Inhibitory Effects of *Stephania tetrandra* S. MOORE on Free Radical-Induced Lysis of Rat Red Blood Cells

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Crude preparations of *Stephania tetrandra* S. MOORE (ST), a traditional herbal medicine, have been used safely for arthritis and silicosis in China. In this study, we demonstrated that ST *in vitro* protects red blood cells from 2,2-azo-bis (2-amidinopropane) dihydrochloride (AAPH)-induced hemolysis. The inhibitory effect was dose-dependent at concentrations of 10 to 1000 $\mu\text{g/ml}$. Moreover, tests were carried out to identify the main ingredient of ST that exerts a scavenging effect on free-radicals. Three representative alkaloids, tetrandrine, fangchinoline, and cyclanoline, isolated from ST, were found to have inhibitory activities against AAPH-induced lysis of red blood cells (RBC). Furthermore, the ingestion of 200 mg of ST extract was associated with a significant increase in free-radical scavenging effect of plasma in rats. These results suggest that ST as antioxidant inhibits AAPH-induced hemolysis of RBC both *in vitro* and *in vivo*.

Key words antioxidant effect; reactive oxygen specie; *Stephania tetrandra*

Stephania tetrandra (ST) has been used as an analgesic drug for arthritis for thousands of years in China. ST has been reported to have anti-inflammatory^{1,2)} and immunosuppressive³⁾ properties *in vitro* and in experimental animals. This study was performed to clarify the role of ST from an antioxidative point of view.

In the present study, we examined the effects of ST on scavenging activities of reactive oxygen species (ROS) by means of electron spin resonance (ESR) method, and on red blood cell (RBC) hemolysis induced by an azo free-radical initiator in healthy volunteers. The ESR method was used for the measurement of O_2^- and $\text{HO}\cdot$ scavenging activities of ST. RBC hemolysis was used for determination of the antioxidative effect of ST, and the influence of ST on RBCs, which have the most important role in hemorrheology.

Furthermore, we hypothesized that ingestion of ST extract (ST.E) would further strengthen the antioxidative effect of plasma, which already has a function of reducing the free radical-induced tissue damage in cerebrovascular disease and ischemic heart disease, if the responsible compounds in ST could be absorbed and circulated in blood. Therefore, the present study examined the effect on plasma antioxidative activity after a gavage-dose of ST.E in rats.

MATERIALS AND METHODS

Preparation of Drugs ST.E was prepared from *Stepha-*

nia tetrandra (Chinese origin) purchased commercially (Uchida Wakanyaku Co. Ltd., Tokyo, Japan). The extract was obtained by boiling the herb in water for 50 min and then freeze-drying into a resultant powder. We obtained 10.67 g of extract from 100 g of raw material.

This powder extract was dissolved in phosphate buffered saline (PBS, pH=7.4).

Reagents Tetrandrine (TET), fangchinoline (FAN) and cyclanoline (CYC) were the products of Tsumura & Co. (Tokyo, Japan). Their contents in ST.E were 2%, 1% and 1%, respectively. The chemical structures of TET, FAN and CYC are illustrated in Fig. 1. The purity of each compound was examined by HPLC using a photodiode array detector.

ESR Assay for O_2^- and $\text{HO}\cdot$ Scavenging Activities of ST.E Measurement of O_2^- and $\text{HO}\cdot$ scavenging activities was performed as described previously⁴⁾ with slight modification. ST.E was diluted to 0.2, 2.0, and 20.0 mg/ml with PBS. O_2^- and $\text{HO}\cdot$ scavenging activities of each sample (50 μl) were assessed by electron spin resonance (ESR) technique⁵⁾ and then IC_{50} (Inhibition Concentration 50%) was calculated.

In this experiment, O_2^- was generated from a hypoxanthine (HPX)–xanthine oxidase (XOD) reaction system in PBS.⁶⁾ Briefly, 15 μl of 9.2 M 5,5-dimethyl-1-pyrroline *N*-oxide (DMPO) (LABOTEC, Tokyo, Japan), 50 μl of 2 mM HPX (Sigma, St. Louis, MO, U.S.A.), 35 μl of 5.5 mM diethylenetriamine-*N,N,N',N'',N'''*-pentaacetic dianhydride (DETA-

Fig. 1. Chemical Structures of the Three Alkaloids

a) TET; b) FAN; c) CYC.

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PAC; Wako Pure Chemical Industries, Tokyo, Japan) and 50 μ l of prepared sample were put into a test tube. After adding 50 μ l of 0.4 U/ml XOD (Roche, Indianapolis, IN, U.S.A.) and quick mixing, 200 μ l of the mixture was transferred to a flat quartz ESR cuvette, which was fixed to the cavity of an ESR spectrometer (JES-FR30, JEOL, Tokyo, Japan). Recordings of the spectra were made at 24 °C and started at 1 min after the mixing of XOD; each scan took 1 min. Data were expressed as the ratio of the peak of the DMPO–OOH signal to the peak of the intrinsic standard, MnO (S/M). The scavenging activity (Scv) was calculated by the following equation:

$$\text{Scv (\%)} = (S/M_{\text{BLANK}} - S/M_{\text{SAMPLE}}) / S/M_{\text{BLANK}} \times 100$$

where S/M_{BLANK} is the intensity of the ESR spectrum of DMPO–OOH spin adduct in PBS as a blank, and S/M_{SAMPLE} is the intensity of the ESR spectrum of DMPO–OOH spin adduct in the sample.

HO $\cdot$ was generated by the Fenton reaction⁷⁾ consisting of 75 μ l of 0.1 mM H₂O₂, 50 μ l of each sample, 20 μ l of 92 mM DMPO and 75 μ l of 0.1 mM FeSO₄. The spectrum of DMPO–OH was measured at 1 min after the addition of H₂O₂. Scavenging activity was calculated in the same manner as described above.

In Vitro Assay for Anti-hemolytic Activity of ST (ST.E, TET, FAN and CYC) Eight male Wistar rats (350 g) were fed commercial feed (type CE-2, CLEA Japan Inc., Tokyo, Japan) for one week. Blood obtained from rats by cardiac puncture was collected into heparinized tubes. RBC was separated from plasma by centrifugation at 1500 *g* for 20 min. Crude RBC was then washed three times with 5 volumes of PBS. Packed RBC was thereafter suspended in 4 volumes of PBS solution.

Hemolysis of RBC mediated by 2,2-azo-bis (2-amidinopropane) dihydrochloride (AAPH) was determined as reported previously.^{8–11)} The addition of AAPH to the suspension of RBC causes the oxidation of lipids and proteins in the cell membrane, thereby inducing hemolysis. It is known that AAPH-induced hemolysis in RBC is a function of incubation time and proportional to the concentration of free radicals.^{12,13)} The inhibitory effect on RBC hemolysis is also proportional to the concentration of antioxidants in the incubation mixture. The AAPH (200 mM) solution was prepared with PBS.

RBC suspension (1 ml) was mixed with 1 ml of PBS solution containing various amounts of ST.E, TET, FAN and CYC. RBC suspension (1 ml) mixed with 1 ml of PBS solu-

tion alone was used as control sample. The AAPH solution (1 ml) was then added to the mixture. The incubation mixture was shaken gently in a water bath at 37 °C for 3 h. After incubation, 4 ml of PBS solution was added to the reaction mixture, followed by centrifugation at 1000 *g* for 10 min. Absorbance (A) of the supernatant at 540 nm was recorded with a UV-visible recording spectrophotometer (UV-265FS, Shimadzu, Kyoto, Japan). Percentage inhibition was calculated in the same way as in the ESR assay.

Assay for Anti-hemolytic Activity of Rat Plasma after Ingestion of ST.E Twelve male Wistar rats (350 g) were fed the same commercial chow as for the *in vitro* assay for one week. They were randomly divided into two groups. After an overnight fast, one group (group A) was gavaged with 2 ml of distilled water containing 200 mg of ST.E, whereas the control group (group B) was gavaged with 2 ml of distilled water only. Thirty min after administration, blood was collected from the heart in heparinized tubes. RBC was separated from plasma by centrifugation at 1500 *g* for 20 min. After removal of white blood cells and platelets, the remaining RBC was mixed with the volume of plasma therefrom. The reconstituted blood (1 ml) was then used for the hemolysis assay by adding 1 ml of AAPH solution and 1 ml of PBS followed by incubation at 37 °C for 2, 3 or 4 h. PBS solution (4 ml) was added to the incubation mixture followed by centrifugation at 1000 *g* for 10 min. Absorbance (A) of the supernatant at 540 nm was measured. The percentage inhibition of a gavage dose of ST.E was calculated in the same way as in the *in vitro* study:

This study conformed to the guidelines for the care and use of animals as approved by the Ethics Committee for Animal Experiments of Toyama Medical and Pharmaceutical University.

Statistical Analysis Data were expressed as mean $\pm$ S.D. Repeated measures ANOVA and repeated measures one-way ANOVA followed by Fisher's PLSD were used for statistical analysis. A value of $p < 0.05$ was accepted as statistically significant.

RESULTS

The O₂^{•-} and HO $\cdot$ scavenging activities of ST.E determined by ESR method are shown in Fig. 2. ST.E inhibited free-radical generation in a dose-dependent manner. The IC₅₀ values for O₂^{•-} and HO $\cdot$ were 1.19 and 2.89 mg/ml, respectively.

The anti-hemolytic effect of ST.E is shown in Fig. 3. The

Fig. 2. ROS Scavenging Activities of ST.E

a) O₂^{•-}; b) HO $\cdot$. Data are expressed as mean $\pm$ S.D. of $n=4$ samples.

water extract of ST *in vitro* inhibited the hemolysis of rat RBC due to AAPH-induced peroxy radicals in a dose-dependent manner.

Figure 4 shows the anti-hemolytic activities of the three alkaloids obtained from the methanol extract of ST. The inhibitory activity of CYC was significantly stronger than those of TET and FAN. However, the inhibition of TET at a concentration of 100 μM was greater than that at 200 μM , suggesting that TET attains cytotoxicity at high concentration.

The results of the *in vivo* tests are presented in Fig. 5. RBC in reconstituted blood obtained from group A rats was more resistant to AAPH-induced hemolysis than that obtained from group B rats (Fig. 5). Together with the findings of the *in vitro* study, these results suggest that ST as antioxidant inhibits AAPH-induced hemolysis of RBC both *in vitro* and *in vivo*.

DISCUSSION

The biomembrane may be most susceptible to free-radical attack due to its content of polyunsaturated fatty acids. The present study revealed that ST.E protects these polyunsaturated fatty acids from oxidation in the membrane of RBC incubated with AAPH. Of our particular interest was the fact that the AAPH-induced lysis of human RBC was more sensitive than the ESR method in the determination of the ROS scavenging activity of ST. The mechanism, however, has still to be clarified. It has been reported that antioxidant enzyme such as superoxide dismutase, catalase, peroxidase and glutathione peroxidase were elevated in RBCs treated with *m*-chloroperbenzoic acid, a novel photosensitizer.¹⁴⁾ Tests are currently being planned to determine whether ST affects the

antioxidant activities of these enzymes or not.

Although seventeen alkaloids in ST are known,¹⁵⁾ their amounts are far from sufficient except for the three alkaloids, TET, FAN and CYC, examined in this study, as shown in Fig. 1. These three different alkaloids isolated from ST protected the RBC membrane from free-radical attack. Further, in this regard, it has been reported that TET has a scavenging effect on reactive oxygen species (ROS).¹⁶⁾ To the best of our knowledge, the present study is the first to demonstrate that FAN and CYC *in vitro* protect RBC from free-radical-induced oxidation.

Moreover, the present study was the first to demonstrate that ST *in vivo* augments the antioxidative effect of plasma.

It has been reported that TET and FAN have other actions, such as inhibition of Ca^{2+} influx,^{17,18)} vasodilating and hypotensive effects,¹⁹⁾ and inhibition of platelet aggregation.²⁰⁾ Together with those previously reported findings, the direct protection of RBC membrane from free-radical attack as observed in the present study would provide an important pathophysiological basis for putting to use the helpful hemorrheological, anti-atherosclerotic and anti-inflammatory effects of TET, FAN, CYC and ST.

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Fig. 3. Inhibitory Effect of ST.E on AAPH-Induced Lysis of Rat Red Blood Cells

Data are expressed as mean $\pm$ S.D. of $n=8$ samples. * $p<0.0001$ vs. control.

Fig. 5. The Time Course of AAPH-Induced Hemolysis of Reconstituted Blood Obtained from Rats 30 min after Oral Ingestion of 2 ml of Distilled Water Containing 200 mg ST.E (group A) or 2 ml of Distilled Water Only (Group B)

Group A (●), group B (○). Data are expressed as mean $\pm$ S.D. of $n=6$ rats. Asterisks indicate significant difference from control group ($p<0.01$).

Fig. 4. Inhibitory Effect of the Three Alkaloids, the Main Ingredients of ST, on AAPH-Induced Lysis of Rat Red Blood Cells

a) TET; b) FAN; c) CYC. Data are expressed as mean $\pm$ S.D. of $n=8$ samples. † $p<0.001$, * $p<0.0001$ vs. control.

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