Radical-scavenging activity of Wen-Pi-Tang and its component crude drugs: with special reference to the effects on nitric oxide, superoxide and peroxynitrite

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Abstract

In renal diseases, active oxygen and free radicals play various roles in the development and progression of the pathological condition. Our previous studies have provided evidence that the Oriental medical prescription Wen-Pi-Tang normalizes the kidney under conditions of increased oxidative stress. In the present study, we examined the antioxidant capacity of Wen-Pi-Tang and its component crude drugs in a nitric oxide, superoxide and peroxynitrite generation system. It was found that the radical-scavenging effect of Wen-Pi-Tang is dose-dependent, and that three of its component crude drugs, i.e., Rhei Rhizoma, Zingiberis Rhizoma and Glycyrrhizae Radix, play important roles in the antioxidant action.

Key words nitric oxide, superoxide, peroxynitrite, Wen-Pi-Tang (温脾湯), Rhei Rhizoma, Zingiberis Rhizoma, Glycyrrhizae Radix.

Abbreviations EDRF, endothelial-derived relaxing factor; H_2O_2, hydrogen peroxide; NO, nitric oxide; O_2^-, superoxide; $ONOO^-$, peroxynitrite; SIN-1, 3-morpholinosydnonimine; SOD, superoxide dismutase.

Introduction

It is well known that antioxidant enzymes and antioxidant substances present in the living body offer protection from oxidative damage, working against active oxygen species that are produced in excess under oxidative stress. In particular, plasma contains antioxidant defense enzymes such as superoxide dismutase (SOD), catalase and glutathione peroxidase, which play major roles in the inactivation of active oxygen, together with water-soluble low-molecular-weight antioxidants such as ascorbic acid and uric acid, lipid-soluble low-molecular-weight antioxidants such as α-tocopherol, β-carotene and bilirubin, and binding proteins such as transferrin, albumin and ferritin.[1-4] However, if the oxidative stress is too great, the body's defense mechanism is unable to cope, and the roles of antioxidants become more important. Therefore, much attention has been focused on antioxidants.

Synthetic t-butyl hydroxyanisole and t-butyl hydroxytoluene have been used as antioxidants. Tocopherol, a natural antioxidant, has also been widely used. However, because of doubts about the safety and efficacy of these agents, new, safer and more effective natural antioxidants have been sought.[5]

Oriental medicines include a variety of antioxidant compounds and are still called for even though conventional drugs are in widespread use. Extensive research is now being conducted in the treatment of chronic diseases which respond poorly to conventional drugs, to determine the actual role of the antioxidants contained in Oriental medicines and medical prescriptions comprising several Oriental medicines, and also to clarify how active oxygen and its effects such as

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lipid peroxidation are involved in various diseases.[6]

The authors have previously demonstrated the usefulness of Wen-Pi-Tang as a conservative treatment for renal failure under an enhanced oxidative conditions in both experimental and clinical settings.[7-21] However, the effects of Wen-Pi-Tang and its component crude drugs on free radicals have remained unclear. Therefore the present study was designed to determine these effects.

Materials and Methods

Wen-Pi-Tang: The composition of Wen-Pi-Tang used in this study was 15 g Rhei Rhizoma (*Rheum officinale* BAILLON), 3 g Ginseng Radix (*Panax ginseng* C. A. MEYER), 9 g Aconiti Tuber (*Aconitum japonicum* THUNBERG), 3 g Zingiberis Rhizoma (*Zingiber officinale* ROSCOE) and 5 g Glycyrrhizae Radix (*Glycyrrhiza glabra* LINN. var. *glandulifera* REGEL et HERDER). Ginseng Radix was produced in Korea, Aconiti Tuber was from Japan and all the other ingredients were from China. As described previously,[22] an extract was obtained by boiling the above mixture of crude drugs gently in 1,000 ml water for 65 min, which yielded about 500 ml of decoction, which was concentrated under reduced pressure to leave a brown residue with a yield of about 30 %, by weight, of the original preparation.

Crude drugs: One hundred grams of each crude drug component of Wen-Pi-Tang was boiled gently in 1,000 ml water for 5~65 min, according to theWen-Pi-Tang preparation procedure described previously,[22] and each extract was concentrated under reduced pressure to leave a residue. The yields of Rhei Rhizoma, Ginseng Radix, Aconiti Tuber, Zingiberis Rhizoma and Glycyrrhizae Radix were 21 %, 32 %, 37 %, 11 % and 20 %, respectively, by weight, of the starting materials.

Reagent: Peroxynitrite was synthesized in a quenched-flow reactor as described elsewhere.[23]

Measurement of nitric oxide (NO): According to the method of Nagase *et al.*,[24] the reaction mixture consisted of 2.0 ml of 20 mM potassium phosphate buffer (pH 7.4) containing 30 mM hydrogen peroxide (H_2O_2) and 20 mM L-arginine, and the required extract at varying concentration was incubated with the mixture for 2 days at 37°C. The same mixture containing an equivalent amount of buffer instead of the tested extract was used as a control. The possible absorbance brought by the tested extract was extinguished by using an equivalent amount of buffer instead of the Griess reagent. The amount of NO produced by the reaction of H_2O_2 and L-arginine was assayed by measuring the accumulation of nitrite in the culture medium by a microplate assay method based on the Griess reaction.[25]

Measurement of superoxide (O_2^-): On the basis of the method of Az-ma *et al.*,[26] O_2^- generation was achieved by adding xanthine oxidase (final concentration 0.1 unit/ml) to xanthine (0.4 mM). Each of 98 wells in a plate contained xanthine oxidase, extract and xanthine, in a total volume of 200 μl of 50 mM phosphate buffer, and then 50 μl of 50 mM phosphate buffer containing 0.4 μM dichlorodihydrofluorescein and 60 μU esterase was added and incubated at 20°C for 20 min. The changes in fluorescence were measured at an excitation wavelength of 485 nm and an emission wavelength of 530 nm.

Measurement of peroxynitrite ($ONOO^-$): All oxidation reactions were carried out in a stirred 3-ml glass cuvette at 37°C according to the method of Crow.[27] Solutions contained 100 μM diethylenetriaminepentaacetic acid in 1 ml 100 mM phosphate buffer (pH 7.4). The reaction mixture was incubated for 1 min. The extract, 100 μM dichlorodihydrofluorescein and 5 μM $ONOO^-$ or 100 μM 3-morpholinosydnonimine (SIN-1) were added, and absorbance measurements at 500 nm were taken over 5 min.

Statistics: All the values presented are means ± S.E. of 5 determinations. Where appropriate, the significance of differences was tested using Dunnett's method. Differences at p values of less than 0.05 were considered statistically significant.

Results

Figure 1 shows that Wen-Pi-Tang and its component crude drugs lowered NO production significantly in a dose-dependent manner. Wen-Pi-Tang and Rhei Rhizoma, its main component, inhibited NO production more effectively than the others. Aconiti Tuber,

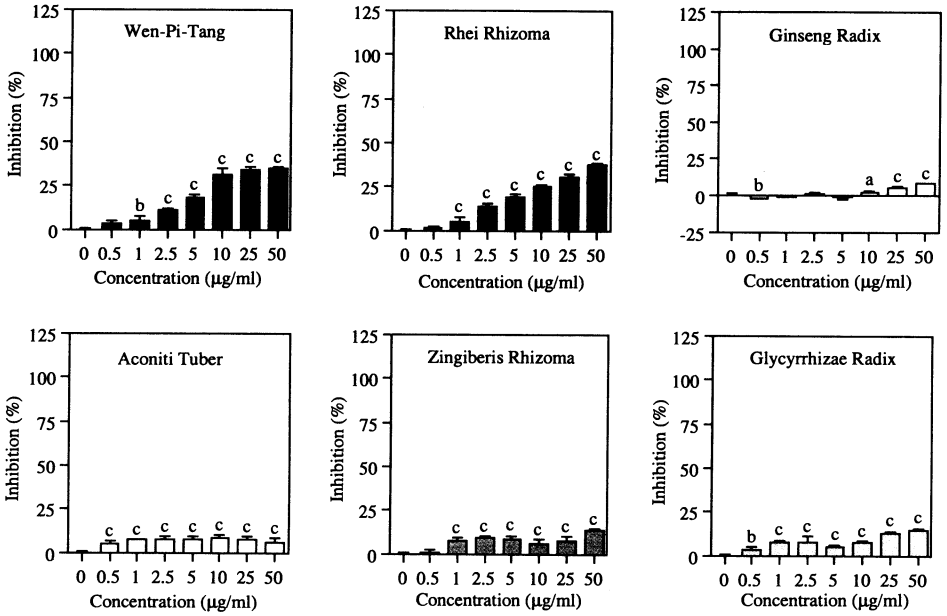

Fig. 1 Effect of Wen-Pi-Tang and its constituents on NO production. Significantly different from the control value : $^{a}p<0.05$, $^{b}p<0.01$, $^{c}p<0.001$.

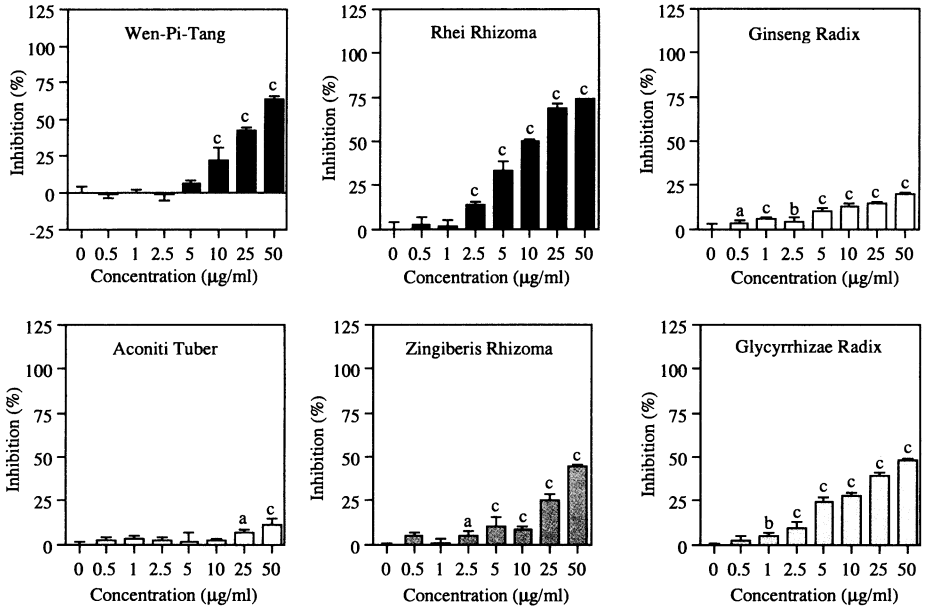

Fig. 2 Effect of Wen-Pi-Tang and its constituents on superoxide. Significantly different from the control value : $^{a}p<0.05$, $^{b}p<0.01$, $^{c}p<0.001$.

Zingiberis Rhizoma and Glycyrrhizae Radix also decreased NO production, whereas they showed neither a marked inhibitory effect on NO production nor a dose-dependent action.

The inhibitory effect on O_2^- formation is represented in Fig. 2. More than 5 μg/ml Wen-Pi-Tang

Table I Effect of Wen-Pi-Tang and its crude drug components on peroxynitrite-scavenging activity.

Material	Concentration (μg/ml)	Fluorescence/min
Control	–	13986±1405
Wen-Pi-Tang	10	1452±195[b]
	50	1175±11[b]
	250	508±4[b]
Rhei Rhizoma	10	2245±180[b]
	50	590±20[b]
	250	301±3[b]
Ginseng Radix	10	16258±846[a]
	50	15415±972
	250	8776±579[b]
Aconiti Tuber	10	15946±803[a]
	50	15020±608
	250	9452±512[b]
Zingiberis Rhizoma	10	12613±325[a]
	50	5502±364[b]
	250	1852±64[b]
Glycyrrhizae Radix	10	12057±530[a]
	50	3903±130[b]
	250	998±20[b]

Significantly different from the control value : [a]$p<0.05$, [b]$p<0.001$.

Table II Effect of Wen-Pi-Tang and its crude drug components on peroxynitrite formation from SIN-1.

Material	Concentration (μg/ml)	Fluorescence/min
Control	–	148.5±2.1
Wen-Pi-Tang	10	45.6±2.1[b]
	50	20.0±0.1[b]
	250	6.6±2.5[b]
Rhei Rhizoma	10	27.7±2.5[b]
	50	8.0±0.7[b]
	250	0.2±4.0[b]
Ginseng Radix	10	138.2±4.7[a]
	50	168.2±1.2[b]
	250	115.8±4.5[b]
Aconiti Tuber	10	159.2±2.5[b]
	50	141.7±2.6[a]
	250	115.7±1.1[b]
Zingiberis Rhizoma	10	141.5±10.8
	50	96.1±4.6[b]
	250	37.5±2.3[b]
Glycyrrhizae Radix	10	142.3±5.7
	50	78.6±5.4[b]
	250	28.7±2.9[b]

Significantly different from the control value : [a]$p<0.01$, [b]$p<0.001$.

had O_2^- scavenging activity, and thus addition of 50 μg/ml inhibited the formation of O_2^- by over 60 %. Rhei Rhizoma, Zingiberis Rhizoma and Glycyrrhizae Radix also exhibited concentration-dependent O_2^- scavenging activity. Rhei Rhizoma had the highest inhibitory effect among the component crude drugs, and treatment at 50 μg/ml scavenged O_2^- to 74 %. On the other hand, Ginseng Radix and Aconiti Tuber had a relatively weak O_2^- scavenging effect.

Table I shows the $ONOO^-$-scavenging activity of Wen-Pi-Tang and its component crude drugs. Wen-Pi-Tang scavenged $ONOO^-$ markedly; at 250 μg/ml, Wen-Pi-Tang scavenged nearly all $ONOO^-$. The component crude drugs of Wen-Pi-Tang also scavenged $ONOO^-$ effectively as their concentration was increased. Among them, Rhei Rhizoma, Zingiberis Rhizoma and Glycyrrhizae Radix showed strong $ONOO^-$-scavenging activity. Rhei Rhizoma had the highest scavenging activity, whereas Ginseng Radix and Aconiti Tuber had relatively low activity.

As shown in Table II, Wen-Pi-Tang and its component crude drugs caused a significant decrease in $ONOO^-$ formation from SIN-1. As the added concentration increased, the formation of $ONOO^-$ from SIN-1 became significantly lower. The formation of $ONOO^-$ from SIN-1 was reduced by Wen-Pi-Tang, and was only 4 % at a concentration of 250 μg/ml. Among the component crude drugs of Wen-Pi-Tang, Rhei Rhizoma had the strongest inhibitory effect on $ONOO^-$ formation from SIN-1. Zingiberis Rhizoma and Glycyrrhizae Radix also had an inhibitory effect. On the other hand, Ginseng Radix and Aconiti Tuber revealed higher $ONOO^-$ formation than other component crude drugs, even though they did lower one than control.

Discussion

Active oxygen and free radicals are produced in the body through various reactions, but it is unclear what triggers their production. In the kidney, NO produced by constitutive NO synthase maintains the renal blood flow through vasodilation, causing changes in microcirculation in the glomeruli and

regulating glomerular blood flow and the glomerular filtration rate. On the other hand, these changes in glomerular microcirculation may induce excessive filtration and result in injury to the glomeruli or uriniferous tubules.[28,29] Narita *et al.*[30] found that NO production by inducible NO synthase in glomerular mesangial cells and macrophages that had infiltrated into glomeruli after stimulation by cytokines and lipopolysaccharide had the potential to cause mesangiolysis, leading to glomerular injury. In addition, Hirata[31] stressed that NO produced through these two pathways was deeply involved not only in regulation of renal circulation but also the pathophysiology of the kidney.

It has been reported that active oxygen species cause toxic or immunity-related renal injury as well as ischemic renal injury. O_2^- produced in ischemia-reperfusion reacts rapidly with NO to produce the more toxic radical $ONOO^-$. Feelish and Noack[32] have reported that if this reaction occurs in the living body, the vasodilative effect of NO decreases because of its reduction in quantity, resulting in enhancement of ischemic injury. On the other hand, it is possible that elimination of O_2^- by NO prevents O_2^--derived injury. However, $ONOO^-$ produced from NO and O_2^- is a strong oxidant of SH groups.[23] It is also an unstable weak acid (pKa=6.8), readily gaining protons around neutrality to be epimerized to nitric acid.[33] As Koppenol *et al.*[34] reported, during this epimerization process, $ONOO^-$ passes through an activated state in which the reactivity is practically equal to that of the hydroxyl radical, inducing lipid peroxidation. In addition, Beckman *et al.*[35] reported that $ONOO^-$ produces NO^{2+} in the presence of transition metal ions including SOD, and that NO^{2+} attacks tyrosine and other aromatic amino acid residues with its strong nitrating action. Based on these findings, the mechanism of defense against the production of NO, O_2^- and $ONOO^-$ through the reaction of NO with O_2^- in the body, and capture and removal of the radicals produced are considered to be key issues in the prevention of diseases or pathological conditions.

Wen-Pi-Tang used in the present study is an Oriental medical prescription which is representative of drugs aiding the body's defense mechanism and eliminating impurities. In China, this prescription is widely used for the treatment of patients with moderate chronic renal failure, achieving relatively good improvement. The first Japanese report of its therapeutic effect was published in 1984.[18] To establish experimentally the scientific basis for the action of Wen-Pi-Tang, whose clinical efficacy is already recognized, we have previously investigated the effects of Wen-Pi-Tang and its component crude drugs using *in vivo* and *in vitro* evaluation systems such as chronic renal failure and ischemia-reperfusion models and cultured cells including mesangial cells and renal epithelial cells.[7-17] The results of our previous studies suggested that Wen-Pi-Tang as a whole and some of its component crude drugs would exert an antioxidant action on the impaired kidney under oxidative stress.

Antioxidants exert their actions by preventing the production of active oxygen radicals or by capture/removal of the produced radicals. We examined the effects of Wen-Pi-Tang and its five component crude drugs on the latter anti-radical process, using the NO, O_2^- and $ONOO^-$ generation system. Wen-Pi-Tang as a whole and Rhei Rhizoma alone showed a dose-dependent NO-eliminating effect. Aconiti Tuber, Zingiberis Rhizoma and Glycyrrhizae Radix also showed inhibitory activity, but their effects were far weaker than those of Wen-Pi-Tang as a whole or Rhei Rhizoma. Against O_2^-, Wen-Pi-Tang and three of its component crude drugs, i.e., Rhei Rhizoma, Zingiberis Rhizoma and Glycyrrhizae Radix showed a dose-dependent inhibitory action; the minimum concentration required for significant inhibition of O_2^- was 1 μg/ml for Glycyrrhizae Radix, 2.5 μg/ml for Rhei Rhizoma and Zingiberis Rhizoma, and 5 μg/ml for Wen-Pi-Tang. On the other hand, Wen-Pi-Tang and Rhei Rhizoma exhibited strong $ONOO^-$-scavenging activity, as in the case of NO and O_2^-, suggesting that the marked inhibitory effect results from elimination of NO and O_2^- radicals. Zingiberis Rhizoma and Glycyrrhizae Radix also scavenged $ONOO^-$. Their effects were weaker than those of Wen-Pi-Tang or Rhei Rhizoma, but became distinct as their concentrations increased. In view of the ratio of the component crude drugs in Wen-Pi-Tang, the inhibitory effect of Wen-Pi-Tang on $ONOO^-$ seems to be attributable to the complex effect of Rhei Rhizoma, Zingiberis Rhizoma and Glycyrrhizae Radix. Similar results

were obtained with regard to the generation of $ONOO^-$ from SIN-1. Thus, Wen-Pi-Tang was shown to inhibit the generation of $ONOO^-$ from NO and O_2^- and eliminate the generated $ONOO^-$, and Rhei Rhizoma, Zingiberis Rhizoma and Glycyrrhizae Radix were proved to play important roles in such activity of Wen-Pi-Tang.

The results of our study demonstrated that Wen-Pi-Tang and its component crude drugs, i.e., Rhei Rhizoma, Zingiberis Rhizoma and Glycyrrhizae Radix, contribute to the capture and removal of $ONOO^-$. $ONOO^-$ tends to exist as the more stable and toxic cis-form below pH 7.9. The risk of $ONOO^-$ accumulating as the trans-form was formerly considered to be preventable if a large amount of SOD was added.[22,33,34] However, Beckman *et al.*[35] reported that the trans-form of $ONOO^-$ tends to be converted to its cis-form, and that the rate of decomposition of $ONOO^-$ does not exceed 9 %. On the other hand, besides its role as an endothelial-derived relaxing factor (EDRF), various biological actions (bactericidal and neurotransmission) of NO are now attracting attention.[36-38] NO reacts well with other free radicals. In particular, NO shows high reactivity with O_2^-, which is, in fact, a radical that cancels the action of NO. In addition, $ONOO^-$ generated as a product of the reaction of NO with O_2^- exerts strong cytotoxicity, as reported by Shi *et al.*[39] Based on these findings, it is believed that elimination of NO and O_2^- and inhibition of $ONOO^-$ generation are essential conditions for correcting oxidative stress. Wen-Pi-Tang has been proved to eliminate these radicals, suggesting the possible further development of medicaments which act *via* oxygen metabolism control.

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和文抄録

腎疾患において活性酸素・フリーラジカルはさまざまな形でその病態の成立，進展に関与しているが，漢方方剤温脾湯が酸化的ストレス状態にある腎を是正する作用をこれまで報告してきた。本研究では温脾湯と構成和漢薬の抗酸化能を NO, O_2^- 並びに $ONOO^-$ 発生系を用い検討し，温脾湯によるこれらラジカル消去作用は用量依存的に認められ，また構成和漢薬では大黄，乾姜，甘草が重要な役割を担っていることが明らかとなった。

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