Refined Qing Kai Ling, Traditional Chinese Medicinal Preparation, Reduces Ischemic Stroke-Induced Infarct Size and Neurological Deficits and Increases Expression of Endothelial Nitric Oxide Synthase

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Refined Qing Kai Ling (RQKL) is an improved injectable multi-component preparation derived from Qing Kai Ling, which could offer the neuroprotection effect in middle cerebral artery occlusion (MCAO) model of rats by relieving the damage of vascular endothelial cell as well as inhibiting the process of inflammation. Herein, we observed whether RQKL could exert influence on the expression of endothelial nitric oxide synthase (eNOS), as a mechanism of its protective effects against ischemia. Sprague-Dawley rat model of focal cerebral ischemia was established by permanent filament occlusion of the left middle cerebral artery. We found that the administration of RQKL could reduce the ischemic infarct size as well as neurological deficit of model rats. Furthermore, it was showed that the eNOS level was consistently increased in endothelium of blood vessels of the ischemic penumbra after 2 to 72 h of permanent MCAO, and the expression of eNOS increases more in animals treated with RQKL. Our results suggested that eNOS levels in penumbral zone were enhanced after permanent focal ischemia, and RQKL could stimulate postischemic eNOS expression, which may be an important mechanism in RQKL’s protection against cerebral ischemia.

Key words cerebral ischemia; endothelial nitric oxide synthase; Refined Qing Kai Ling

Production of nitric oxide (NO) by nitric oxide synthase (NOS) has been confirmed as a major mechanism in physiological and pathological processes in the central nervous system.1,2) In cerebral ischemia, because of the different cellular sources and the different stages of the ischemic process, the role of NO can be protective or destructive.3) Studies have shown that NO produced by endothelial nitric oxide synthase (eNOS) plays a prominent role in maintaining cerebral blood flow and preventing neuronal injury as well as inhibiting platelet and leukocyte adhesion, and thus protects against stroke.4–6)

Consequently, the protective role of NO in brain ischemia underlines the necessity for selective therapeutic approaches to augment eNOS. Recently, several therapeutic modalities to upregulate and/or activate eNOS have been proved that it might mediate NO-dependent stroke-protective effects,7) such as statins could improve stroke outcome through the eNOS-dependent mechanism.8–10) Particularly, an extract of Ginkgo biloba, which has been reported to be able to alleviate cerebrovascular problems, can significantly enhance the level of eNOS mRNA as well.11)

Many therapeutics and remedies of traditional Chinese medicine are proved effective for stroke through years of practice. Refined Qing Kai Ling (RQKL) is an improved injection derived from the combination of effective and safety components in Qing Kai Ling injection, which has clinically been practiced as a useful injection to treat acute stroke.12) We have previously demonstrated that some components of RQKL could relieve the damage of vascular endothelial cell and inhibit the process of inflammation, offering the neuroprotection effect in middle cerebral artery occlusion (MCAO) model of rats.13) Herein, we investigated whether its cerebroprotective effects are correlated with the expression of eNOS under ischemic condition.

MATERIALS AND METHODS

Animal Model All experiments were performed on male SD rats (Experimental Animal Company of Beijing Vital, Certificate No. 19-053), weighing 280 to 320 g. The animal experiments were conducted in accordance with the UK Animals (Scientific Procedures) Act 1986 and associated guidelines. Focal cerebral ischemia was induced by the filament model according to the method of Longa et al.14) In short, animals were anesthetized with 10% chloral hydrate (0.35 g/kg, intra-peritoneally (i.p.)). After median incision of the neck skin, the left external carotid artery (ECA) was carefully dissected. An 18.5±0.5 mm length of nylon suture (d=0.25 mm, with one end rounded by heat and d<0.3 mm) was introduced into transected lumen of ECA and gently advanced into the internal carotid artery to block the origin of the left middle cerebral artery. Afterward, retracted soft tissues were replaced, wounds were sutured, and the rats were put back into their cages. Body temperature was kept at 37°C with a heat lamp and a piece of heating pad during operation until animal regained consciousness. After awakening, a brief behavioral assessment was performed on rats. The rats without right forelimb paresis were regarded unsuccessful MCAO, and these animals were excluded from further study. Thereafter, rectal temperature of rats with right forelimb paresis was checked every 10 to 15 min during the following 2 h and, if necessary, it was corrected to 37°C by placing a heating pad under the cage. Sham-operated animals underwent identical procedures except for only brief insertion of the filament into the ECA stump. Sham-operated animals served as controls in all experiments mentioned.

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Treatment With RQKL  The concentrated solution of RQKL was offered by Pharmaceutical Factory of Beijing University of Chinese Medicine. The individual components of RQKL are cholic acid (7 g/l), jasminoidin (12.5 g/l), baicalin (6.25 g/l), and the hydrolysis fluid of nacre (50 g/l). The working solution of RQKL was diluted 1:3 with saline solution. RQKL at a dose of 3 mL·kg⁻¹ was injected i.p. for the treatment group according to the clinical practice daily dose of patient. All rats had prophylactic administration an hour before the operation. Four hours after the first medication, there was a second administration. Continuous injection was administrated at every 12 h interval until the animal was decapitated.

Determination of Neurological Deficits  Before sacrifice, rats were examined for neurological sensory-motor deficits at 72 h after surgery (model group, sham-operated group and RQKL treatment group; n=6 per group) by an observer blinded to the treatment. Neurological evaluations were performed on a scale from 0 (no deficit) to 10 (severe deficit), as previously described with minor modifications. An animal with a maximal deficit scored ten points and an animal with no deficit scored 0 points.

Percentage of Infarct Volume  Rats were anesthetized and decapitated at 72 h after surgery (n=6 per group). Brains were rapidly removed and sliced to 2 mm-thick coronal sections. Slices were stained with 1% of 2,3,5-triphenyltetrazolium chloride (TTC, Sigma) in 0.1 M PBS (pH 7.4) for 30 min, room temperature (RT), and fixed in 10% buffered formalin overnight. They were photographed on line with an image acquisition system HPIAS-1000 (Beijing Kong Hai Company). The infarct volumes were blindly quantified with analysis software (Image-Pro Plus 5.1) and calculated directly by summing the infarct volume of each section or indirectly to correct for brain edema by subtracting the volume of the undamaged ipsilateral hemisphere from the contralateral hemisphere. And the percentage of infarct volume was calculated by a division of infarct volume to total volume.

Immunohistochemistry/Hematoxylin and Eosin (HE) Staining  Rats in each group were anesthetized and decapitated at 2, 6, 12, 24, 48, and 72 h after MCAO (n=6 per group) by an observer blinded to the treatment. The brains were immersed in 10% formalin and then embedded in paraffin. Coronal sections of 8 μm thickness were cut for HE staining and immunohistochemistry. Sections were hydrated and rinsed in 20 mM PBS, and then incubated with 3% H₂O₂, 10% methanol for 20 min, RT, to block endogenous peroxidase. Slides were sequentially incubated with 5% bovine serum albumin (BSA) (Bioeas Inc.) for 30 min, a mouse anti-rat eNOS monoclonal antibody (dilution 1:1000, Sigma-Aldrich Inc.) overnight, and biotinylated goat anti-mouse IgG (Jackson ImmunoResearch Laboratories Inc., diluted 1:1000 in 2% BSA) for 1 h. Subsequently, SABC Reagent (Boster Company) and DAB were used for section coloration. The primary antibodies were replaced with PBS in negative control and immunohistochemical studies were performed blindly. Adjacent sections to eNOS-stained ones were chosen for HE staining as well as eNOS/hematoxylin double staining. To differentiate infarcted tissue and penumbra tissue, areas of infarct were superimposed on thresholded and contrast-enhanced images of eNOS staining.

Semiquantitative Analysis of Immunostaining  eNOS-stained sections were digitized using a camera mounted on an Eclipse 50i microscope (Nikon) and the image analysis system (Image-Pro plus, version 5.1). To quantify side differences, 3 chosen high magnifications fields in the peri-infarct and the corresponding area in the contralateral hemisphere after MCAO were assessed. These 6 visual fields of each animal were adjusted using the same threshold for both hemispheres to get rid of staining intensity differences. The semiquantitative parameter “percentage of area above threshold” using thresholded grayscale images, which reflects both extent and density of eNOS staining, was calculated for each side.

To compare differences in eNOS immunoactivity of the ischemic hemisphere versus contralateral hemisphere among rats of model and RQKL treatment groups, the ratios of “percentage of area above threshold” of the ischemic hemisphere versus contralateral hemisphere were calculated.

SDS-PAGE/Western Blot  Three of the rats in each group were sacrificed as described above. Left cerebrum was homogenized in 20 mM Tris–HCl, 100 mM NaCl, 1 mM ethylendiamine tetraacetate acid (EDTA), and 1 mM phenylmethylsulfonylfluoride (PMSF) at 4 °C. Samples were centrifuged at 10000 rpm, 4 °C, 10 min. Eighty micrograms of protein was loaded and separated by 9% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Proteins were then transferred to polyvinylidene difluoride (PVDF) membrane. And the membrane was incubated with a monoclonal mouse anti-rat eNOS antibody (Sigma-Aldrich Inc., 1:1000 dilution), or with a monoclonal mouse anti-rat actin antibody (Bioeas Inc., 1:5000 dilution), and then with a goat anti-mouse IgG secondary antibody (Jackson ImmunoResearch Laboratories Inc., 1:5000 dilution). A chemiluminescent system (SuperECL Plus, Applygen Technologies Inc.) and Kodak autoradiographic film were used to visualize the bands. For quantification, all blots were digitized with a scanner. Optical density of bands was measured using a Microsoft-based image processing system (Image Master Total Lab version 1.00; Amersham pharmacia biotech). The antibody.

Statistical Analysis  The data are expressed as mean±S.D. We used one-way ANOVA followed by post hoc analysis for significance with the Student–Newman–Keuls multiple comparison test. A probability value <0.05 was regarded as statistically significant.

RESULTS  RQKL Treatment Reduces the Ischemic Infarct Size as Well as Neurological Deficit of MCAO Rats  To determine whether RQKL administration confers protection against ischemic stroke, the cerebra ischemia infarct volume after 72 h of MCAO were determined by TTC staining (Fig. 1A). The infarctions of model rat showed extensive area, while rat treated with RQKL displayed decreased infarction area. That is to say, the infarction volume was smaller in the animals of RQKL treatment group than that of animals of model group.

The cerebral infarct volumes were later on measured by computer image analysis of TTC-stained 2-mm brain sections (Fig. 1B). In model rats, percentage of infarct volume
was 29.024±8.223 at 72 h (mean±S.D., n=6), showing the presence of brain injury in MCAO animals. However, the percentage of infarct volume of rats treated with RQKL was 18.273±5.901 at 72 h after MCAO (n=6, p<0.05), reduced by 37.04% compared with model group. No evidence of infarction was observed on the TTC slices in sham-operated rats (data not shown), indicating the CNS integrity.

The accompanying motor deficit was next measured to see the RQKL protection (Fig. 1C). In model group, the neurological deficit was 6.25±1.488 at 72 h after MCAO (mean±S.D., n=6). Whereas the neurological deficit of rats treated with RQKL was 4.833±0.753 when evaluated at corresponding time (n=6, p<0.05), reduced by 22.7% compared with model group. No neurological deficits were observed in the sham-operated rats throughout the observation period (data not shown).

We investigated the infarct volume and neurological scores at 2, 6, 12, 24 and 48 h after MCAO as well to evaluate the beneficial effects of the treatment with RQKL. The protection effect of RQKL was observed at other time points after MCAO (data not shown) but not as significantly as that of 72 h, and it has shown no statistical difference with the untreated group.

**RQKL Treatment Increases the Immunoactivity of**

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Fig. 1. **RQKL Treatment Reduces the Ischemic Infarct Size as Well as Neurological Deficit of MCAO Rats**

(A) The cerebral ischemia infarctions of 72 h of MCAO by TTC staining. The ivory white area represents ischemia brain tissue where cellular metabolic activity stopped, and the red area represents non-ischemia brain tissue where metabolic activity went on. The upper line showed TTC staining of model rat, the infarction area was extensive, including cortical layer and diencephalons of one side brain, with fuzzy boundary and significant swelling of brain tissue; the lower line was TTC staining of rat treated with RQKL. The decreased infarction area confined to limited cortical layer, with distinct boundary and slight swelling. (B) The effects of RQKL on cerebral infarct volume after 72 h of MCAO compared with animals of model group. Cerebral infarction volume was determined quantitatively from TTC staining. Treatment with RQKL reduced cerebral infarct size by 37.04% (n=6 per group, *p<0.05). (C) The effects of RQKL on neurological deficits after 72 h of MCAO compared with animals of model group. Deficits were evaluated blindly using an established rating system ranging from 0 (no deficit) to 10 (severe). Neurological deficits were reduced 22.7% in RQKL-treated animals. (n=6 per group, *p<0.05).

Fig. 2. **The immunostaining Studies of Regional Distribution of eNOS Protein in the Model and RQKL Treatment Group**

Coronal sections of 8-mm thickness from rat brains of 24 h and 72 h after ischemia were stained with hematoxylin (blue) and costained with eNOS (brown) antibody in the selected areas of ischemic and nonischemic sides. Bar=50 μm. Note that an increase in signal intensity of eNOS was found in the endothelial cells of all kinds of vessels of ischemic hemispheres after 24 h of MCAO (A), and 24 h of MCAO with RQKL treatment (B). The increase was showed on the sections of 72 h of MCAO (C) and 72 h MCAO with RQKL treatment (D) as well. No changes in signal intensity of eNOS were detected in the contralateral (right) side in either 24 h of MCAO (E) or 72 h of MCAO with RQKL treatment (F). Similar results were duplicated in two other sets of animals.
It was reported that cerebral ischemia itself affects NOS activity and expression. Herein, immunohistochemistry was conducted to examine the regional expression of eNOS. We found that the expression level of eNOS was increased remarkably after MCAO (Fig. 2). For the rats of model group, superimposing of adjacent HE and eNOS/hematoxylin double stained sections revealed increased immunostaining mainly in border areas of infarct (Fig. 2A, C). After 24 h of MCAO, the increase appeared in endothelium of blood vessels of all sizes, including microvessels and relatively large arteries as well as arterioles and veins, but mainly in endothelial cells of microvessels of ischemic hemispheres (Fig. 2A). The increase was also showed on the sections of 72 h of MCAO (Fig. 2C). In infarcted areas, however, eNOS was not obviously stained on the nonischemic side at any examined time point (Fig. 2E). Similar staining patterns were observed on the rats of RQKL treatment group (Figs. 2B, D, F).

Semiquantitative analysis of immunostaining was conducted thereafter. We primarily quantified eNOS expression in the penumbra tissue because it is the most important area for rescue after stroke and eNOS expression was most consistently prominent inside the infarct borders in MCAO model. It was showed that the percentages of positive area of eNOS protein immunostaining were consistently increased in the ischemic penumbra compared with the nonischemic side after 2 to 72 h MCAO (Fig. 3), in both of model (Fig. 3A) and RQKL treatment group (Fig. 3B). It exhibited that eNOS protein expression is increased very rapidly after focal ischemia, and the expression was higher in the ischemic compared with the nonischemic hemisphere in MCAO group (Fig. 3A) as well as in RQKL treatment animals (Fig. 3B). We next compared the eNOS protein of the untreated and RQKL-treated groups directly. Ratio of the positive area percentage of eNOS protein immunostaining of ischemic versus that of nonischemic side showed significant difference between animals treated and untreated ones (Fig. 3C), suggesting an augmentation of the eNOS-side difference after treatment with RQKL.

In sham-operated animals, eNOS was expressed at low levels. Little differences of eNOS immunostaining between the ischemic hemisphere and nonischemic hemisphere were observed, suggesting no substantial effect of the surgical procedure on eNOS expression (data not shown).

**RQKL Treatment Increases Expression Levels of the eNOS Protein after MCAO**

According to the result of immunohistochemistry, eNOS mainly increased in ischemic brains of untreated animals or treated with RQKL. To further examine the potential effect of RQKL increasing postischemic eNOS expression, we compared eNOS protein levels in left cerebrum of RQKL treated animals with that of untreated ones (Fig. 4). All eNOS was showed only in a single band at the appropriate molecular weight range on the blots.
Quantitative western blot to measure expression levels of the eNOS protein was conducted. The blot of actin, a universal protein in the endothelial cells, was used as a loading control and the relative expression of eNOS was normalized by the amount of actin (Fig. 4B). Even though there were some variations among different animals, the bands of eNOS protein on the ischemic side of rats in RQKL treated groups were always greater than those of model groups at individual time points (Fig. 4). Thus, it was confirmed that consistently enhanced eNOS expression in the RQKL treated animals. Duplicate blots demonstrated excellent reproducibility of band intensities as well as treatment effects.

**DISCUSSION**

Our previous studies have shown that the administration of RQKL after an ischemic insult reduces the extent of the brain damaged areas.13) In the present study, we found that its cerebroprotective effects are correlated with the expression of eNOS under ischemic condition.

eNOS protein expression is believed to be an important protective mechanism against cerebral ischemia. The cellular sources and the molecular mechanisms of the stimuli involved in posts ischemic eNOS upregulation are still unknown. However, based on most studies since 1993, vascular eNOS protein expression is believed to increase very rapidly after focal ischemia. Although there were differences between experimental paradigms caused by different animal categories and/or models, in most studies, eNOS expression was higher in the ischemic compared with the nonischemic hemisphere. However, there was different opinion that ischemia did not influence the expression of eNOS17) as well. Herein, in agreement with most previous studies, we found that eNOS expression was significantly increased after MCAO, from 2 h after ischemia, and consistently upregulated to 72 h after ischemia.

We found that eNOS staining occurs in endothelium of blood vessels in ischemic pnumbra after 2 to 72 h of MCAO. The immunostaining appeared in endothelial cells of all sizes blood vessels, mainly in endothelial cells of microvessels. Our results showed that increased eNOS was relatively restricted to penumbral zone, indicating an important role of enhanced eNOS levels in endothelium of penumbral zone microvessels after ischemia. There are also some reports coincidently supported this idea. Hashiguchi reported that there was a simultaneous compensatory response through eNOS activation within the endothelium of blood vessels after ischemia, which mediates vasodilation and hence increases blood flow to the damaged brain area.18) Recently, researchers found that the increase of activation of eNOS occurred in endothelial cells of microvessels after ischemic episodes with temporal expression of VEGF, which was suggested to be a contribution to the autoregulation of posts ischemic brain damage.19) Another research pointed out that mice lacking eNOS expression showed a greater degree of hemodynamic compromise after MCAO and suggested that the product of eNOS may protect brain after focal cerebral ischemia, possibly by improving blood flow within the penumbral zone.20)

We also demonstrated that the cerebroprotective effect of RQKL was accompanied by eNOS upregulation in cerebral blood vessels. Compared with untreated animals, eNOS level in the ischemic penumbra of rats treated with RQKL increased as early as 2 h after MCAO, and prolonged upregulation was observed for at least 72 h, the longest time point we examined. Thus, we hypothesize that RQKL could increase eNOS expression and subsequently increased NO production after ischemia stroke, and therefore, protects some of the tissue otherwise destined to infarction, limiting or even reducing brain tissue damage.

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