

Increase of radical in rats with adenine-induced renal failure is suppressed by Wen-Pi-Tang

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Abstract

We analyzed the free radical reaction in the body *in vivo* under the conditions of renal failure, using an L-band electron spin resonance apparatus. In rats with adenine-induced renal failure, the attenuation velocity of 3-carbamoyl-2,2,5,5-tetramethylpyrrolidine-N-oxyl was lowered in comparison with normal rats, indicating that they were in a state of augmented oxidation. In contrast, the attenuation velocity was higher in rats given Wen-Pi-Tang, showing a shift toward reduction. In the kidney of rats given Wen-Pi-Tang, we also found that a significant decrease in glutathione disulfide (GSSG) level caused an increase in the glutathione/GSSG ratio. In addition, there were significant reductions of increased thiobarbituric acid-reactive substance level and decreased superoxide dismutase and increased glutathione peroxidase activities, suggesting a decreased hydrogen peroxide production, which presumably drove the glutathione redox cycle toward reduction. The results of the present study suggest the possibility that Wen-Pi-Tang exerts an antioxidant effect through regulation of the redox cycle.

Key words *in vivo* ESR, free radical, glutathione redox cycle, Wen-Pi-Tang.

Introduction

Free radicals including active oxygen species are produced inside and outside the living body, affecting various biological functions. Their relation with aging, which is critical for longevity, and with various inflammatory diseases, carcinogenesis, and intractable diseases attracted much attention.¹⁻³⁾ The true entity of free radicals is difficult to understand because they generally have short half-lives. However, *in vivo* evaluation of free radical reactions in the body has been attempted in recent years.⁴⁾

Living organisms have effective defense mechanisms to protect themselves from attacks by active oxygen or other free radicals and the resultant disorders and diseases.⁵⁻⁷⁾ However, if there is excessive oxidative stress, it may be beyond the capability of the defense mechanisms, and then the role of antioxidants assumes greater importance. For these reasons, antioxidants are

of great interest, with particular attention focused on the search for natural antioxidants.

Oriental medicines are known to contain many antioxidants of various types, and the elucidation of their effects in relation to the involvement of active oxygen in various diseases, including lipid peroxidation, has progressed.⁸⁾ Wen-Pi-Tang is an Oriental medical prescription that has empirically been used in China for the treatment of chronic renal failure. We have previously studied the effects of this medical prescription in various renal failure models.⁹⁻¹¹⁾ Among such models, our adenine-induced renal failure model most closely resembles human chronic renal failure, and it is characterized by a higher production of the strongest nephrotoxic agent methylguanidine (MG) in comparison with other currently available renal failure models. We have demonstrated that MG is produced from creatinine in the presence of hydroxyl radical ($\cdot\text{OH}$) through creatol *via* creatone A and creatone B.¹²⁻¹⁴⁾ We have also found by measurement using X-band electron spin resonance (ESR) that

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the peak representing 5,5-dimethyl-1-pyrroline-N-oxide is markedly elevated in rats with adenine-induced renal failure,¹⁵⁾ and this is accompanied by a marked increase in •OH-adducted 8-hydroxydeoxyguanosine.¹⁶⁾ This finding suggests that active oxygen is increased in chronic renal failure, accelerating the aggravation of the pathological condition. On the other hand, some reports have provided data against the involvement of free radicals,¹⁷⁾ so that no consensus has yet been reached. This discrepancy seems to be derived from the fact that many studies drew inferences about the complicated free radical reaction in the body from their *in vitro* effects and the effects of antioxidants.

To investigate this issue, we carried out a real-time observation of the free radical reaction in the body under the conditions of renal failure, using *in vivo* ESR, and examined redox control by glutathione. In addition, we administered Wen-Pi-Tang to rats who were in an enhanced oxidative condition, and evaluated its usefulness in their treatment.

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,¹⁸⁾ an extract was obtained by boiling the above crude drugs gently in 1,000 ml water for 65 min. This 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.

Animal treatment and quantification by *in vivo* ESR: All experimental studies using animals were conducted in accordance with "Recommendations on the Establishment of Animal Experimental Guidelines" approved at the Toyama Medical and Pharmaceutical University. Male rats of the Wistar strain, with a body weight of 200-210 g, were kept in an animal room at an ambient temperature of $24 \pm 1^\circ\text{C}$ under a 12-h dark-light cycle. They

were allowed an adaptation period of several days, during which they were fed on a commercial feed (type CE-2, CLEA Japan Inc., Tokyo, Japan). They were then fed *ad libitum* on an 18% casein diet containing 0.75% adenine, which produced the experimental renal failure. In rats with renal failure induced by adenine, it has been confirmed previously, both histologically and biochemically, that the renal failure progresses as the period of adenine-feeding is prolonged.¹⁹⁾ During the adenine-feeding period, an aqueous solution of Wen-Pi-Tang extract was dissolved in water, and given to rats orally every day as drinking water. The dose was adjusted to 100 mg/kg body weight/day by regulating the concentration in relation to water consumption. Control rats were given a corresponding amount of water. Throughout the experimental period, there were no statistically significant differences between the controls and rats treated with extract as regards changes in body weight. The food intake of each rat was essentially proportional to its weight change, and no case of diarrhoeal symptoms was found. Six rats were used for each experimental group. After 20 consecutive days of administration, rats were anesthetized by intraperitoneal administration of 50 mg/kg body weight of sodium pentobarbital. 3-Carbamoyl-2,2,5,5-tetramethylpyrrolidine-N-oxyl (C-PROXYL) 100 nmol/kg body weight was injected into the caudal vein, and the attenuation velocity of C-PROXYL in the surroundings of the kidney was determined using an L-band ESR apparatus (ES-LLBA2, JEOL). The conditions of ESR measurement were as follows: microwave power, 4 mW; center field, 10.8 mT; field modulation width, 1 mT; sweep time, 1 min; receiver gain, $\times 500$; time constant, 0.3 sec. Measurements were carried out at room temperature. The peak intensity was determined four times at 30-sec intervals, and the mean value of the measurements was analyzed using the compartment model concept. The elimination rate constant (k) and the elimination half-life ($t_{1/2}$) were also calculated. The elimination rate constant was obtained from the gradient of the regression line of phase α , after plotting time on the X axis and the natural logarithm of the peak intensity on the Y axis, while the elimination half-life was calculated from the equation $t_{1/2} = \ln 2/k$. Afterwards blood samples were obtained by cardiac puncture, and the serum was separated immediately by centrifugation. The kidney was subsequently extirpated

from each rat, and immediately frozen in liquid nitrogen. The tissue was kept at -80°C until analysis.

Glutathione (GSH) and glutathione disulfide (GSSG) assays : According to the method of Floreani *et al.*,²⁰⁾ the tissue (about 250 mg) was homogenized in 1 ml of 25% metaphosphoric acid plus 3.75 ml of 0.1 M sodium phosphate-5 mM EDTA buffer (pH 8.0), and then centrifuged at $105,000 \times g$ for 30 min at 4°C . The determination of GSH and GSSG in the supernatant was performed by the method of Hissin and Hilf,²¹⁾ using *o*-phthalaldehyde as the fluorescent reagent.

Enzyme assays : The tissue was homogenized with a 9-fold volume of ice-cold physiological saline, and the activities of enzymes in the homogenate were determined. The activity of superoxide dismutase (SOD) was measured according to the nitrous acid method described by Elstner and Heupel²²⁾ and Oyanagui,²³⁾ based on inhibition of nitrite formation from hydroxylamine in the presence of superoxide (O_2^-) generators. Catalase activity was measured by following the decomposition of hydrogen peroxide (H_2O_2) directly by the decrease in extinction at 240 nm. The difference in extinction (ΔE_{240}) per unit time was then used as a measure of catalase activity.²⁴⁾ Glutathione peroxidase (GSH-Px) activity was obtained by colorimetry with 2-nitro-5-thiobenzoic acid, a compound produced through the reaction between glutathione and 5,5'-dithiobis(2-nitrobenzoic acid).²⁵⁾

Determinations of thiobarbituric acid (TBA)-reactive substance : TBA-reactive substance in serum was measured using the method of Naito and Yamanaka,²⁶⁾ and that in kidney tissue was assayed according to the method of Uchiyama and Mihara.²⁷⁾

Determination of protein : Protein was determined by the method of Itzhaki and Gill²⁸⁾ with bovine serum albumin as a standard.

Statistics : Values are presented as the mean $\pm$ S.E. Differences among the groups were analyzed by Dunnett's test. Significance was accepted at $p < 0.05$.

Results

The elimination rate of C-PROXYL

Table I shows the results of the spin clearance rate constant (k) and half-life ($t_{1/2}$) of C-PROXYL. The k value in normal rats was $2.48 \times 10^{-3} \text{ sec}^{-1}$, but the adenine-fed control rats showed a decreased k value as compared with the normal rats. However, the oral administration of Wen-Pi-Tang extract caused a significant increase of the k value. As a consequence, the $t_{1/2}$ value was markedly increased in adenine-fed control rats. When adenine-fed rats were treated with Wen-Pi-Tang extract, significant reversal effects were observed. Typical clearance curves of the 3 groups are shown in Fig. 1. The decrease of C-PROXYL in the 3 groups almost approximated an exponential function, although the

Table I Spin clearance rate constant and half-life of C-PROXYL in normal rats and adenine-fed rats with and without Wen-Pi-Tang administration.

Group	k (sec^{-1})	$t_{1/2}$ (sec)
Normal rats	$2.48 \times 10^{-3} \pm 6.29 \times 10^{-5}$	280.6 ± 7.4
Adenine-fed rats		
Control	$1.96 \times 10^{-3} \pm 1.50 \times 10^{-4a}$	361.3 ± 25.5^a
Wen-Pi-Tang extract	$2.40 \times 10^{-3} \pm 1.70 \times 10^{-4b}$	294.9 ± 21.6^b

Statistical significance: ^a $p < 0.001$ vs. normal rats, ^b $p < 0.01$ vs. adenine-fed control rats.

Table II Glutathione levels and GSH/GSSG ratio in kidney of normal rats and adenine-fed rats with and without Wen-Pi-Tang administration.

Group	GSH ($\mu\text{mol/g}$ kidney)	GSSG ($\mu\text{mol/g}$ kidney)	GSH/GSSG
Normal rats	0.929 ± 0.045	0.157 ± 0.003	5.94 ± 0.28
Adenine-fed rats			
Control	1.163 ± 0.018^a	0.171 ± 0.003^a	6.81 ± 0.09^a
Wen-Pi-Tang extract	1.211 ± 0.026^a	0.158 ± 0.002^{ab}	$7.65 \pm 0.15^{a,b}$

Statistical significance: ^a $p < 0.001$ vs. normal rats, ^b $p < 0.001$ vs. adenine-fed control rats.

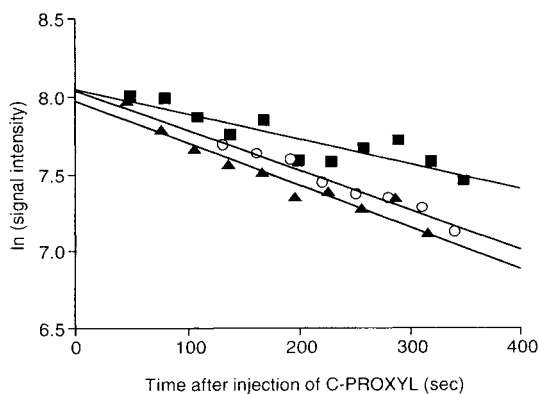


Fig. 1 Signal intensity measured by *in vivo* ESR in normal rats and adenine-fed rats with and without Wen-Pi-Tang administration after intravenous injection of C-PROXYL. ○, Normal rat; ■, adenine-fed control rat; ▲, adenine-fed rats with Wen-Pi-Tang administration.

rate of decrease was smaller in adenine-fed control rats.

Glutathione redox cycle in the kidney

As shown in Table II, the levels of GSH and GSSG were significantly elevated in adenine-fed control rats, being about 25% of the normal value for the former, and 9% for the latter. In addition, the GSH/GSSG ratio was increased from 5.94 to 6.81. In contrast, administration of Wen-Pi-Tang extract efficiently suppressed the oxidation of GSH in renal failure rats. The level of the oxidized form, GSSG, was decreased, and the GSH/GSSG

ratio in adenine-fed rats given Wen-Pi-Tang extract was elevated by 12% as compared with that in adenine-fed rats without extract.

Detection of the related enzyme activities showed significant decreases in both SOD and catalase in the kidney from adenine-induced renal failure rats, while a significant increase in the activity of GSH-Px was observed, as shown in Table III. The administration of Wen-Pi-Tang extract significantly decreased the decreased SOD and attenuated the increased GSH-Px activities. The SOD activity decreased from a mean value of 15.45 U/mg protein to 12.74 U/mg protein, and GSH-Px from 301.1 to 257.1 U/mg protein. In contrast to these enzymes, the alteration of catalase activity by the Wen-Pi-Tang administration was less marked, and was not statistically significant.

TBA-reactive substance in the serum and kidney

In comparison with normal rats, the level of TBA-reactive substance was significantly elevated in the serum, but not the kidney, of adenine-fed rats. As shown in Table IV, the level in the serum of normal rats was 3.38 nmol/mg protein, while that level of adenine-fed rats was 4.58 nmol/mg protein and showed an increase of about 36% when compared with the normal rats. Wen-Pi-Tang extract significantly decreased the TBA-reactive substance level in the serum and kidney of adenine-fed rats (Table IV).

Table III Renal antioxidant enzyme activities in kidney of normal rats and adenine-fed rats with and without Wen-Pi-Tang administration.

Group	SOD (U/mg protein)	Catalase (U/mg protein)	GSH-Px (U/mg protein)
Normal rats	34.42 ± 1.98	199.1 ± 10.6	138.1 ± 4.4
Adenine-fed rats			
Control	15.45 ± 0.85 ^a	80.8 ± 13.9 ^a	301.1 ± 26.5 ^a
Wen-Pi-Tang extract	12.74 ± 1.05 ^{a,b}	83.1 ± 4.2 ^a	257.1 ± 11.1 ^{a,c}

Statistical significance: ^a $p < 0.001$ vs. normal rats, ^b $p < 0.05$, ^c $p < 0.01$ vs. adenine-fed control rats.

Table IV TBA-reactive substance levels in serum and kidney of normal rats and adenine-fed rats with and without Wen-Pi-Tang administration.

Group	Serum TBA-reactive substance (nmol/mg protein)	Kidney TBA-reactive substance (nmol/mg protein)
Normal rats	3.38 ± 0.43	0.356 ± 0.014
Adenine-fed rats		
Control	4.58 ± 0.22 ^b	0.384 ± 0.017
Wen-Pi-Tang extract	3.48 ± 0.09 ^c	0.299 ± 0.021 ^{a,c}

Statistical significance: ^a $p < 0.01$, ^b $p < 0.001$ vs. normal rats, ^c $p < 0.001$ vs. adenine-fed control rats.

Discussion

Free radicals are atoms or molecules having one or more unpaired electrons, which include many active oxygen species such as O_2^- . In recent years, it has become apparent that these free radicals are involved in various diseases and pathological conditions. ESR, which targets substances with unpaired electrons, is a technique essential for studying active oxygen species.²⁹⁾ The advent of L-band ESR (*in vivo* ESR) using L-band microwaves (1-2 GHz) has enabled real-time *in vivo* evaluation of the radical reactions in the body, which were formerly studied only in the *in vitro* situation, and studies of various diseases and pathological conditions using this technique are now being conducted. However, the sensitivity of *in vivo* ESR is about several hundred-fold lower than that of the conventional X-band ESR, with a particularly low sensitivity for the abdominal region of the animal, where microwaves are apt to be absorbed. Therefore, at present it is difficult to quantify free radicals in the body, and current evaluation of the radical reaction uses changes in signals of stable radicals administered into the body as a spin probe.³⁰⁾ In the present study, we used the nitroxide radical C-PROXYL as the spin probe. This nitroxide radical is reduced to a hydroxylamine through reaction with various oxidation-reduction enzymes, antioxidants, or active oxygen species, losing magnetism and thus causing the signal to disappear.^{31,32)} Utilizing this property, we analyzed the free radical reaction in the body using *in vivo* ESR. As a result, we found that the elimination rate of C-PROXYL was significantly lower in rats with adenine-induced renal failure than in normal rats, which shows a significant prolongation of the radical half-life in rats with renal failure.

Utsumi³⁰⁾ stated that, although the major factor that determines the attenuation velocity is the reduction of nitroxide radicals in the blood at the early stages after administration, the attenuation velocity itself is an index suggestive of the body's ability to reduce radicals because it is modified by the organ distribution characteristics as well as renal clearance and is based on the disappearance of paramagnetism due to one-electron reduction of nitroxide radicals. It is thought that the cytochrome system of hepatic microsomes (cytochrome

P-450 and cytochrome P-450 reductase), the mitochondrial respiratory chain, and antioxidants such as vitamin C and E are involved in the reduction of nitroxide radicals in the body.³³⁾ On the other hand, under the conditions of aging³⁴⁾ or ischemia-reperfusion,³⁵⁾ reactions with the active oxygen series may also take place, and the reduction velocity is reported to vary according to the balance between the free radical generation system and the antioxidation system.³⁶⁾ Thus, the facts on the reduction of radicals have yet to be clarified. However, Takeshita *et al.*³²⁾ concluded that GSH is an electron donor since the reduction velocity of the spin probe were lowered when the GSH level was decreased. In addition, they suggested the possibility that the action of glutathione reductase and other SH compounds might be involved. Ajima,³⁷⁾ who carried out studies of ischemia-reperfusion in the kidney, pointed out that decreases in the activity and quantity of glutathione synthetase, as well as the GSH level are involved in the decreased ability of the body to reduce nitroxide radicals. They found that the ability to reduce nitroxide radicals in the kidney was lower in animals given pretreatment with SOD than in those not given the enzyme, and inferred that SOD administration facilitated a disproportionate reaction of O_2^- to stimulate the production of H_2O_2 , resulting in a further decrease in the reduction of nitroxide radicals. In our experiment, we investigated the effect of Wen-Pi-Tang on glutathione redox cycle in the kidney of adenine-fed rats. As a result, the levels of both reduced and oxidized glutathiones were increased in the kidney of rats given adenine, and a movement in the direction of oxidized glutathione production was found in terms of the GSH/GSSG ratio, consistent with the results reported by Takeshita *et al.*³²⁾ However, we speculated that GSH-Px was activated as a mechanism of H_2O_2 disposal, accompanied by accumulation of GSSG and lipid peroxides, since there were marked decreases in SOD and catalase activities together with a marked increase in GSH-Px activity. In contrast, in adenine-fed rats given Wen-Pi-Tang, the elimination rate and half-life of C-PROXYL were shifted to the normal range, with an elevation of the renal GSH/GSSG ratio resulting from a significant decrease in the renal GSSG level. In addition, the accompanying reductions of increased TBA-reactive substance level and decreased SOD and increased GSH-Px activities in the kidney of adenine-fed rats treated with Wen-

Pi-Tang suggested a decreased production of H_2O_2 in the tissue. Thus, we speculated that a decreased H_2O_2 production drove the glutathione redox cycle toward reduction in the kidney of adenine-fed rats with Wen-Pi-Tang administration.

We have already demonstrated that Wen-Pi-Tang has the ability to eliminate radicals.³⁸⁻⁴¹⁾ Based on the finding of the present study, we consider that Wen-Pi-Tang exerts its antioxidant activity through a cross talk between free radicals and the redox state.

和文抄録

L-バンド ESR (生体計測用 ESR) 装置を用い、腎不全における生体内フリーラジカル反応を解析した。アデニン誘発腎不全ラット腎では、正常ラットより 3-carbamoyl-2,2,5,5-tetramethylpyrrolidine-N-oxyl の減衰速度が遅延し、酸化亢進状態にあった。これに対し、温脾湯投与群では減衰速度が速やかとなり、還元方向に変換していた。一方、温脾湯投与群の腎臓では増加したグルタチオンジスルフィドレベルの有意な低下からグルタチオン/グルタチオンジスルフィド比の上昇が認められ、また増加したチオバルビツール酸反応物質レベル、低下したスーパーオキシドジスムターゼと上昇したグルタチオンレダクターゼ活性の有意な低下から、過酸化水素の産生低下が示唆され、グルタチオン酸化還元サイクルを還元方向に作動しているものと考えられた。以上より、温脾湯は酸化還元サイクル調節を介して抗酸化作用を発揮する可能性が示唆された。

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References

- Halliwell, B.: Oxidants and human disease: some new concepts. *FASEB J.* **1**, 358-364, 1987.
- Sies, H.: Role of reactive oxygen species in biological processes. *Klin. Wochenschr.* **69**, 965-968, 1991.
- Beckman, K.B. and Ames, B.N.: The free radical theory of aging matures. *Physiol. Rev.* **78**, 547-581, 1998.
- Feldman, A., Wildman, E., Bartolinini, G. and Piette, L.H.: *In vivo* electron spin resonance in rats. *Phys. Med. Biol.* **20**, 602-612, 1975.
- Reiter, R.J.: Oxidative processes and antioxidative defense mechanisms in the aging brain. *FASEB J.* **9**, 526-533, 1995.
- Sies, H.: Oxidative stress: oxidants and antioxidants. *Exp. Physiol.* **82**, 291-295, 1997.
- Evans, P. and Halliwell, B.: Free radicals and hearing. Cause, consequence, and criteria. *Ann. New York Acad. Sci.* **884**, 19-40, 1999.
- 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.
- 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.
- Yokozawa, T.: Effectiveness of Wen-Pi-Tang in renal failure. *Kidney and Dialysis* **41**, 501-508, 1996.
- Yokozawa, T., Dong, E. and Chen, C.P.: Protection of the kidney by Wen-Pi-Tang against ischemia-reperfusion injury. *Phytomedicine* **7**, 185-189, 2000.
- Nakamura, K., Ienaga, K., Yokozawa, T., Fujitsuka, N. and Oura, H.: Production of methylguanidine from creatinine via creatol by active oxygen species. Analyses of the catabolism *in vitro*. *Nephron* **58**, 42-46, 1991.
- Ienaga, K., Nakamura, K., Yamakawa, M., Toyomaki, Y., Matuura, H., Yokozawa, T., Oura, H. and Nakano, K.: The use of ^{13}C -labelling to prove that creatinine is oxidized by mammals into creatol and 5-hydroxy-1-methylhydantoin. *J. Chem. Soc., Chem. Commun.* 509-510, 1991.
- Yokozawa, T., Fujitsuka, N., Oura, H., Ienaga, K. and Nakamura, K.: *In vivo* effect of hydroxyl radical scavenger on methylguanidine production from creatinine. *Nephron* **75**, 103-105, 1997.
- Yokozawa, T., Fujitsuka, N., Oura, H., Mori, A. and Kashiwagi, H.: Determination of radical species in the kidney of rats with chronic renal failure by the spin trapping method. *Nephron* **70**, 382-384, 1995.
- Yokozawa, T., Fujioka, K. and Oura, H.: Increase in kidney 8-hydroxydeoxyguanosine level with the progression of renal failure. *Nephron* **61**, 236-237, 1992.
- Gamelin, L.M. and Zager, R.A.: Evidence against oxidant injury as a critical mediator of postischemic acute renal failure. *Am. J. Physiol.* **255**, F450-F460, 1988.
- 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.
- Yokozawa, T., Zheng, P.D., Oura, H. and Koizumi, F.: Animal model of adenine-induced chronic renal failure in rats. *Nephron* **44**, 230-234, 1986.
- Floreani, M., Petrone, M., Debetto, P. and Palatini, P.: A comparison between different methods for the determination of reduced and oxidized glutathione in mammalian tissues. *Free Rad. Res.* **26**, 449-455, 1997.
- Hissin, P.J. and Hilf, R.: A fluorometric method for determination of oxidized and reduced glutathione in tissues. *Anal. Biochem.* **74**, 214-226, 1976.
- Elstner, E.F. and Heupel, A.: Inhibition of nitrite formation from hydroxylammonium chloride: A simple assay for superoxide dismutase. *Anal. Biochem.* **70**, 616-620, 1976.
- Oyanagui, Y.: Reevaluation of assay methods and establishment of kit for superoxide dismutase activity. *Anal. Biochem.* **142**, 290-296, 1984.
- Aebi, H.: Catalase. In "Methods of Enzymatic Analysis" (Ed. by Bergmeyer, H.U.), Verlag Chemie, New York, pp.673-684, 1974.
- Ellman, G.L.: Tissue sulfhydryl groups. *Arch. Biochem. Biophys.* **82**, 70-77, 1959.
- Naito, C. and Yamanaka, T.: Atherosclerotic disorder and lipid

- peroxidation. *Jap. J. Geriatr.* **15**, 187-191, 1978.
- 27) Uchiyama, M. and Mihara, M.: Determination of malonaldehyde precursor in tissues by thiobarbituric acid test. *Anal. Biochem.* **86**, 271-278, 1978.
 - 28) Itzhaki, R.F. and Gill, D.M.: A micro-biuret method for estimating proteins. *Anal. Biochem.* **9**, 401-410, 1964.
 - 29) Kohno, M.: Free radical measurement. In "Kidney and Free Radical" (Ed. by Koide, H., Ishida, M., Sugisaki, T., Tomino, Y., Owada, S. and Ito, S.), Tokyo Igakusya, Japan, pp.28-34, 1994.
 - 30) Utsumi, H.: Clinical application of in vivo ESR. In "Kidney and Free Radical" (Ed. by Koide, H., Ishida, M., Sugisaki, T., Tomino, Y., Owada, S. and Ito, S.), Tokyo Igakusya, Japan, pp.48-56, 1994.
 - 31) Utsumi, H., Muto, E., Masuda, S. and Hamada, A.: In vivo ESR measurement of free radicals in whole mice. *Biochem. Biophys. Res. Commun.* **172**, 1342-1348, 1990.
 - 32) Takeshita, K., Hamada, A. and Utsumi, H.: Mechanisms related to reduction of radical in mouse lung using an L-band ESR spectrometer. *Free Rad. Biol. Med.* **26**, 951-960, 1999.
 - 33) Iannone, A., Bini, A., Swartz, H.M., Tomasi, A. and Vannini, V.: Metabolism in rat liver microsomes of the nitroxide spin probe tempol. *Biochem. Pharmacol.* **38**, 2581-2586, 1989.
 - 34) Gomi, F., Utsumi, H., Hamada, A. and Matsuo, M.: Aging retards spin clearance from mouse brain and food restriction prevents its age-dependent retardation. *Life Sci.* **52**, 2027-2033, 1993.
 - 35) Utsumi, H., Takeshita, K., Miura, Y., Masuda, S. and Hamada, A.: In vivo ESR measurement of radical reaction in whole mice-influence of inspired oxygen and ischemia-reperfusion injury on nitroxide reduction. *Free Rad. Res. Commun.* **19**, S219-S225, 1993.
 - 36) Miura, Y., Utsumi, H. and Hamada, A.: Antioxidant activity of nitroxide radicals in lipid peroxidation of rat liver microsomes. *Arch. Biochem. Biophys.* **300**, 148-156, 1993.
 - 37) Ajima, I.: A study of ischemic acute renal failure using in vivo ESR. *St. Marianna Med. J.* **24**, 337-347, 1996.
 - 38) Yokozawa, T., Dong, E., Oura, H., Nishioka, I., Kawai, Y. and Gamba, M.: Protective effects of Wen-Pi-Tang against cultured renal epithelial cellular injury. *Phytomedicine* **4**, 245-250, 1997.
 - 39) 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.
 - 40) Yokozawa, T., Sekiya, M., Rhyu, D.Y., Hattori, M. and Chung, H.Y.: Radical-scavenging activity of Wen-Pi-Tang and its component crude drugs: with special reference to the effects on nitric oxide, superoxide and peroxynitrite. *J. Trad. Med.* **17**, 41-47, 2000.
 - 41) Cho, E.J., Yokozawa, T., Rhyu, D.Y., Mitsuma, T., Terasawa, K. and Park, J.C.: Protective activity from hydrophilic and lipophilic free radical generators of Wen-Pi-Tang and its crude drug extracts in LLC-PK₁ cells. *J. Trad. Med.* **17**, 245-252, 2000.