0106)*	1.72±0.25 (p<0.0001)*	3.12±0.71 (p=0.0001)*	10.11±0.46 (p<0.0001)*
YLS polysaccharide (low)	0.15	88.88±13.98 (p=0.0435)*	2.17±0.28 (p=0.0243)*	4.32±0.64 (p=0.0151)*	12.25±0.66 (p=0.0104)*
Nimodipine	0.02	111.26±13.07 (p=0.0002)*	1.29±0.17 (p<0.0001)*	2.36±0.46 (p<0.0001)*	8.75±0.42 (p<0.0001)*

*versus ischemia/reperfusion group, U/mgprot - milligrams of protein

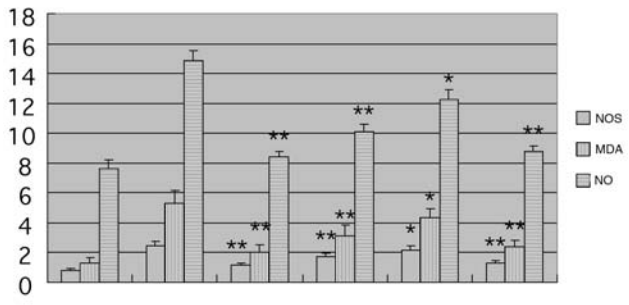

Figure 1 - Effect of YLS polysaccharide on the activities of SOD, NOS, and the contents of MDA, and NO in cerebral ischemia reperfusion rats. Versus ischemia/reperfusion group: *p<0.05,**p<0.01. YLS - Yulangsan, SOD - super oxide dismutase, NOS - nitric oxide synthase, MDA - malondialdehyde, NO - nitric oxide

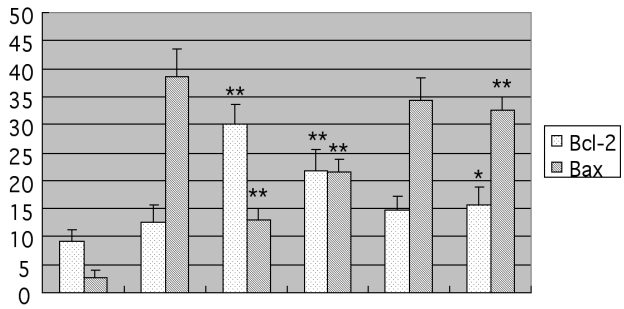

Figure 3 - Effect of YLS polysaccharide on expression of Bcl-2 and Bax in cerebral ischemia reperfusion rats. Versus ischemia/reperfusion group: *p<0.05,**p<0.01. YLS - Yulangsan, Bcl-2 - B-cell lymphoma/leukemia-2, Bax - Bcl-2-associated X protein

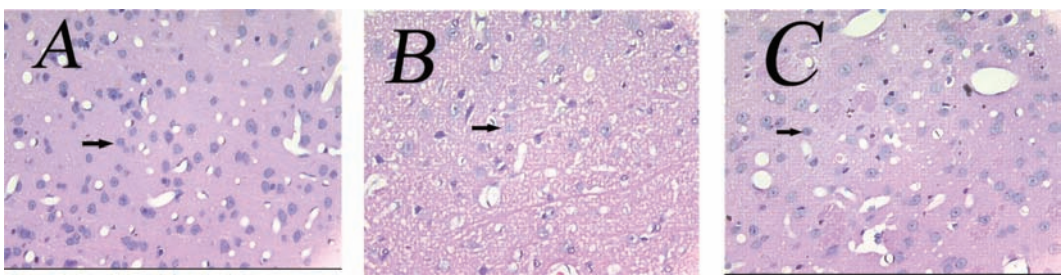

Figure 2 - Representative photographs of cerebral cortex after ischemia reperfusion in rats (Hematoxylin-eosin staining x400). **A:** sham-operated group; **B:** ischemia reperfusion group; **C:** Yulangsan polysaccharide 0.6g·kg^{-1} group.

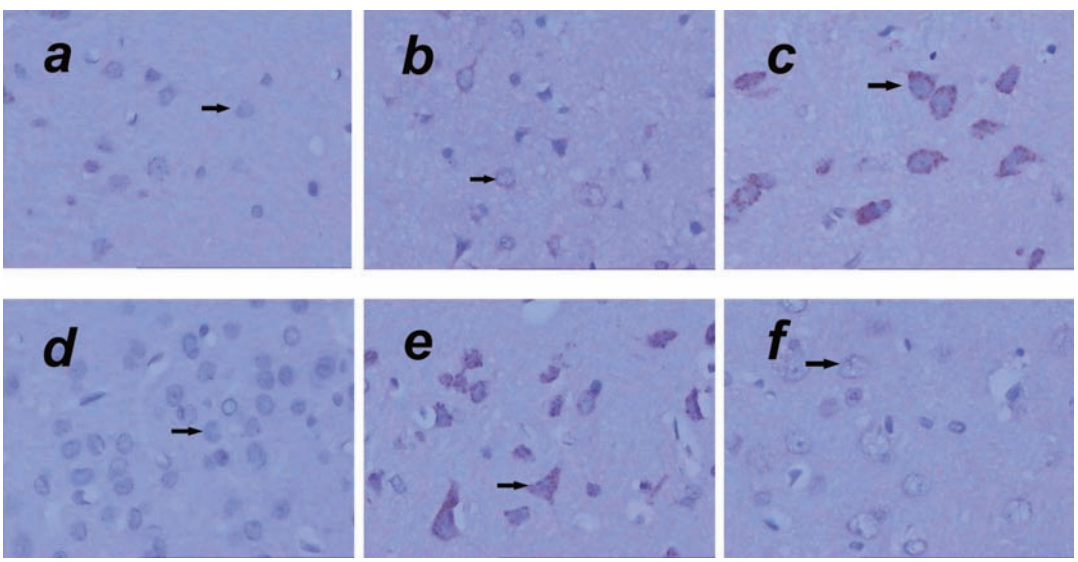

Figure 4 - Representative photographs of expression of Bcl-2 and Bax expression in cerebral cortex after ischemia reperfusion in rats (x400). **a, b, c**: expression of Bcl-2 **a:** sham-operated group; **b:** ischemia reperfusion group; **c:** Yulangsan polysaccharide 0.6g·kg^{-1} group. **d, e, f:** expression of Bax **e:** sham-operated group; **d:** ischemia reperfusion group; **f:** Yulangsan polysaccharide 0.6g·kg^{-1} group.

Table 3 - Effect of Yulangsan (YLS) polysaccharide on expression of Bcl-2 and Bax in cerebral ischemia reperfusion rats.

Group	Dose ($g \cdot kg^{-1}$)	Bcl-2	Bax
Sham		9.1±2.13	2.6±1.51
Ischemia/reperfusion		12.6±2.99	38.5±4.89
YLS polysaccharide (high)	0.6	30.0±3.65 (p<0.0001)*	12.9±2.13 (p<0.0001)*
YLS polysaccharide (medium)	0.3	21.8±3.74 (p<0.0001)*	21.5±2.25 (p<0.0001)*
YLS polysaccharide (low)	0.15	14.7±2.50	34.4±3.86 (p=0.0243)*
Nimodipine	0.02	15.8±3.05 (p=0.0318)*	32.5±2.42 (p=0.0068)*

*versus ischemia/reperfusion group

the model group the expression of Bax was higher than those in the sham operation group (p<0.0001), but the expression of Bcl-2 showed no remarkable difference. The YLS 0.6 and 0.3 $g \cdot kg^{-1}$ treatment groups showed a significant decrease in the positive expression of Bax, and an increase in the positive expression of Bcl-2. There was no significant difference in the positive expression of Bcl-2 and Bax between the YLS 0.15 $g \cdot kg^{-1}$ and I/R groups.

Discussion. In this study, we have shown that the YLS polysaccharide has a positive effect on experimental cerebral ischemia in rats, as the mean infarct size, water content, and neurological score of rats treated with YLS polysaccharide were significantly lower than that of the control group.

The reactive-oxygen species are inevitably formed as byproducts of various, normal cellular processes involving interactions with oxygen. These reactive-oxygen species damage macromolecules in the cells, and therefore, sometimes make significant contributions to the several pathological processes of I/R injury. As the final product of lipid peroxidase, MDA was highly reactive and responsible for cytotoxic effects and neuronal death. Indeed, numerous studies[1,2] have demonstrated that antioxidant enzymes can provide substantial protection against oxidative stresses. Also, recombinant enzymes such as SOD have been used to protect against oxidative stresses. With the reperfusion occurrence and development of reperfusion, the large burst of free radicals are yielded in the penumbra, which contributes to lipid peroxidation, membrane damage, as well as inflammation, and apoptosis. The evidence that free radical scavengers used after cerebral ischemia are still effective indicates that free radicals are initiators in ischemic cerebral injury and subsequent reperfusion injury,[13] NO in living beings is also a free radical synthesized by L-arginine and O_2. The NOS in the brain exists in the vascular endothelia cell, peripheral vascular nerve, and is distributed in the neurons, therefore, NO is probably involved in the incidence of cerebral ischemia. Studies[14,15] have indicated that NO has toxic effects in ischemic cerebral injury. The activated NOS synthesize NO, which produces neurotoxicity by means of a compound of NO-ferrum, oxygenation of protein sulfhydryl, superoxide anion, and so forth. In the present experiment, we demonstrated that the YLS polysaccharide significantly raised SOD activity, decreased MDA and NO levels, and NOS activity, and showed a strong anti-oxidative effect.

Cell apoptosis refers to programmed cell death (PCD), which is an initiative suicide process after the cells receive a signal or are stimulated through interaction with some other related gene, characterized by deoxyribonucleic acid degradation in an earlier period. It is an initiative cell suicide process of multi-celled organisms controlled by genes. The purpose is to regulate organism development and keep homeostasis, which is different from the necrosis of cells.[16,17] This PCD occurs during cerebral I/R injury. The mitochondrion plays a significant role in the process of apoptosis, and determines necrosis, or apoptosis. The Bcl-2 is the earliest related gene with apoptosis that has been researched. The Bcl-2 gene family is distributed to the mitochondrial outer membrane, and they are divided into suppressing or enhancement apoptosis proteins, the former like Bcl-2, Bcl-xL, Bcl-W, Mcl-1, the latter like Bax, Bak, Bok.[18] To maintain stabilization of the mitochondrion, the suppressing apoptosis protein of the Bcl-2 gene family might be implemented by regulating permeability and transmembrane pressure of the mitochondrial membrane. It was previously reported[4,5,19] that following detection of the normal expression of Bcl-2 production in the human body and various cells, the expressed content of Bcl-2 had a positive correlation with the length of cell life. Thus, the Bcl-2 gene may prolong the length of cell life by regulation of PCD.[19] In the present experiment, we demonstrated that high and medium dose YLS polysaccharide significantly reduced the expression of Bax and increased the expression of Bcl-2.

A major limitation of the present study is that the YLS polysaccharide was administered 7 days prior to MCAO. From a therapeutic standpoint, such a treatment regimen would be of certain clinical value in the prevention of human stroke patients. In future experiments, we will test whether the YLS polysaccharide

is capable of providing benefits when administered after reperfusion.

In conclusion, our results suggest that the YLS polysaccharide has significant protection against cerebral I/R injury via the mechanism of up-regulating Bcl-2 and down-regulating Bax, and increasing the Bcl-2/bax ratio and their antioxidant activity.

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