Antioxidant Potential of Qizhu Tang, a Chinese Herbal Medicine, and the Effect on Cerebral Oxidative Damage after Ischemia Reperfusion in Rats

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A traditional Chinese herbal medicine, Qizhu Tang (QZT) was studied for its in vitro antioxidant activity and the effect on cerebral oxidative damage after forebrain ischemia followed by reperfusion in rats. The QZT decoction was shown to have strong hydroxyl radical (·OH) scavenging activity (approx. 0.1 mM as Trolox equivalent) when determined by ESR using DMPO as a spin trap reagent and H2O2/UV as the ·OH source. When the QZT decoction was injected into rat duodenum 2 h before cerebral ischemia, the oxidative brain damage after 45 min reperfusion was strongly inhibited in terms of two biochemical indications, thioarbituric acid reactive substance formation and the loss of glutathione peroxidase. Since the QZT formula consists of 4 herbal constituents (Rhizoma atractylodis, Poria, Radix notoginseng and Radix astragali), each of the component herbs and their combinations were also examined for their protective effects on the cerebral ischemia/reperfusion injury and the effects were compared with their in vitro antioxidant potential. Although some of the incomplete formulas showed as strong antioxidant activities as complete QZT in vitro, only the complete QZT formula was effective in preventing cerebral oxidative injury in rats, and other preparations showed limited activity in vivo.

Key words Traditional Chinese medicine; antioxidant herb medicine; cerebral oxidative damage; ischemia-reperfusion; Qizhu Tang; composite formula

It is now apparent that the oxidative stress induced by reactive oxygen and nitrogen species is implicated in aging, carcinogenesis and many other pathophysiological conditions as a causative factor. Antioxidant therapy thus has been proposed for the treatment of these disorders. However, the single use of antioxidants is usually less effective than expected. The therapeutic potential of traditional herbal medicines however, is attracting attention as an alternative medicine for treating diseases related to lifestyle. Since reactive oxygen species are implicated in these diseases, antioxidative Chinese traditional medicines would be a reliable target of study for their potential to prevent and repair oxidative stress-related pathological conditions. Usually, the formula for a Chinese herbal medicine consists of several components with different functions. Therefore, it seems to be a good model for an antioxidant-based composite therapy, in that the combination of several components with different functions is designed to ameliorate the pathophysiological conditions related to oxidative stress.

Brain oxidative damage is one of the targets for the antioxidant-based composite therapy, because lipid peroxidation is implicated as a major step involved in the progression of brain damage after ischemia/reperfusion. Several trials thus have been carried out using a small molecular antioxidant such as lipico acid for the intervention of damage progression in the brain. However, the results were limited, probably because several steps occur in pathogenesis in the brain after ischemia/reperfusion besides lipid peroxidation, such as the release of excitable amine, Ca2+, cytokines and NO.

In the present studies, we focused our attention on one formula of Chinese traditional medicine, Qizhu Tang (QZT). QZT consists of four herbal components; Rhizoma atractylodis macrocephalae, Poria, Radix notoginseng and Radix astragali seu hedysari. It has been used to treat syndromes related to Qi depression in the spleen and stomach such as dyspepsia, water stool, lassitude of limbs, slow and weak pulse and pale complexion. It is also used to promote blood circulation to remove blood stasis and has especially been prescribed for enhancing the immune function of the body. QZT can enhance celiac macrophage phagocytic function in mice and enhances serum IgG in humans. It also facilitates the restoration of thymus atrophy and hypofunction, which are induced by malnutrition.

We previously observed the marked protective and reparative effects of another Qi and immune stimulating Chinese traditional medicine, Shengmai san (SMS), on cerebral ischemia/reperfusion damage in rats. Although the component herbs are completely different from each other, the wide spectrum of QZT effects, including its Qi and immune modulating activity, allow us to evaluate the protective effect of QZT on brain oxidative injury after forebrain ischemia/reperfusion in rats as another example of antioxidant-based composite formula.

MATERIALS AND METHODS

Chemicals and Herbal Materials 2,2'-Azobis (2-amidino-propane) dihydrochloride (AAPH), glutathione reduced form (GSH), 2-thiobarbituric acid (TBA) and 1,1-diphenyl-2-picrylhydrazyl (DPPH) were purchased from Wako Pure Chemical Ind. Disodium EDTA was obtained from Kanto Chemical Co., Ltd., Sodium dodecyl sulfate (SDS) from nacalai tesque Co., Ltd., β-NADPH and GSH reductase from Sigma Co., Ltd. U.S.A. 5,5-Dimethyl-1-pyrroline-N-oxide (DMPO) was purchased from LABOTEC Co., Ltd., Tokyo. All other chemicals were of analytical grade.

Dried herbal materials, including Rhizoma atractylodis macrocephalae (B), Poria (F), Radix notoginseng (S), and Radix astragali seu hedysari (H) were obtained from Magiya Pharmacy Co., Ltd., Niigata, Japan. B, S and H are products of Hei Long Jiang Sheng, Yun Nan Sheng and Si Chuan Sheng in China, respectively. F is a product of Korea.

QZT and Their Related Preparations QZT was pre-
pared by gently boiling a mixture of B (24 g), F (18 g), S (36 g) and H (36 g) in 250 ml of distilled water for 30 min to reduce the volume to 100 ml. The herb mixture was soaked for 1 h at room temperature before boiling. The decoction was then filtered through delipidated gauze and stored in a refrigerator until use. The concentration of the decoction was 1.14 g dried herb mixture/ml. Similarly, the preparations containing each QZT component alone or the combinations lacking one or two components from the complete QZT formula were decocted in the same way as above, using the same amount of component herb(s) as in the QZT formula.

**Hydroxyl Radical Scavenging Assay by the ESR Spin Trapping Method** Hydroxyl radical (·OH) scavenging activity of QZT was determined by a spin trapping ESR using DMPO as a spin trap reagent according to the method described previously. Both the Fenton reaction and H2O2/UV systems were used as the ·OH source.

Comparative study on the ·OH scavenging activity of QZT and its related decoctions was carried out in the presence of liver homogenate using the Fenton system as ·OH generator. Briefly, rat liver homogenate (0.923 mg protein) was previously incubated with an aliquot of QZT or its related decoctions at 37 °C for 30 min. The homogenate (100 μl) was mixed with 160 μl of distilled water, 30 μl of 100 mM H2O2 and 30 μl of 200 mM DMPO. Then, 30 μl of 2 mM FeSO4 was finally added to the mixture to initiate the Fenton reaction. The DMPO–OH adduct formation was measured in a silica flat cell 2 min after the addition of FeSO4 using a JEOL JES-TE 200 electron spin resonance (ESR) spectrometer (X-Band Microwave Unit). The spectrometer settings were as follows: microwave power, 8 mW; microwave frequency, 9.20 GHz; modulation amplitude, 0.1 mT; time constant, 0.03 sec; sweep time, 30 sec; center fields, 332.6/322.6 mT.

**In Vitro Radical Scavenging Activity Determined by DPPH Quenching** The DPPH radical scavenging activity of QZT and its related decoctions was determined according to the method by Yoshida et al. QZT sample (0.1 ml) was incubated with 0.2 ml of rat liver homogenate (0.923 mg as protein) prepared in 1.15% (w/v) KCl for 30 min at room temperature. Then, 0.1 ml of aqueous DPPH (1.5 × 10–4 M) was added, shaken vigorously, and allowed to stand for 30 min at room temperature. Unreacted DPPH was determined by the absorbance at 520 nm. For the control, physiological saline solution was added instead of DPPH sample.

**In Vitro Lipid Peroxidation Assay by AAPH-Induced TBARS Formation in Rat Liver Homogenate** AAPH-initiated lipid peroxidation was determined by TBA reactive substances (TBARS) formation in normal rat liver homogenate according to the method by Ohkawa et al. Briefly, the reaction mixture containing 0.1 ml liver homogenate (0.923 mg as protein), 0.1 ml AAPH (1 mM aqueous solution), and 0.1 ml QZT and its related decoctions was incubated aerobically at 37 °C for 30 min. Then, 0.2 ml of 8.1% SDS, 1.5 ml of 20% acetic acid and 1.5 ml of 0.8% aqueous TBA were added. The mixture was finally made up to 4.0 ml with distilled water and heated at 95 °C for 60 min. After adding 1.0 ml of distilled water, the solution was extracted with 5.0 ml of n-butanol and pyridine (15:1 v/v). TBARS was determined by the absorbance at 532 nm in the organic layer after centrifugation.

**A Rat Model of Cerebral Ischemia-Reperfusion** Male Wistar rats (6 weeks old and 160—182 g body weight) purchased from SLC Inc., Japan were conditioned for one day by allowing free access to pelleted diet and water before the experiment.

The forebrain ischemic treatment and other experimental conditions were given previously. Briefly, a small incision was made in the abdomen under anesthesia to expose the duodenum and then QZT and its related decoctions were administered directly into the lumen of the duodenum with a syringe at a dose of 4 ml/rat 2 h before the cerebral ischemic operation. Anesthesia was induced with diethyl ether and maintained with pentobarbital. For saline control, rats were administered physiological saline solution instead of a test preparation. The normal control rats were not subjected to any treatment.

For ischemic operation, a middle ventral incision was made in the neck. Both right and left common carotid arteries were exposed and occluded using nontraumatic aneurysm clips. After 85 min of ischemia, the clips were removed to restore blood flow (reperfusion).

**Brain Sample Collection and Biochemical Assay** After reperfusion for 45 min, the rats were decapitated under anesthesia. The whole brain was removed quickly, rinsed with saline, and then frozen in a freezer (–80 °C) until used. The tissue was suspended in cold 0.05 M phosphate buffer containing 1.15% (w/v) KCl (1 g wet tissue/9 ml), and homogenized using a glass homogenizer at 0 °C.

TBARS was determined as described above but without adding AAPH. GPX activity was determined according to the method of Albrecht. Briefly, an aliquot of the brain homogenate (4 mg wet tissue) was mixed in a quartz cuvette with 935 μl of the coupling solution containing 33.6 mg disodium EDTA, 6.5 mg NaN3, 30.7 mg of GSH, 16.7 mg NADPH and 100 units of GSH reductase in 100 ml of 50 mM Tris–HCl (pH 7.6). Kinetic decay of NADPH fluorescence (Ex. 355 nm/Em. 465 nm) was measured after the addition of 25 μl of 1 mM H2O2 as a substrate using a Hitachi model 650-60 fluorescence spectrophotometer.

**RESULTS**

**In Vitro Antioxidant Activities of QZT and Its Components** First, we examined ·OH scavenging activity of QZT by ESR using DMPO as a spin trap reagent. As shown in Fig. 1, the DMPO–OH signal produced by the Fenton reaction was reduced by the addition of QZT in a concentration-dependent manner. The 50% inhibitory concentration (ID50) of QZT for DMPO–OH formation was compared to that obtained for Trolox as a reference radical scavenger. The QZT decoction was found to have a strong ·OH scavenging activity comparable to that by 0.03 mM Trolox. In order to know whether the observed ·OH scavenging activity of the QZT was due to its direct scavenging of ·OH or due to the inhibition of ·OH generation in the Fenton reaction, the inhibition of DMPO–OH formation by QZT was also examined using a low concentration of DMPO as the spin trapping reagent (2 mM, 1/10 of the above condition). The result was that the dose-response curve shifted to the left without changing shape significantly. The ID50 obtained was, however, approximately 1/6.4 of that observed with high DMPO concentration (20 mM) (Fig. 2), indicating that QZT not only scavenges...
OH directly but also inhibits the ·OH generation in the Fenton reaction. This was further confirmed when the ·OH scavenging activity of QZT was determined in the H2O2/UV system as ·OH source. In this system, the Trolox equivalence of the QZT decoction was calculated as approx. 0.1 mM.

Effect of QZT on Cerebral Oxidative Damage Induced by Forebrain Ischemia/Reperfusion in Rats

The experimental conditions described in the Methods section were essentially the same as our previous study on SMS which showed marked prevention and repair of cerebral oxidative damage after ischemia/reperfusion treatment.20) The whole brain homogenate prepared from the brain removed after 45 min reperfusion was used for determining both TBARS formation as an index of lipid peroxidation and glutathione peroxidase (GPX) activity as an index of scavenging potential toward lipid peroxides to evaluate the oxidative damage to the brain. As is shown in Fig. 3a, TBARS formation in the damaged brain was significantly inhibited. The TBARS level in the damaged brain was increased only to 16% of the normal control. QZT thus inhibited approximately 86% of the ischemia/
reperfusion-induced damage in the rat’s brain \((n=3, \ p<0.05)\).

At the same time, GPX activity was measured to evaluate the defense potential of the cell against the oxidative stress. A considerable loss of GPX activity was observed in the saline control brain after ischemia/reperfusion (approx. 40% of normal control was retained), but the loss of GPX activity was markedly prevented by the QZT preadministration (Fig. 3b). The GPX activity was maintained at 80% of the normal level in the QZT pretreated rats which corresponds to approximately 40% protection of the damage induced.

**In Vitro Antioxidant Activities of QZT Component and Their Combinations** The ·OH scavenging activities of QZT components and their combinations *in vitro* were determined through the ESR spin trapping method in the presence of rat liver homogenate with which the decoctions were preincubated for 30 min at 37 °C (Fig. 4). The scavenging activity of the HBS combination was the strongest among the preparations tested and was comparable to that of the complete QZT decoction. The HBF, HS and HB combinations followed. Relatively weak but significant inhibition was also found in H, B alone and in HFS, BS and HF combinations. However, F did not show any scavenging activity and even behaved as a prooxidant under the experimental conditions. Likewise, the BF combination showed weak prooxidant activity. The same trends were obtained when the scavenging activities were determined in distilled water without liver homogenate (data not shown).

DPPH radical quenching activities were also examined *in vitro*. Only complete QZT formula and HBS combination showed marked quenching activity for DPPH radical (100 and 100.5%, respectively). No other combinations nor the component alone showed significant quenching except HS and S which showed moderate quenching activities (approximately 25 and 22%, respectively) (data not shown).

The inhibitory effects of QZT components and their combinations on AAPH-induced TBARS formation were further evaluated in the rat liver homogenate. Complete QZT formula showed the highest activity (100%), followed by the HBS combination (approx. 79%). Considerable inhibition was also achieved with B, S and HB (58, 58 and 49%, respectively). Other decoctions showed only limited inhibition, and both the HBF and HF combinations even behaved as prooxidant (data not shown).

**In Vivo Antioxidant Activities of QZT Components and Their Combinations** To understand the rationale of the composite formula in preventing the cerebral oxidative damage, the effect on the ischemia/reperfusion damage in a rat brain was examined for QZT and some of the QZT-related decoctions which have shown as strong ·OH scavenging activity *as QZT in vitro* (Fig. 5). F was also examined as a reference because it did not show any ·OH scavenging activity, even enhanced DMPO–OH signal. The complete QZT formula showed the most effective prevention of TBARS formation in the damaged brain followed by the HBS combination (Fig. 5a). Although the HBS combination showed rather stronger *in vitro* DPPH and ·OH quenching activities than QZT (approx. 101 and 109% of complete QZT, respectively), the *in vivo* activity was only 46% of QZT. Likewise, the HBF and HS combinations which also showed comparable ·OH scavenging activity to QZT did not significantly inhibit TBARS formation *in vivo*. F was not effective at all. The same trend was observed in their action on GPX activity in the damaged brain. The most effective protection was attained by QZT but not by other decoctions except HBS, which showed a moderate protective effect (Fig. 5b).

**DISCUSSION**

Traditional Chinese herbal medicines have recently at-
Chinese traditional medicines are usually formulated with several herbal components having different physiological activity. Therefore, it is known that the synergism among the component herbs plays an important role in the physiological action such that combination of two herbs enhances each original function or decreases the toxic effect of the partner herb.\(^{27}\) In the present study, we analyzed in vitro whether such synergism also occurred among the component herbs of QZT in terms of antioxidant activities.

When the \(\cdot\)OH scavenging activity was determined for all the QZT components and their combinations, H and B were found considerably active followed by S. However, F enhanced DMPO–OH formation rather than decreased it (Fig. 4). This type of enhancement of DMPO–OH formation is reported in several chemical systems involving ion chelate formation as well as in our previous report on ferric iron/lactate complex.\(^{28}\)

When each component herb was mixed with another, essentially an additive effect of the component herb was observed among the H, B, S in terms of \(\cdot\)OH scavenging activity. However, F, which has a prooxidant nature itself, behaved differently. For example, F stimulated the antioxidant activity of HB but negatively affected on the HS and BS combinations to reduce their activities. Therefore, HBS combination showed the maximal \(\cdot\)OH scavenging activity comparable to complete QZT followed by the HBF and HS combinations.

When the \(\cdot\)OH scavenging activity of each decoction was correlated to other in vitro antioxidant parameters (TBARS and DPPH quenching activities) in Fig. 6, the activity was not correlated with either TBARS inhibitory activity or DPPH quenching activity among the decoctions. In the figure, however, it was noticed that all the F containing decoctions except complete QZT showed quite low TBARS inhibitory action regardless of their \(\cdot\)OH scavenging potency. Thus no correlation was established between TBARS and \(\cdot\)OH scavenging activities among the F containing decoctions. In contrast, a rather good correlation was observed among the decoctions containing H. These results suggest that each component behaves differently in each antioxidant assay system. Indeed, only the HBS combination showed the strong antioxidant potential comparable to QZT in all three antioxidant assays, but none of the other decoctions showed this trend in their antioxidant potential in any of these three in vitro assays (data not shown).

Therefore, we selected the decoctions that showed almost the same \(\cdot\)OH scavenging activity as QZT and examined their protective effect on cerebral ischemia/reperfusion damage in rats, together with F as a negative reference.

The most effective prevention of TBARS formation in vivo was accomplished by complete QZT followed by the HBS combination. However, the activity of the HBS combination was only half that of complete QZT. Other combinations, HBF and HS showed only limited activity. F did not show any inhibitory action at all, as expected. GPX activity was also preserved most effectively by complete QZT followed by the HBS combination. However, the activity of the HBS combination was only 33% of QZT. All other preparations afforded significant protective activity in vivo. This trend observed in in vivo action was not consistent with any of the in vitro antioxidant indications examined above. However, the present studies revealed the propriety of the idea that Chinese traditional medicine is an interesting model for antioxidant-based combina-
tion therapy. When the in vivo effectiveness was compared for the QZT and HBS combinations, both showed comparable in vitro antioxidant activity. The complete QZT formula inhibited both TBARS formation and GPX loss but HBS was only effective in preventing the TBARS formation. This type of differential effect on these two in vivo indications was also found between the different formulas of QZT and SMS. SMS was previously shown not only to protect against cerebral oxidative damage by its preadministration but also to ameliorate the damage even when administered long after ischemia/reperfusion. Both formulas effectively and comparably inhibited the TBARS formation in the rat brain after ischemia/reperfusion but the preventive activity of GPX loss was stronger by SMS than by QZT (60% inhibition vs. 40%).

In this sense, it is interesting to note the unique role of F in the composite formula, which itself behaved as prooxidant rather than an antioxidant, both in the ·OH scavenging activity and TBARS formation in vitro. When F was combined with HBS to make the complete QZT formula, the protective effect on the oxidative damage in vivo was remarkably enhanced. Although further study is needed, it is suggested that F plays a key role in modulating the antioxidant property of the composite formula so that it is active in the brain.

Although the present experimental results suggest that the in vitro antioxidant activity is not the sole factor determining the in vivo effectiveness of a composite formula with different component herbs, we recently observed a clear correlation between the in vitro antioxidant activities and the in vivo protective effects on the ischemia/reperfusion injury in the rat brain among different decoctions of SMS (manuscript in preparation). Thus, the in vitro antioxidant potential might be a reasonable indication with which to evaluate the relative in vivo effectiveness of the same composite formula.

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