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Prevention of Peroxynitrite-induced Renal Injury through Modulation of Peroxynitrite Production by the Chinese Prescription Wen-Pi-Tang

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The effect of Wen-Pi-Tang extract on renal injury induced by peroxynitrite ($ONOO^-$) production was investigated using rats subjected to intravenous lipopolysaccharide (LPS) injection and then renal ischemia followed by reperfusion. The plasma level of 3-nitrotyrosine, a marker of cytotoxic $ONOO^-$ formation *in vivo*, was enhanced markedly in control rats subjected to LPS plus ischemia-reperfusion, but was significantly reduced by the oral administration of Wen-Pi-Tang extract, at doses of 62.5 and 125 mg/kg body weight/day, for 30 days prior to LPS plus ischemia-reperfusion. The activities of inducible nitric oxide synthase (iNOS) and xanthine oxidase (XOD) in renal tissue of control and Wen-Pi-Tang extract-treated rats did not change significantly, while those of the antioxidant enzymes, superoxide dismutase, catalase and glutathione peroxidase, were significantly increased by the administration of Wen-Pi-Tang extract, indicating that Wen-Pi-Tang improved the defense system by scavenging free radicals, not by directly inhibiting nitric oxide and superoxide production by iNOS and XOD. In addition, the levels of the hydroxylated products, *m*- and *p*-tyrosine, declined, whereas that of phenylalanine increased, after oral administration of Wen-Pi-Tang extract. Furthermore, the elevated plasma urea nitrogen and creatinine levels resulting from LPS plus ischemia-reperfusion process were significantly reduced by Wen-Pi-Tang extract, implying amelioration of renal impairment. The present study indicates that Wen-Pi-Tang extract contributes to the regulation of $ONOO^-$ formation and plays a beneficial role against $ONOO^-$-induced oxidative injury and renal dysfunction *in vivo*.

Keywords: Peroxynitrite; Lipopolysaccharide; Ischemia-reperfusion; Wen-Pi-Tang; 3-Nitrotyrosine; Superoxide dismutase; Catalase; Glutathione peroxidase; Urea nitrogen; Creatinine

INTRODUCTION

The incidence of renal failure and number of dialysis patients have been increasing worldwide, which implies that we have no effective strategy to halt the progression of renal diseases. Dialysis and dietary protein restriction are the most commonly employed management strategies for these diseases.[1] However, the dialysis procedure carries the risk of bleeding and hemorrhage from the site of vascular access.[2] Therefore, a new approach to prevent and treat effectively renal diseases and their associated complications is highly desirable.

Although several causes of renal failure have been demonstrated, in recent years, numerous clinical and experimental studies have indicated that increased oxidative stress is mainly responsible for renal failure.[3,4] In particular, peroxynitrite ($ONOO^-$), which can be formed by the reaction between the superoxide anion (O_2^-) and nitric oxide (NO), is considered to be a major factor in the pathogenesis of renal ischemia-reperfusion injury. $ONOO^-$-induced tyrosine nitration alters the structure and function of proteins[5–7] and inflicts damage on biological molecules with spontaneous decomposition to the hydroxyl radical (·OH).[8] Therefore, antioxidants that play a beneficial role in ameliorating renal diseases and their complications by scavenging O_2^-, ·OH and $ONOO^-$, and inhibiting their generation would be expected to be useful therapeutic agents.

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Unfortunately, only a few antioxidant intervention studies on renal patients have been published.

Recently, Chinese traditional prescriptions have attracted a lot of attention as therapeutic agents for oxidative stress-related diseases. Wen-Pi-Tang is one of the traditional prescriptions used clinically as a medicine to treat renal failure. In previous studies, we demonstrated that Wen-Pi-Tang exhibited a protective action on the impaired kidney under oxidative stress[9–14] as well as free radical-scavenging activity in $ONOO^-$, NO and O_2^- generation systems *in vitro*.[15–17] On the basis of these findings, Wen-Pi-Tang would be expected to ameliorate renal damage induced by $ONOO^-$ *in vivo*. Beneficial effects of Wen-Pi-Tang under *in vivo* conditions of excessive levels and simultaneous generation of NO, O_2^- and $ONOO^-$ responsible for renal failure have not been demonstrated yet. In biological system, nitrated and/or hydroxylated products from tyrosine and/or phenylalanine serve as indicators of $ONOO^-$ formation. Therefore, we measured the levels of 3-nitrotyrosine and hydroxylated products to investigate the generation of $ONOO^-$ and its decomposition products under the animal model that subjected to lipopolysaccharide (LPS) plus ischemia-reperfusion, and also observed whether Wen-Pi-Tang extract scavenges or inhibits radicals produced by the process and whether such activities ameliorate renal impairment.

MATERIALS AND METHODS

Materials

LPS (from *Escherichia coli* serotype 055: B5), DL-*o*-, *m*- and *p*-tyrosine, 3-nitro-L-tyrosine, pepstatin A, chymostatin, aprotinin, phenylmethylsulfonyl fluoride, flavin adenine dinucleotide (FAD), tetrahydrobiopterin, dithiothreitol (DTT), β-nicotinamide-adenine dinucleotide phosphate (reduced, NADPH), lactate dehydrogenase (LDH) and sodium pyruvate were purchased from Sigma Chemical Co. (St. Louis, MO, USA). DL-Phenylalanine, phenazine methosulfate and nitro blue tetrazolium were purchased from Wako Pure Chemical Industries Ltd. (Osaka, Japan).

Preparation of Wen-Pi-Tang Extract

The Wen-Pi-Tang prescription was prepared with 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). Aconiti Tuber was obtained from Japan, Ginseng Radix was produced in Korea and the other elements were from China. As previously described,[9] an extract was manufactured by boiling the above crude drugs gently in 1,000 ml water for 60 min and the mixture was concentrated under reduced pressure, resulting in a yield of about 30%, by weight, of the original preparation. For analysis of the Wen-Pi-Tang components, the aqueous extract was filtered and subjected to treatment with an Alumina cartridge (Bond Elute Co., Ltd.). HPLC using a TSK GEL ODS-80Ts column (250 × 4.6 mm) with a LC-10AD pump (Shimadzu, Tokyo, Japan) and a SPD-M10A_{vp} absorbance detector was performed. The solvents were (A) 0.05 M AcOH–$AcONH_4$ (pH 3.6) and (B) 100% CH_3CN, and the column was eluted with a linear gradient of 90% (v/v) A and 10% (v/v) B that changed over 60 min to 100% B. The flow rate was 1.0 ml/min and the effluent from the column was monitored with a UV detector. The three-dimensional HPLC profile of Wen-Pi-Tang extract is shown in Fig. 1.

Animals and Treatments

The "Guidelines for Animal Experimentation" approved by Toyama Medical and Pharmaceutical University were followed in these experiments. Five-week-old male Wistar rats (120–130 g) were obtained from Japan SLC, Inc. (Hamamatsu, Japan). They were kept in wire-bottomed cages at constant temperature (about 25°C) and humidity (about 60%) with a 12 h light–dark cycle and free access to a commercial chow (CLEA Japan Inc., Tokyo, Japan; comprising 24.0% protein, 3.5% lipid and 60.5% carbohydrate) and water. Following several days of adaptation, the rats were divided into four groups, avoiding any intergroup differences in body weight. Two groups (control and sham) were given water to drink and the other two were given Wen-Pi-Tang extract dissolved in their drinking water. The dose was adjusted to 62.5 or 125 mg/kg body weight/day by regulating the concentration in response to water consumption. After administration for 30 consecutive days, the rats were anesthetized with pentobarbital sodium (50 mg/kg). Using aseptic technique, bilateral flank incisions were made to expose the kidneys, both renal pedicles were isolated and occluded for 60 min with microvascular clamps. Then, the clamps were released and the incisions were closed with skin staples. Fifty minutes after the cessation of ischemia, all the rats received an intravenous LPS injection (5 mg/kg). The sham group underwent sham surgery (incisions were made to expose the kidneys, but the renal pedicles were not clamped). Six hours after the LPS challenge, blood was collected by cardiac puncture and centrifuged to prepare plasma. Subsequently,

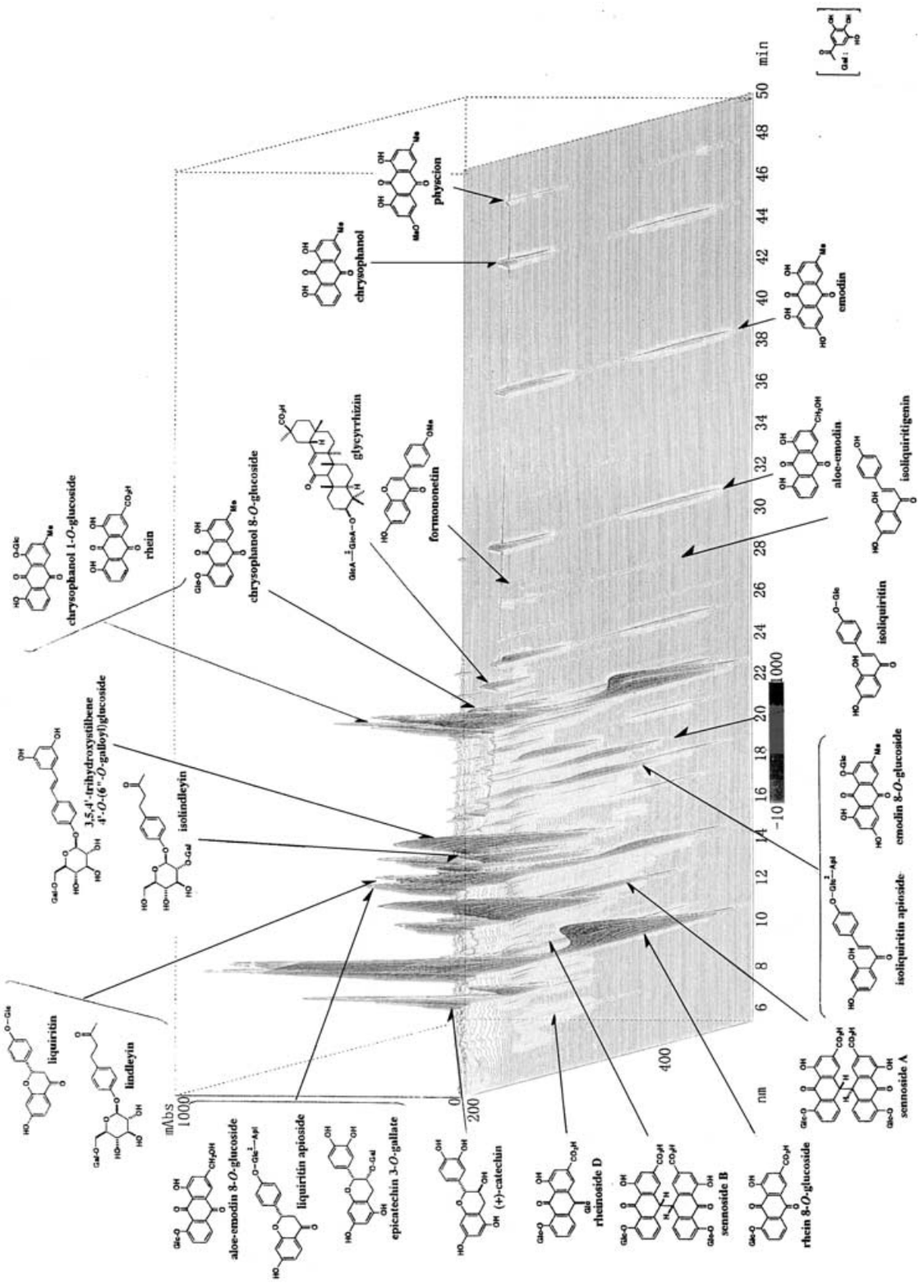

FIGURE 1 Three-dimension HPLC profile of Wen-Pi-Tang extract.

the renal arteries of each rat were perfused with ice-cold perfusion buffer comprising 50 mM sodium phosphate, 10 mM EDTA–2Na and 120 mM NaCl, the kidneys were removed, quickly frozen and kept at −80°C until analysis.

HPLC Analysis of 3-Nitrotyrosine, and *o*-, *m*- and *p*-Tyrosine

The amounts of 3-nitrotyrosine, and *o*-, *m*- and *p*-tyrosine, markers of proteins nitrated and hydroxylated by $ONOO^-$ and ·OH, formed were determined by HPLC analysis according to the methods of van der Vleit *et al.*[18] and Kaur *et al.*[19] with a slight modification. Briefly, the blood samples were centrifuged at 14,000 rpm for 15 min, the resulting plasma was incubated with proteinase K (1 U/10 mg protein) for 18 h at 55°C, then centrifuged again at 14,000 rpm for 15 min and passed through a 10,000-Da molecular mass cut-off filter. The resulting samples were loaded onto a reversed-phase column (Nucleosil 5 μC-18, 250 × 46 mm) at 25°C and eluted with 50 mM KH_2PO_4–H_3PO_4 (pH 3.01) containing 10% methanol (v/v) at a flow rate of 0.8 ml/min. Detection of the amino acid derivatives was accomplished by monitoring ultraviolet absorbance 274 and 365 nm. The peaks were identified by comparing their retention times with those of authentic standards added to additional samples and quantified according to their peak areas relative to known amounts of the external standards.

Determination of Enzyme Activities

Inducible nitric oxide synthase (iNOS) activity, an initiator of NO production, was measured using the method described by Suh *et al.*[20] Kidney tissue was homogenized in cold 40 mM Tris–HCl (pH 8.0) containing 5 μg/ml pepstatin A, 1 μg/ml chymostatin, 5 μg/ml aprotinin and 100 μM phenylmethylsulfonyl fluoride. The homogenate was centrifuged at 12,000 *g* for 5 min at 4°C, the precipitate containing the nuclei and cell debris was discarded, and aliquots of the supernatant fraction were used in the experiments described below. Triplicate aliquots (10–20 μg protein) of each supernatant were incubated in 20 mM Tris–HCl (pH 7.9) containing 4 μM FAD, 4 μM tetrahydrobiopterin, 3 mM DTT, 2 mM L-arginine and 2 mM NADPH in a 96-well plate for 180 min at 37°C. Residual NADPH was oxidized enzymatically with 10 U/ml LDH and 5 mM sodium pyruvate in a final volume of 130 μl and quantified by the Griess assay. Xanthine oxidase (XOD) activity was evaluated by the method of Bergmeyer *et al.*[21] Briefly, kidney tissue was homogenized in nine volumes of 250 mM sucrose solution, the reaction was initiated by adding sodium phosphate buffer (pH 7.4), EDTA and hypoxanthine, the mixture was incubated for 60 min at 37°C and the XOD activity was determined by measuring the absorbance at 292 nm. Superoxide dismutase (SOD) activity was determined according to the nitrous acid method described by Elstner and Heupel[22] and Oyanagui,[23] which is based on the inhibition of nitrite formation by hydroxylamine in the presence of O_2^- generators. Catalase activity was evaluated by following the decomposition of hydrogen peroxide (H_2O_2) directly by monitoring the decrease in extinction at 240 nm.[24] Glutathione peroxidase (GSH-Px) activity was measured by a colorimetric assay of the concentration of 2-nitro-5-thiobenzoic acid, a compound produced by the reaction between glutathione and 5,5′-dithiobis (2-nitrobenzoic acid).[25] Protein levels were determined by the micro-biuret method with bovine serum albumin as the standard.[26]

Determination of Plasma Urea Nitrogen and Creatinine (Cr) Levels

Plasma urea nitrogen and Cr levels were determined using the commercial reagents BUN Kainos and CRE-EN Kainos, respectively (Kainos Laboratories, Inc., Tokyo, Japan).

Data Analysis

The results are expressed as mean ± S.E. values. The statistical significance of differences among the four groups was evaluated using Dunnett's test. In all cases, differences at $p < 0.05$ were considered significant.

RESULTS

Plasma Concentrations of 3-Nitrotyrosine

Figure 2 shows the plasma concentrations of 3-nitrotyrosine after stimulation with LPS plus ischemia-reperfusion process. The plasma 3-nitrotyrosine level of the control group subjected to LPS plus ischemia-reperfusion was elevated markedly to 609 pmol/ml, whereas no nitrated tyrosine was detected in the plasma of the sham group. In contrast, the oral administration of Wen-Pi-Tang extract at doses of 62.5 and 125 mg/kg body weight/day for 30 days significantly and dose-dependently reduced the plasma 3-nitrotyrosine levels to 452 and 317 pmol/ml, respectively, compared with the control group value, 609 pmol/ml.

Plasma Concentrations of *o*-, *m*- and *p*-Tyrosine

As shown in Table I, the plasma concentrations of *o*-, *m*- and *p*-tyrosine, which were identified as

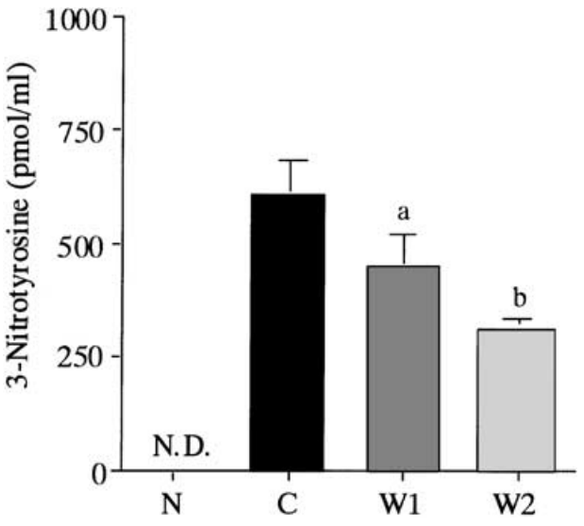

FIGURE 2 Effect of Wen-Pi-Tang extract on 3-nitrotyrosine level in plasma. N, sham treatment; C, LPS plus ischemic-reperfused control; W1, LPS plus ischemic-reperfused Wen-Pi-Tang extract (62.5 mg/kg B.W./day); W2, LPS plus ischemic-reperfused Wen-Pi-Tang extract (125 mg/kg B.W./day). [a]$p < 0.05$, [b]$p < 0.001$ vs. LPS plus ischemic-reperfused control values.

hydroxylated products, were markedly elevated in the LPS plus ischemia-reperfusion group, whereas the phenylalanine level decreased. However, the level of *m*-tyrosine in rats given Wen-Pi-Tang extract was significantly lower than that of the control rats subjected to LPS plus ischemia-reperfusion. In addition, the plasma phenylalanine level that was reduced by the experimental process showed a significant increase toward the normal value after the oral administration of Wen-Pi-Tang extract at a dose of 125 mg/kg body weight/day for 30 days.

Enzyme Activities in Renal Tissues

The activities of iNOS, XOD and antioxidant enzymes in renal tissues are shown in Fig. 3. The iNOS activity of normal rats was 1.73 pmol/mg protein/min, while that of the LPS plus ischemia-reperfusion control rats showed a significant increase to 2.81 pmol/mg protein/min and the oral administration of Wen-Pi-Tang extract did not reduce this activity. In addition, there were no significant differences among the renal tissue XOD activities of the normal, control and Wen-Pi-Tang extract-treated groups, although the activity of the LPS plus ischemia-reperfusion control group increased slightly in comparison with that of normal group. The activities of the antioxidant enzymes, SOD, catalase and GSH-Px, in the kidney were significantly suppressed by the LPS plus ischemia-reperfusion process, by 40%, 35% and 35%, respectively, relative to the normal group's values. However, the oral administration of Wen-Pi-Tang extract significantly increased the activities of these oxygen species-scavenging enzymes in a dose-dependent manner. The SOD activity of the LPS plus ischemia-reperfusion control rats was 27.0 U/mg protein, whereas the levels of the rats given Wen-Pi-Tang extract at doses of 62.5 and 125 mg/kg body weight/day for 30 days were 30.1 and 36.8 U/mg protein, respectively. Similarly, catalase activity was inhibited by the experimental process, but it was dose-dependently enhanced by Wen-Pi-Tang extract nearly normal value after oral administration of 125 mg/kg body weight/day for 30 days. In addition, the administration of Wen-Pi-Tang extract at doses of 62.5 and 125 mg enhanced the GSH-Px activity from 157.7 to 198.0 U/mg protein (26% increase, $p < 0.001$) and 202.1 U/mg protein (28% increase, $p < 0.001$), respectively.

Plasma Urea Nitrogen and Cr Levels

Table II shows the effects of Wen-Pi-Tang extract on parameters of renal function. The plasma urea nitrogen and Cr levels of the LPS plus ischemia-reperfusion control rats were significantly augmented about 4.3- and 4.8-fold, respectively, in comparison with those of the normal (sham) group. In contrast, the urea nitrogen levels of rats given Wen-Pi-Tang extract 62.5 and 125 mg/kg body weight/day for 30 days prior to LPS and ischemia-reperfusion declined from 65.4 to 51.9 mg/dl (21% decrease, $p < 0.001$) and to 48.8 mg/dl

TABLE I Effects of Wen-Pi-Tang extract on plasma *o*-, *m*-, *p*-tyrosine and phenylalanine levels

Group	Tyrosine (nmol/ml)			Phenylalanine (nmol/ml)
	o-	*m*-	*p*-	
Sham treatment	91.2 ± 6.9	15.2 ± 0.6	5896 ± 312	8756 ± 352
LPS plus ischemia-reperfusion				
Control	217.9 ± 9.1[c]	'39.9 ± 3.5[c]	6730 ± 460[a]	6218 ± 483[b]
Wen-Pi-Tang extract (62.5 mg/kg B.W./day)	217.1 ± 43.6[c]	25.7 ± 3.6[b,d]	6733 ± 396[a]	7746 ± 760
Wen-Pi-Tang extract (125 mg/kg B.W./day)	232.6 ± 41.4[c]	25.7 ± 6.9[b,d]	6431 ± 285	9225 ± 1798[d]

[a]$p < 0.05$, [b]$p < 0.01$, [c]$p < 0.001$ vs. sham treatment values, [d]$p < 0.001$ vs. LPS plus ischemia-reperfusion control values.

(25% decrease, $p < 0.001$), respectively. Similarly, the Cr level was markedly elevated by the experimental process. However, in the rats given Wen-Pi-Tang extract prior to the process, the Cr level decreased significantly after both the 62.5 (20% decrease, $p < 0.001$) and 125 mg (25% decrease, $p < 0.001$) doses, compared with the control group level.

DISCUSSION

$ONOO^-$ has been recognized as a major mediator responsible for tissue or cellular injury in various models of free radical-induced damage.[27,28] In addition, $ONOO^-$ is becoming a new focus of interest, as a variety of pathologic conditions *in vivo*,

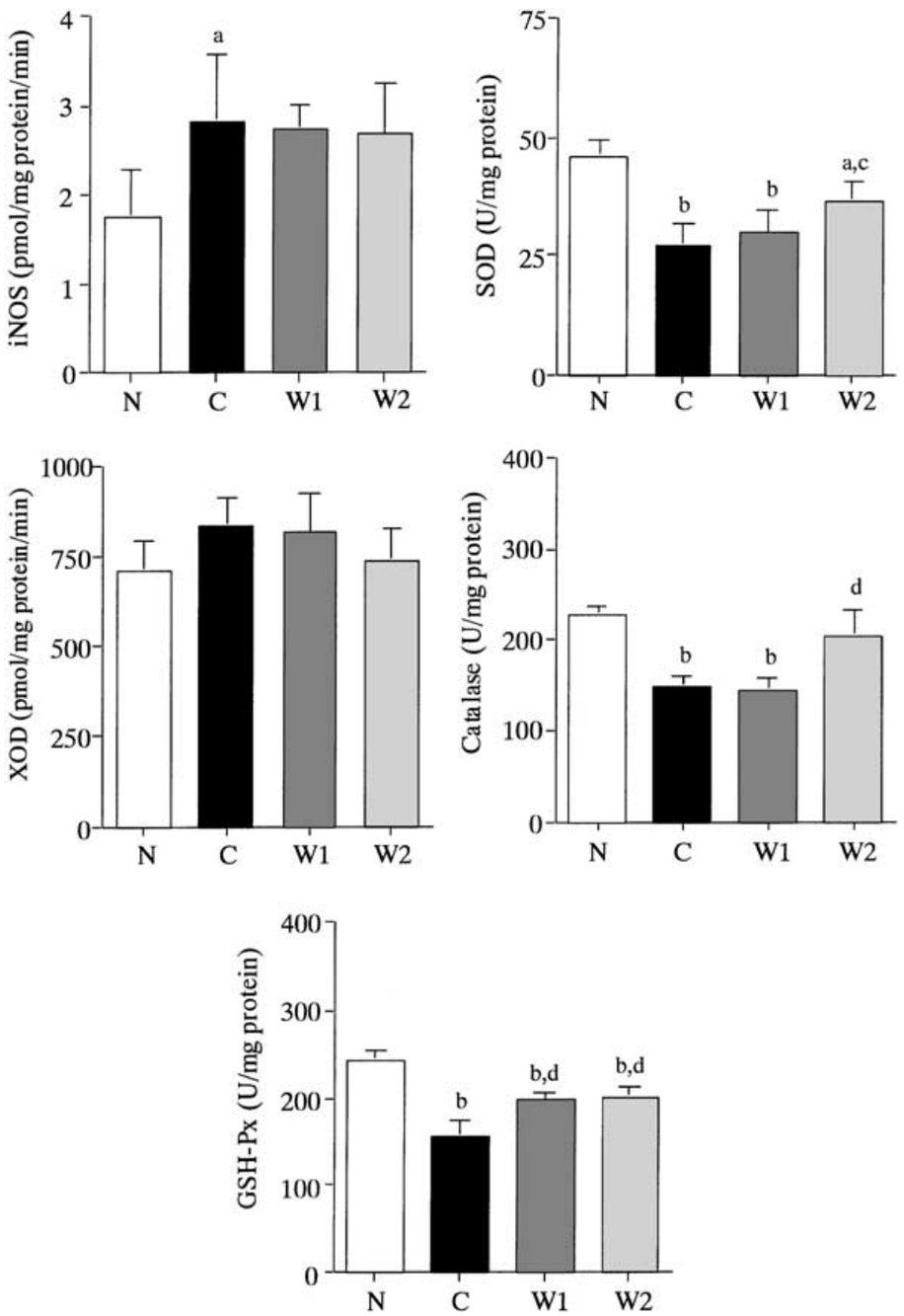

FIGURE 3 Effect of Wen-Pi-Tang extract on iNOS, XOD, and radical scavenging enzyme activities in renal tissue. N, sham treatment; C, LPS plus ischemic-reperfused control; W1, LPS plus ischemic-reperfused Wen-Pi-Tang extract (62.5 mg/kg B.W./day); W2, LPS plus ischemic-reperfused Wen-Pi-Tang extract (125 mg/kg B.W./day). [a]$p < 0.05$, [b]$p < 0.001$ vs. sham treatment values, [c]$p < 0.01$, [d]$p < 0.001$ vs. LPS plus ischemic-reperfused control values.

TABLE II Effects of Wen-Pi-Tang extract on plasma urea nitrogen and creatinine levels

Group	Urea-N (mg/dl)	Cr (mg/dl)
Sham treatment	15.2 ± 1.2	0.33 ± 0.04
LPS plus ischemia-reperfusion		
Control	65.4 ± 0.1[a]	1.59 ± 0.05[a]
Wen-Pi-Tang extract (62.5 mg/kg B.W./day)	51.9 ± 2.6[a,b]	1.27 ± 0.07[a,b]
Wen-Pi-Tang extract (125 mg/kg B.W./day)	48.8 ± 1.9[a,b]	1.20 ± 0.05[a,b]

[a]$p < 0.001$ vs. sham treatment values, [b]$p < 0.001$ vs. LPS plus ischemia-reperfusion control values.

such as inflammatory lung diseases, atherosclerosis and rheumatoid arthritis, have been attributed to $ONOO^-$ formation.[29–31] Under experimental conditions of ischemia-reperfusion of the heart, lung or kidney and endotoxin-induced renal oxidative injury, induction of NOS, increased O_2^- production and formation of $ONOO^-$, a particularly potent oxidizing agent, have been demonstrated.[32–34] However, due to the instability and reactivity of $ONOO^-$ in biological systems, the formation of enough $ONOO^-$ to enable its cytotoxicity under the conditions of renal failure to be evaluated should be accompanied by the simultaneous and excessive generation of NO and O_2^-. Therefore, we employed a LPS plus ischemia-reperfusion animal model to evaluate whether Wen-Pi-Tang extract can protect the kidney against damage caused by NO, O_2^-, $ONOO^-$ and ·OH generated by the decomposition of $ONOO^-$ *in vivo*.

$ONOO^-$ in biological fluids was detected by identifying nitrated tyrosine as a marker of $ONOO^-$ formation *in vivo* or a stable end-product of $ONOO^-$ oxidation. The formation of 3-nitrotyrosine in human tissues and animal models of various diseases is a remarkable observation, since traditionally, nitration has been viewed as a chemical modification that can be used to investigate the functional roles of tyrosine residues in enzymatic activity and protein function.[35] Recently, high levels of 3-nitrotyrosine have been found in the plasma of patients with chronic renal failure,[36] rheumatoid arthritis[31] and septic shock,[37] whereas 3-nitrotyrosine is generally not detectable in the plasma of healthy subjects.[31,36,37] Noiri *et al.*[38] observed that suppression or scavenging of $ONOO^-$ in ischemic acute renal failure improved renal function, consequently preventing lipid peroxidation and oxidative DNA damage. In our study, the significant increase of 3-nitrotyrosine caused by the pathological process of LPS plus ischemia-reperfusion declined after the oral administration of Wen-Pi-Tang extract prior to the process (Fig. 2). Therefore, our results suggest that Wen-Pi-Tang extract would ameliorate $ONOO^-$ mediated-renal damage by inhibiting $ONOO^-$ generation.

$ONOO^-$ decomposes to generate a potent oxidant, ·OH, which may cross cell membranes through anion channels and cause greater toxicity to tissues than $ONOO^-$. Van der Vliet *et al.*[18] demonstrated the formation of hydroxylated products after the reaction of $ONOO^-$ with phenylalanine by trapping the ·OH. Therefore, aromatic hydroxylation of phenylalanine is a specific detector of ·OH, and reaction of phenylalanine with $ONOO^-$ leads to the generation of tyrosine isomers such as *o*-, *m*- and *p*-tyrosine. On the basis of their report, we measured the levels of tyrosine isomers in order to investigate the formation of ·OH resulting from the decomposition of $ONOO^-$. Our results revealed that the high levels of the tyrosine isomers *o*-, *m*- and *p*-tyrosine produced by hydroxylation under condition of an $ONOO^-$ generation system *in vivo* were reduced by Wen-Pi-Tang extract (Table I). Moreover, the ·OH scavenging activity of Wen-Pi-Tang extract was confirmed by electron spin resonance analysis of kidney homogenates subjected to the Fenton reaction (data not shown). These findings provide direct evidence that Wen-Pi-Tang extract modulates the development of $ONOO^-$ and ·OH as secondary reactive end-products stimulated by LPS plus ischemia-reperfusion. Such a protective effect against $ONOO^-$ and ·OH may play an important role in preventing and reversing oxidative damage of tissue, and improving renal function.

NO, O_2^- and NO-derived oxidants, such as $ONOO^-$, have been proposed to be responsible for the tissue or cellular injury in various models, including ischemia-reperfusion,[8] and iNOS and XOD are major sources of NO and O_2^-, respectively. While the activity of iNOS was elevated in the LPS plus ischemia-reperfusion control group compared with that of rats subjected to sham operation, the XOD activities of these two groups were not significantly different (Fig. 3). In general, it is accepted that the levels of NO and O_2^- generated by iNOS and XOD increase during the ischemia-reperfusion process. However, several workers[39–42] demonstrated that, although XOD activity initially increased during ischemia, a decline in XOD activity occurred during reperfusion following the burst of reperfusion-associated $ONOO^-$ generation, suggesting that $ONOO^-$ could feed back and inhibit XOD. Our results support this, as following the postischemic burst of $ONOO^-$ generation, the XOD

activity did not increase during the period of reperfusion, implying that $ONOO^-$ in biological systems can feed back and down-regulate XOD activity. Wen-Pi-Tang extract inhibited $ONOO^-$ formation (Fig. 2), whereas it inhibited neither iNOS nor XOD activity (Fig. 3), indicating that the protective property of Wen-Pi-Tang extract was attributable not to the inhibition of NO and O_2^- but to direct scavenging of $ONOO^-$ and its highly reactive decomposition product ·OH, both of which were involved in the development of oxidative injury and renal dysfunction.

NO has been shown to inhibit catalase and GSH-Px, which might lead to elevated H_2O_2 levels and a subsequent increase in $ONOO^-$ production.[43,44] In addition, $ONOO^-$ itself inhibits these enzymes. There is a requirement for cellular defense against excessive $ONOO^-$ generation to protect against oxidative damage. Our present results showed that the activities of SOD, catalase and GSH-Px in renal tissue were all significantly suppressed by LPS plus ischemia-reperfusion process, which resulted in marked $ONOO^-$ generation (Fig. 3). However, these enzyme activities were effectively increased by the administration of Wen-Pi-Tang extract. SOD is well known to reduce O_2^- to levels low enough to prevent it reacting with NO to form $ONOO^-$[45,46] and inhibition of either molecular species by SOD was associated with improved renal function and decreased oxidative damage. In the light of this evidence, Wen-Pi-Tang extract may ameliorate renal oxidative damage through the enhancement of SOD activity. SOD may play a role as an O_2^- scavenger before O_2^- reacts with NO to form $ONOO^-$. SOD scavenges O_2^- to form H_2O_2, but subsequently, H_2O_2 can be detoxified by catalase and GSH-Px. Therefore, we also measured the activities of catalase and GSH-Px to confirm whether Wen-Pi-Tang extract actually detoxifies the radical damage induced by $ONOO^-$, O_2^- and ·OH. Wen-Pi-Tang extract increased the activity of catalase, which is specifically involved in the elimination of H_2O_2 and inhibition of ·OH production. LPS plus ischemia-reperfusion process generated $ONOO^-$ and its decomposition product ·OH, which is well known to be a highly toxic and reactive oxygen free radical that is mainly responsible for oxidative damage. Therefore, the increase of catalase activity by Wen-Pi-Tang extract in this experimental model indicates that Wen-Pi-Tang may reverse the oxidative damage caused by ·OH as well as $ONOO^-$. GSH-Px removes H_2O_2 by coupling its reduction to H_2O with oxidation of reduced glutathione, GSH. Therefore, loss of the GSH-Px activity would reduce the detoxification of lipid hydroperoxides as well as H_2O_2. Sies *et al.*[47] provided evidence for a protective function of GSH-Px against $ONOO^-$. The decline in GSH-Px activity due to $ONOO^-$ generation was significantly reversed by Wen-Pi-Tang extract (Fig. 3), suggesting that Wen-Pi-Tang extract may afford protection against $ONOO^-$. This result demonstrates that the destroyed defense system against excessive $ONOO^-$ recovered after the administration of Wen-Pi-Tang extract, resulting in improvement of the pathological condition induced by $ONOO^-$.

The oxidative stress caused by the generation of $ONOO^-$ accompanies acute renal ischemia and contributes to the pathophysiology of renal damage. Our present investigation showed that the levels of urea nitrogen and Cr were increased by LPS plus ischemia-reperfusion process (Table II), indicating that renal damage and dysfunction resulted from this process. Wen-Pi-Tang extract reduced the urea nitrogen and Cr levels, indicating that it ameliorated the renal dysfunction induced by $ONOO^-$ produced by this process. However, a protective action against the renal dysfunction was weaker than that of $ONOO^-$ level, which implied that some other factors were also involved in the renal dysfunction. Indeed, pathogenesis of renal dysfunction in living system is extremely complicated. We intend to carry out further detailed investigations on this aspect.

These results described that LPS plus ischemia-reperfusion process results in the production of $ONOO^-$ and ·OH, and destruction of defense mechanism associated with antioxidative enzymes in biological system, which are mainly responsible for the renal injury. Therefore, the damage induced by this process could be attenuated by scavenging the radicals and elevating the activities of antioxidative enzymes, SOD, catalase and GSH-Px. From this investigation, we could confirm that Wen-Pi-Tang extract scavenges $ONOO^-$ itself, not the precursor of $ONOO^-$, NO and O_2^-, and it also increases the antioxidative enzyme activities. Furthermore, it ameliorated renal dysfunction through decreasing the levels of urea nitrogen and Cr. This study suggests that Wen-Pi-Tang would be expected to be a therapeutic agent for $ONOO^-$ associated pathological renal disorders.

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