Protective activity from hydrophilic and lipophilic free radical generators of Wen-Pi-Tang and its crude drug extracts in LLC-PK_1 cells

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Abstract

We investigated Wen-Pi-Tang and its crude drug extracts to determine their protective effect from oxidative stress caused by the hydrophilic and lipophilic free radical generators, 2,2'-azobis(2-amidinopropane) dihydrochloride (AAPH) and 2,2'-azobis(2,4-dimethylvaleronitrile) (AMVN) in LLC-PK_1 renal tubular epithelial cells. In response to AAPH and AMVN treatment, cell viability decreased significantly and significantly enhanced thiobarbituric acid-reactive substances (TBARS) formation was observed. However, Wen-Pi-Tang and its crude drug extracts showed scavenging of peroxyl radicals, which were generated by AAPH and AMVN, resulting in greater cell viability and lower TBARS formation than controls treated only with free radical generators. In particular, Wen-Pi-Tang, Rhei Rhizoma and Ginseng Radix demonstrated high protective activity, whereas Aconiti Tuber, Zingiberis Rhizoma and Glycyrrhizae Radix showed relatively low activity. This result suggests that the antioxidant activity of Wen-Pi-Tang was attributable to the crude extracts, and that both act as hydrophilic and lipophilic antioxidants.

Key words Wen-Pi-Tang, Rhei Rhizoma, Ginseng Radix, LLC-PK_1, 2,2'-azobis(2-amidinopropane) dihydrochloride, 2,2'-azobis(2,4-dimethylvaleronitrile).

Introduction

It is well accepted that free radical-mediated oxidative stress results in a variety of pathological conditions.[1] Although several possible sources and reactions of free radicals are known, it remains unclear how free radicals are initially generated *in vivo*, and the time, site and damage of oxygen radical formation have not been definitively established. Therefore, *in vitro* model experiments should be performed to clarify our understanding of the mechanisms and dynamics of oxidations taking place *in vivo*, while carefully considering what differences if any exist between the *in vitro* and *in vivo* systems.

To generate free radicals at a controlled and well-defined rate, azo compounds are widely employed, since they produce free radicals without biotransformation in response to spontaneous thermal decomposition. Noguchi *et al.*[2] have used azo compounds successfully *in vitro* and *in vivo* not only on the actions of free radicals upon biological molecules but also on the protective effects of antioxidants. As free radicals can be generated in either the aqueous or lipid phase,[3] the efficiency of free radical scavenging should be determined in these two phases for hydro-

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philic and lipophilic antioxidants.

In recent years, a great deal of attention has been focused on traditional Chinese medicines which have been recognized as antioxidant agents for radical-scavenging activity, as well as on the various physiological functions of crude drugs frequently prescribed in clinical practice. Chinese herbal medicines have been regarded as promising new antioxidants and have thus generated a great deal of research interest due to the fact that antioxidant therapy apparently offers protection against a wide range of free radical-induced diseases.[4] Of these, Wen-Pi-Tang, the Chinese traditional prescription composed of Rhei Rhizoma as the main ingredient, together with Ginseng Radix, Aconiti Tuber, Zingiberis Rhizoma and Glycyrrhizae Radix, is reported to have a radical scavenging action.[5-7]

The present study uses azo compounds, 2,2'-azobis(2-amidinopropane) dihydrochloride (AAPH) and 2,2'-azobis(2,4-dimethylvaleronitrile) (AMVN) as hydrophilic and lipophilic free radical generators, respectively, to investigate the protective activity of Wen-Pi-Tang and its crude drug extracts on the LLC-PK_1 renal tubular epithelial cell line, which is susceptible to oxidative stress.

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 other ingredients were from China. As described previously,[8] an extract was obtained by boiling the above crude drugs gently in 1,000 ml water for 65 min, which yielded approximately 500 ml of decoction, which was then 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 the Wen-Pi-Tang preparation procedure described previously,[8] 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 original preparation.

Reagents and medium : AAPH and AMVN were obtained from Wako Pure Chemical Industries, Ltd. (Osaka, Japan) and 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H tetrazolium bromide (MTT) was purchased from Sigma Chemical Co. (St. Louis, MO, USA). Dulbecco's modified Eagle medium/nutrient mixture F-12 (D-MEM/F-12) and fetal calf serum (FCS) were purchased from Life Technologies, Inc. (Grand Island, NY, USA) and Cell Culture Laboratories (Cleveland, OH, USA), respectively.

Cultured cells experiment : Commercially available LLC-PK_1 cells were maintained at 37°C in a humidified atmosphere of 5 % CO_2 in air (routine conditions) in culture plates with 5 % FCS-supplemented D-MEM/F-12 medium. After confluence was reached, the cells were seeded into 96-well culture plates at 10^4 cells per well. Two hours later, 1 mM of AAPH or AMVN treatment was performed, and test samples were then added. The plates were incubated under routine conditions for 24 h. Fifty microliters of MTT (1 mg/ml) solution was added to each well. After incubation for 4 h at 37°C, the MTT solution was removed from the medium. The resultant formazan crystals in the renal cells were solubilized with 100 μl of dimethylsulfoxide. Absorbance at 540 nm of each well was then read using a Microplate Reader (Model 3550-UV, BIO-RAD, Tokyo, Japan). The level of lipid peroxidant released from the cultured cells was estimated as thiobarbituric acid-reactive substances (TBARS) according to the methods of Yagi [9] and Yokode *et al.*[10] with a slight modification. One aliquot of medium was mixed with 1.5 ml of 0.67 % TBA aqueous solution and 1.5 ml of 20 % trichloroacetic acid, and boiled at 95-100°C for 45 min. The mixture was cooled with water and shaken vigorously with 3.0 ml of n-butanol. After centrifugation at 4,000 g for 10 min, the n-butanol layer was removed, and the fluorescence was mea-

sured on a fluorescence spectrophotometer (Model RF-550, SHIMADZU, Kyoto, Japan).

Statistics : Results were presented as the mean± S.E. of 5 determinations. The data were analyzed for statistical significance using Dunnett's test. Significance was accepted at $p<0.05$.

Table I Effect of Wen-Pi-Tang and its crude drug extracts on viability of cells treated with AAPH.

Material	Concentration (μg/ml)	Cell viability (%)
Normal	−	100.0 ± 2.1
Control	−	75.1 ± 3.4[c]
Wen-Pi-Tang	0.5	84.0 ± 3.3[c,e]
	1	84.6 ± 3.6[c,e]
	2.5	89.0 ± 3.5[b,f]
	5	92.7 ± 3.2[a,f]
	10	94.1 ± 3.9[f]
	25	95.6 ± 5.5[f]
Rhei Rhizoma	0.5	88.0 ± 3.9[c,f]
	1	94.0 ± 2.7[a,f]
	2.5	95.3 ± 4.2[f]
	5	95.8 ± 3.0[f]
	10	96.1 ± 1.6[f]
	25	98.4 ± 1.9[f]
Ginseng Radix	0.5	99.9 ± 5.4[f]
	1	102.9 ± 6.7[f]
	2.5	106.4 ± 6.1[f]
	5	109.9 ± 5.7[f]
	10	112.8 ± 7.6[a,f]
	25	112.8 ± 9.4[a,f]
Aconiti Tuber	0.5	76.0 ± 2.3[c]
	1	81.4 ± 0.8[c]
	2.5	84.6 ± 5.1[c,d]
	5	85.7 ± 6.4[c,d]
	10	86.2 ± 6.5[c,e]
	25	86.2 ± 4.9[c,e]
Zingiberis Rhizoma	0.5	81.9 ± 1.5[c,f]
	1	83.7 ± 0.8[c,f]
	2.5	84.3 ± 1.6[c,f]
	5	84.4 ± 2.0[c,f]
	10	87.3 ± 1.3[c,f]
	25	88.1 ± 0.8[c,f]
Glycyrrhizae Radix	0.5	80.5 ± 1.7[c,f]
	1	82.1 ± 0.8[c,f]
	2.5	82.3 ± 0.5[c,f]
	5	82.3 ± 0.4[c,f]
	10	83.7 ± 1.0[c,f]
	25	87.3 ± 0.7[c,f]

Statistical significance: [a]$p<0.05$, [b]$p<0.01$, [c]$p<0.001$ vs. normal values, [d]$p<0.05$, [e]$p<0.01$, [f]$p<0.001$ vs. AAPH-treated control values.

Results

AAPH

Table I shows the effect of Wen-Pi-Tang and its crude drug extracts on the viability of LLC-PK_1 renal epithelial cells treated with AAPH, which is a hydrophilic free radical generator. The viability of LLC-PK_1 cells declined to 75.1 % following AAPH treatment. However, we found that the cytotoxic effect of AAPH could be attenuated by incubation with Wen-Pi-Tang, Rhei Rhizoma and Ginseng Radix while significantly preserving cell survival in comparison with the control. In particular, Ginseng Radix produced greater cell viability when compared with non AAPH-treated cells. On the other hand, Aconiti Tuber, Zingiberis Rhizoma and Glycyrrhizae Radix produced relatively low cell viability in comparison with the other extracts, although they did maintain greater viability than the control.

As shown in Table II, AAPH enhanced lipid peroxidation in LLC-PK_1 renal tubular epithelial cells, while Wen-Pi-Tang, Rhei Rhizoma and Ginseng Radix significantly decreased the formation of TBARS by AAPH. As the treatment concentration was increased, the peroxidation by AAPH declined exponentially. Aconiti Tuber, Zingiberis Rhizoma and Glycyrrhizae Radix showed only relatively low inhibitory effects on TBARS formation.

AMVN

Table III shows the protective activity of Wen-Pi-Tang and its crude drug extracts against cellular damage mediated by AMVN. AMVN induced a loss of LLC-PK_1 renal tubular epithelial cells, and a decrease in cell viability to 59.9 %. However, the presence of Wen-Pi-Tang and its crude drug extracts produced a dose-dependent recovery of viability of LLC-PK_1 cells. Of these, Wen-Pi-Tang (25 μg/ml) showed the highest protective activity, producing nearly 100 % cell viability. Moreover, Rhei Rhizoma and Ginseng Radix also have strong antioxidant activity against peroxyl radicals generated by AMVN. On the other hand, Aconiti Tuber, Zingiberis Rhizoma and Glycyrrhizae Radix showed relatively weak effects.

Table II Effect of Wen-Pi-Tang and its crude drug extracts on the formation of TBARS by AAPH.

Material	Concentration (μg/ml)	TBARS (nmol/well)
Normal	–	0.072 ± 0.004
Control	–	0.140 ± 0.006[b]
Wen-Pi-Tang	0.5	0.142 ± 0.010[b]
	1	0.134 ± 0.006[b]
	2.5	0.130 ± 0.006[b]
	5	0.118 ± 0.008[b,d]
	10	0.108 ± 0.010[b,e]
	25	0.098 ± 0.008[b,e]
Rhei Rhizoma	0.5	0.136 ± 0.004[b]
	1	0.130 ± 0.004[b,c]
	2.5	0.124 ± 0.006[b,e]
	5	0.112 ± 0.004[b,e]
	10	0.100 ± 0.006[b,e]
	25	0.092 ± 0.004[b,e]
Ginseng Radix	0.5	0.134 ± 0.008[b]
	1	0.132 ± 0.006[b]
	2.5	0.126 ± 0.006[b]
	5	0.112 ± 0.006[b,e]
	10	0.098 ± 0.008[b,e]
	25	0.090 ± 0.004[a,e]
Aconiti Tuber	0.5	0.142 ± 0.004[b]
	1	0.140 ± 0.008[b]
	2.5	0.140 ± 0.008[b]
	5	0.134 ± 0.004[b]
	10	0.130 ± 0.006[b]
	25	0.122 ± 0.006[b,d]
Zingiberis Rhizoma	0.5	0.138 ± 0.006[b]
	1	0.140 ± 0.004[b]
	2.5	0.134 ± 0.006[b]
	5	0.128 ± 0.004[b,c]
	10	0.122 ± 0.006[b,e]
	25	0.118 ± 0.006[b,e]
Glycyrrhizae Radix	0.5	0.136 ± 0.004[b]
	1	0.138 ± 0.006[b]
	2.5	0.132 ± 0.006[b]
	5	0.126 ± 0.008[b,d]
	10	0.120 ± 0.006[b,e]
	25	0.114 ± 0.004[b,e]

Statistical significance: [a]$p<0.01$, [b]$p<0.001$ vs. normal values, [c]$p<0.05$, [d]$p<0.01$, [e]$p<0.001$ vs. AAPH-treated control values.

Table III Effect of Wen-Pi-Tang and its crude drug extracts on viability of cells treated with AMVN.

Material	Concentration (μg/ml)	Cell viability (%)
Normal	–	100.0 ± 0.7
Control	–	59.9 ± 1.3[b]
Wen-Pi-Tang	0.5	82.4 ± 3.4[b,d]
	1	90.2 ± 0.8[b,d]
	2.5	91.4 ± 2.7[b,d]
	5	93.0 ± 2.4[b,d]
	10	94.1 ± 3.4[a,d]
	25	98.1 ± 1.6[d]
Rhei Rhizoma	0.5	75.0 ± 0.1[b,d]
	1	79.4 ± 1.1[b,d]
	2.5	85.6 ± 1.1[b,d]
	5	85.8 ± 1.3[b,d]
	10	88.9 ± 1.7[b,d]
	25	92.6 ± 2.8[b,d]
Ginseng Radix	0.5	83.5 ± 1.5[b,d]
	1	83.9 ± 3.7[b,d]
	2.5	85.7 ± 3.6[b,d]
	5	90.3 ± 3.3[b,d]
	10	94.0 ± 3.0[a,d]
	25	95.7 ± 3.4[d]
Aconiti Tuber	0.5	72.1 ± 1.5[b,d]
	1	73.9 ± 1.8[b,d]
	2.5	77.3 ± 1.5[b,d]
	5	78.4 ± 1.6[b,d]
	10	79.7 ± 1.7[b,d]
	25	82.6 ± 1.7[b,d]
Zingiberis Rhizoma	0.5	61.7 ± 0.7[b]
	1	63.7 ± 0.6[b,c]
	2.5	68.2 ± 0.7[b,d]
	5	70.7 ± 0.6[b,d]
	10	72.2 ± 1.3[b,d]
	25	73.4 ± 2.7[b,d]
Glycyrrhizae Radix	0.5	69.9 ± 2.2[b,d]
	1	71.1 ± 1.9[b,d]
	2.5	79.9 ± 3.0[b,d]
	5	80.5 ± 2.4[b,d]
	10	81.8 ± 1.1[b,d]
	25	83.8 ± 5.6[b,d]

Statistical significance: [a]$p<0.05$, [b]$p<0.001$ vs. normal values, [c]$p<0.01$, [d]$p<0.001$ vs. AMVN-treated control values.

The protective activity from lipid peroxidation against AMVN of the various extracts is shown in Table IV. AMVN enhanced lipid peroxidation in LLC-PK_1 cells, and significantly increased TBARS formation. Wen-Pi-Tang and its crude drug extracts demonstrated significant protective activity against AMVN-mediated lipid peroxidation. In a similar manner, Wen-Pi-Tang, Rhei Rhizoma and Ginseng Radix inhibited lipid peroxidation more significantly than the other extracts. In particular, at a concentration of 25 μg/ml, Ginseng Radix extract combined with AMVN treatment resulted in the formation of 0.136 nmol/well of TBARS, whereas 0.204 nmol/well

Table IV Effect of Wen-Pi-Tang and its crude drug extracts on the formation of TBARS by AMVN.

Material	Concentration (μg/ml)	TBARS (nmol/well)
Normal	−	0.076 ± 0.004
Control	−	0.204 ± 0.012[a]
Wen-Pi-Tang	0.5	0.198 ± 0.006[a]
	1	0.192 ± 0.008[a]
	2.5	0.184 ± 0.012[a,b]
	5	0.174 ± 0.004[a,d]
	10	0.160 ± 0.008[a,d]
	25	0.152 ± 0.008[a,d]
Rhei Rhizoma	0.5	0.184 ± 0.008[a,c]
	1	0.180 ± 0.006[a,d]
	2.5	0.170 ± 0.006[a,d]
	5	0.164 ± 0.008[a,d]
	10	0.156 ± 0.006[a,d]
	25	0.146 ± 0.008[a,d]
Ginseng Radix	0.5	0.188 ± 0.008[a,b]
	1	0.178 ± 0.006[a,d]
	2.5	0.166 ± 0.008[a,d]
	5	0.158 ± 0.008[a,d]
	10	0.144 ± 0.006[a,d]
	25	0.136 ± 0.004[a,d]
Aconiti Tuber	0.5	0.208 ± 0.004[a]
	1	0.202 ± 0.008[a]
	2.5	0.198 ± 0.008[a]
	5	0.194 ± 0.004[a]
	10	0.184 ± 0.008[a,c]
	25	0.176 ± 0.004[a,d]
Zingiberis Rhizoma	0.5	0.206 ± 0.004[a]
	1	0.200 ± 0.008[a]
	2.5	0.196 ± 0.006[a]
	5	0.184 ± 0.004[a,c]
	10	0.180 ± 0.006[a,d]
	25	0.172 ± 0.008[a,d]
Glycyrrhizae Radix	0.5	0.206 ± 0.004[a]
	1	0.200 ± 0.008[a]
	2.5	0.198 ± 0.008[a]
	5	0.186 ± 0.006[a,c]
	10	0.180 ± 0.004[a,d]
	25	0.170 ± 0.004[a,d]

Statistical significance: [a]$p < 0.001$ vs. normal values, [b]$p < 0.05$, [c]$p < 0.01$, [d]$p < 0.001$ vs. AMVN-treated control values.

of TBARS was produced in wells treated with AMVN alone.

Discussion

Reactive oxygen metabolites, including free radicals, play an important role in tissue injury.[11] *In vitro* and *in vivo* studies have implicated reactive oxygen metabolites in various forms of toxic nephropathy.[12-16] The putative site of injury in most forms of toxic nephropathy is the epithelial cells lining the tubules.[17] Although various kinds of free radical initiators are known, azo compounds have been used easily and successfully as radical initiators, since they generate free radicals at a measurable and constant rate *via* thermal decomposition without biotransformation. The free radicals generated from azo compounds react with oxygen radicals, attack other lipid molecules to form lipid hydroperoxide and new lipid radicals. This reaction, which induces physiochemical alterations and cellular damage takes place repeatedly with a resultant attack upon various biological molecules.[18,19] The free radicals ultimately cause a diverse array of pathological changes.

Under hydrophilic and lipophilic conditions, generators of free radicals may be located in two different cellular compartments, namely the cytosol and membrane. These areas represent aqueous and hydrophobic phases, respectively.[20] Therefore, we chose AAPH and AMVN as the source of hydrophilic and lipophilic free radical initiators, since unlike most experimental systems suitable for the induction of oxidative stress, these compounds do not require the addition of potentially interferring cofactors and transition metals. For these reasons, they are useful tools for studying the damage induced by free radicals on biological systems. Hydrophilic AAPH added to the aqueous phase generates radicals in the aqueous region, whereas lipophilic AMVN located in the lipid region of micelles or membrane initially generates radicals within the lipid region.[18,21-26]

We confirmed that both AAPH and AMVN caused oxidative damage in LLC-PK_1 renal tubular epithelial cells, resulting in the loss of cell viability. One mM AAPH and AMVN caused declines in cell viability to 75.1 % and 59.9 %, respectively. In the present experiment, further evidence was obtained that cells damaged by oxidative stress from free radicals peroxidize more rapidly, resulting in the formation of lipid peroxidation end products, such as TBARS, that provide a good index of cell destruction. Lipid peroxidation in LLC-PK_1 cells was increased by

AMVN, a lipophilic free radical generator, more significantly than by AAPH, a hydrophilic generator. The TBARS formation was enhanced from 0.072 to 0.140 nmol/well and from 0.076 to 0.204 nmol/well in response to AAPH and AMVN treatment, respectively. These results demonstrate that the oxidative damage caused by AMVN is relatively more severe than that by AAPH.

Antioxidants play a major role in protecting tissue from cellular loss and lipid peroxidation caused by reactive intermediates formed and released during oxidative stress. Oriental medicines, which include a variety of antioxidant compounds, are still much in demand despite the widespread use of conventional medicines. Research is now being conducted in the treatment of chronic diseases that respond poorly to conventional drug therapy, to determine the actual role of the antioxidants in oriental medicines and medical prescriptions comprised of a combination of several oriental medicines. This research also attempts to clarify how active oxygen and its effects, such as lipid peroxidation, are involved in various diseases. Of the numerous prescription Chinese medicines, the authors previously demonstrated the usefulness of Wen-Pi-Tang as a conservative treatment for renal failure under enhanced oxidative conditions in both experimental and clinical settings.[27-34,6] To experimentally establish the scientific basis for the action of Wen-Pi-Tang, we further examined the antioxidant capacity of Wen-Pi-Tang and its component crude drugs both *in vivo* and *in vitro*. The results of our previous studies suggested that Wen-Pi-Tang and some of its component crude drugs exert an antioxidant action on the impaired kidney under oxidative stress.[5,7]

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. Recently, the importance of different types of antioxidants, water-soluble or lipid-soluble, has been the focus of considerable research attention. Several reports suggest that scavenging of aqueous and lipid peroxyl radicals at the surface of membranes as well as within the membranes, plays a considerable role in antioxidative activity.[35-40] Consequently, the localization of antioxidants and the site of radicals generated should be taken into account when assessing their antioxidant activities. In other words, hydrophilic antioxidants scavenge the aqueous radicals efficiently, but cannot scavenge lipophilic radicals within the lipid region. On the other hand, lipophilic antioxidants scavenge lipophilic radicals within the liposomal membranes, not hydrophilic radicals within the aqueous region. With this in mind, the free radical scavenging activity of Wen-Pi-Tang and each of its component crude drugs were investigated using LLC-PK_1 cells, which are susceptible to oxidative stress. Wen-Pi-Tang scavenged both AAPH and AMVN-generated free radicals, suggesting that it plays a role both as a hydrophilic and lipophilic antioxidant. Rhei Rhizoma and Ginseng Radix, two of the component crude drugs, had strong activity, while Aconiti Tuber, Zingiberis Rhizoma and Glycyrrhizae Radix produced a relatively weak effect. Thus, Wen-Pi-Tang was shown to inhibit the scavenging activity of peroxyl radicals generated by AAPH and AMVN, resulting in higher cell viability and lower MDA formation. Two of its component crude drugs, Rhei Rhizoma and Ginseng Radix, were shown to play important contributory roles in such activity of Wen-Pi-Tang.

和文抄録

水溶性アゾ化合物の AAPH (2,2'-azobis(2-amidinopropane)dihydrochloride) と脂溶性アゾ化合物の AMVN (2,2'-azobis(2,4-dimethylvaleronitrile)) で腎上皮細胞の LLC-PK_1 に酸化的ストレスを惹起させ，温脾湯と5種類の構成和漢薬の効果を検討した。AAPH と AMVN で処理した場合，細胞生存率が著しく低下し，チオバルビツール酸反応物質の生成が著しく上昇したが，温脾湯と各構成和漢薬エキスをそれぞれ添加した場合，温脾湯と大黄，薬用人参では高い抗酸化活性を示した。しかし附子，乾姜，甘草では相対的に低い活性であった。このことから，温脾湯の抗酸化活性は構成和漢薬に起因し，また温脾湯は水溶性抗酸化物と脂溶性抗酸化物の両方の特徴を有していることが示唆された。

References

1) Halliwell, B.: Oxidants and human disease: some new concepts.

FASEB J. **1**, 358-364, 1987.

2) Noguchi, N., Yamashita, H., Gotoh, N., Yamamoto, Y., Numano, R. and Niki, E.: 2,2'-Azobis(4-methoxy-2,4-dimethylvaleronitrile), a new lipid-soluble azo initiator: application to oxidations of lipids and low-density lipoprotein in solution and in aqueous dispersions. *Free Radic. Biol. Med.* **24**, 259-268, 1998.

3) Niki, E.: Free radical initiators as source of water- or lipid-soluble peroxyl radicals. *Meth. Enzymol.* **186**, 100-108, 1990.

4) Okuda, T.: Crude drugs. In "Antioxidants. Free Radicals and Biological Defense" (Ed. by Niki, E., Shimasaki, H. and Mino, M.), Japan Scientific Societies Press, Japan, pp.263-275, 1994.

5) Yokozawa, T., Dong, E., Oura, H., Nishioka, I., Kawai, Y. and Gemba, M.: Protective effects of Wen-Pi-Tang against cultured renal epithelial cellular injury. *Phytomedicine* **4**, 245-250, 1997.

6) Yokozawa, T., Dong, E., Yasui, T. and Muraguchi, A.: Protective effect of Wen-Pi-Tang against apoptosis of cultured renal epithelial cells. *Phytother. Res.* **12**, 135-137, 1998.

7) Ninomiya, H., Mitsuma, T., Takara, M., Yokozawa, T., Terasawa, K. and Okuda, H.: Effects of the Oriental medical prescription Wen-Pi-Tang in patients receiving dialysis. *Phytomedicine* **5**, 245-252, 1998.

8) Oura, H., Zheng, P.D. and Yokozawa, T.: Effect of Wen-Pi-Tang in rats with chronic renal failure. *J. Med. Pharm. Soc. WAKAN-YAKU* **1**, 209-217, 1984.

9) Yagi, K.: A simple fluorometric assay for lipoperoxide in blood plasma. *Biochem. Med.* **15**, 212-216, 1976.

10) Yokode, M., Kita, T., Kikawa, Y., Ogorochi, T., Narumiya, S. and Kawai, C.: Stimulated arachidonate metabolism during foam cell transformation of mouse peritoneal macrophages with oxidized low density lipoprotein. *J. Clin. Invest.* **81**, 720-729, 1988.

11) Baud, L. and Ardaillou, R.: Reactive oxygen species: production and role in the kidney. *Am. J. Physiol.* **251**, F765-F776, 1986.

12) Paller, M.S., Hoidal, J.R. and Ferris, T.F.: Oxygen free radicals in ischemic acute renal failure in the rat. *J. Clin. Invest.* **74**, 1156-1164, 1984.

13) Walker, P.D. and Shah, S.V.: Gentamicin enhanced production of hydrogen peroxide by renal cortical mitochondria. *Am. J. Physiol.* **253**, C495-C499, 1987.

14) Walker, P.D. and Shah, S.V.: Evidence suggesting a role for hydroxyl radical in gentamicin-induced acute renal failure in rats. *J. Clin. Invest.* **81**, 334-341, 1988.

15) Paller, M.S.: Hemoglobin- and myoglobin-induced acute renal failure in rats: role of iron in nephrotoxicity. *Am. J. Physiol.* **255**, F539-F544, 1988.

16) Shah, S.V.: Role of reactive oxygen metabolites in experimental glomerular disease. *Kidney Int.* **35**, 1093-1106, 1989.

17) Brezis, M., Rosen, S. and Epstein, F.H.: Acute renal failure. In "The Kidney" (Eds. by Brenner, B.M. and Rector, F.C.), WB Saunders, Philadelphia, pp.993-1061, 1991.

18) Yamamoto, Y., Niki, E., Eguchi, J., Kamiya, Y. and Shimasaki, H.: Oxidation of biological membranes and its inhibition. Free radical chain oxidation of erythrocyte ghost membranes by oxygen. *Biochim. Biophys. Acta* **819**, 29-36, 1985.

19) Terao, K. and Niki, E.: Damage to biological tissues induced by radical initiator 2,2'-azobis(2-amidinopropane) dihydrochloride and its inhibition by chain-breaking antioxidants. *Free Radic. Biol. Med.* **2**, 193-201, 1986.

20) Ko, F.N., Liao, C.H., Kuo, Y.H. and Lin, Y.L.: Antioxidant properties of demethyldiisoeugenol. *Biochim. Biophys. Acta* **1258**, 145-152, 1995.

21) Yamamoto, Y., Haga, S., Niki, E. and Kamiya, Y.: Oxidation of lipids. V. Oxidation of methyl linoleate in aqueous dispersion. *Bull. Chem. Soc. Jpn.* **57**, 1260-1264, 1984.

22) Barclay, L.R.C., Locke, S.J., MacNeil, J.M. and VanKessel, J.: Autoxidation of micelles and model membranes. Quantitative kinetic measurements can be made by using either water-soluble or lipid-soluble initiators with water-soluble or lipid-soluble chain-breaking antioxidants. *J. Am. Chem. Soc.* **106**, 2479-2481, 1984.

23) Yamamoto, Y., Niki, E., Kamiya, Y. and Shimasaki, H.: Oxidation of lipids 7. Oxidation of phosphatidylcholines in homogeneous solution and in water dispersion. *Biochim. Biophys. Acta* **795**, 332-340, 1984.

24) Doba, T., Burton, G.W. and Ingold, K.U.: Antioxidant and co-antioxidant activity of vitamin C. The effect of vitamin C, either alone or in the presence of vitamin E or a water-soluble vitamin E analogue, upon the peroxidation of aqueous multilamellar phospholipid liposomes. *Biochim. Biophys. Acta* **835**, 298-303, 1985.

25) Miki, M., Tamai, H., Mino, M., Yamamoto, Y. and Niki, E.: Free-radical chain oxidation of rat red blood cells by molecular oxygen and its inhibition by α-tocopherol. *Arch. Biochem. Biophys.* **258**, 373-380, 1987.

26) Niki, E., Komuro, E., Takahashi, M., Urano, S., Ito, E. and Terao, K.: Oxidative hemolysis of erythrocytes and its inhibition by free radical scavengers. *J. Biol. Chem.* **263**, 19809-19814, 1988.

27) Zheng, P.D., Yokozawa, T., Oura, H. and Nakada, T.: Effect of orally administered Wen-Pi-Tang to rats with chronic renal failure on blood flow in renal tissue, blood pressure, and hormone levels in blood. *J. Med. Pharm. Soc. WAKAN-YAKU* **3**, 37-44, 1986.

28) Yokozawa, T., Wu, X.Q., Lee, T.W. and Oura, H.: Wen-Pi-Tang administration increases renal function in rats with renal failure. *J. Med. Pharm. Soc. WAKAN-YAKU* **5**, 179-183, 1988.

29) Yokozawa, T., Wu, X.Q. and Oura, H.: Wen-Pi-Tang prevents the progression of renal failure. *J. Med. Pharm. Soc. WAKAN-YAKU* **6**, 147-151, 1989.

30) Yokozawa, T., Wu, X.Q., Lee, T.W. and Oura, H.: Effects of Wen-Pi-Tang on the urinary levels of prostaglandin and kallikrein in rats with renal failure. *J. Med. Pharm. Soc. WAKAN-YAKU* **6**, 188-192, 1989.

31) Yokozawa, T., Zheng, P.D., Oura, H., Hattori, M., Kuwayama, N. and Takaku, A.: Wen-Pi-Tang relieves acidosis in rats given adenine. *J. Med. Pharm. Soc. WAKAN-YAKU* **9**, 55-58, 1992.

32) Yokozawa, T., Maeda, K., Mitsuma, T., Toriizuka, K. and Oura, H.: Effects of Wen-Pi-Tang on renal anemia and platelet aggregation activity. *J. Med. Pharm. Soc. WAKAN-YAKU* **9**, 137-142, 1992.

33) Yokozawa, T., Oura, H., Hattori, M., Iwano, M. and Dohi, K.: Effects of Wen-Pi-Tang and its crude drug extracts on proliferation of cultured mouse mesangial cells. *Phytother. Res.* **8**, 170-173, 1994.

34) Mitsuma, T., Yokozawa, T., Oura, H., Terasawa, K. and Narita, M.: Clinical evaluation of Kampo medication, mainly with Wen-Pi-Tang, on the progression of chronic renal failure. *Jpn. J. Nephrol.* **41**, 769-777, 1999.

35) Frei, B., Stocker, R. and Ames, B.N.: Antioxidant defenses and lipid peroxidation in human blood plasma. *Proc. Natl. Acad. Sci. USA* **85**, 9748-9752, 1988.

36) Frei, B., England, L. and Ames, B.N.: Ascorbate is an outstanding antioxidant in human blood plasma. *Proc. Natl. Acad. Sci. USA* **86**, 6377-6381, 1989.

37) Sato, K., Niki, E. and Shimasaki, H.: Free radical-mediated chain

oxidation of low density lipoprotein and its synergistic inhibition by vitamin E and vitamin C. *Arch. Biochem. Biophys.* **279**, 402-405, 1990.

38) Dooley, M.M., Sano, N., Kawashima, H. and Nakamura, T.: Effects of 2,2'-azobis (2-amidinopropane) hydrochloride *in vivo* and protection by vitamin E. *Free Radic. Biol. Med.* **9**, 199-204, 1990.

39) Kagan, V.E., Tsuchiya, M., Serbinova, E., Packer, L. and Sies, H.: Interaction of the pyridoindole stobadine with peroxyl, superoxide and chromanoxyl radicals. *Biochem. Pharmacol.* **45**, 393-400, 1993.

40) Terao, J., Piskula, M. and Yao, Q.: Protective effect of epicatechin, epicatechin gallate, and quercetin on lipid peroxidation in phospholipid bilayers. *Arch. Biochem. Biophys.* **308**, 278-284, 1994.