Beneficial Effects of *Sanguisorbae Radix* in Renal Dysfunction Caused by Endotoxin *in Vivo*

Cui Ping CHEN,^a Takako YOKOZAWA,^{*,a} and Kenichi KITANI^b

Institute of Natural Medicine, Toyama Medical and Pharmaceutical University,^a 2630 Sugitani, Toyama 930–0194, Japan and National Institute for Longevity Sciences,^b 36–3 Gengo, Morioka-cho, Obu 474–0031, Japan.

Received June 18, 1999; accepted September 7, 1999

The effect of *Sanguisorbae Radix* extract, a traditional crude drug, was investigated in renal dysfunction induced by lipopolysaccharide (LPS) endotoxin. Injection of LPS in rats resulted in a sharp rise in the serum levels of urea nitrogen and creatinine (Cr), indicating impairment of renal function. Nitrite and nitrate levels and the activity of inducible nitric oxide synthase (iNOS), an enzyme which participates in NO synthesis, were also significantly increased in the serum of LPS-treated rats compared with normal rats. In rats pretreated with *Sanguisorbae Radix* extract, renal dysfunction was attenuated and the increases in serum urea nitrogen and Cr induced by LPS were significantly reduced. The administration of *Sanguisorbae Radix* extract also effectively lowered serum nitrite/nitrate level. A similar effect was observed on the iNOS activity. These results indicate that *Sanguisorbae Radix* extract contributes to the regulation of renal function under conditions where there is excessive generation of NO.

Key words *Sanguisorbae Radix*; nitric oxide; inducible nitric oxide synthase; renal function; lipopolysaccharide; rat

Nitric oxide (NO) is an interesting and important molecule with diverse functions, exerting a variety of both beneficial and detrimental effects in different tissues including the kidney. It has been demonstrated that NO plays an important role in renal function by participating in the regulation of renal hemodynamics, sodium excretion and renin release, tubuloglomerular feedback, pressure natriuresis, and tubular function.^{1,2)} However, excessive NO produced under certain pathological conditions may also produce cytotoxic effects and aggravate renal dysfunction. It still remains debatable whether NO is beneficial or harmful in acute and chronic renal failure or other renal diseases, and whether NO or NO synthase inhibitors should be used to regulate these conditions. Conflicting results have been reported in studies on humans and in animal experimental models.^{3–5)}

Lipopolysaccharide (LPS) releases many mediators and produces the condition known as endotoxemia. These mediators include interleukins, tumor necrosis factor, oxygen free radicals, toxic eicosanoids, platelet activating factor and NO.⁶⁾ Endotoxemia gives rise to a multiple organ dysfunction syndrome involving failure of the circulatory system, liver, lungs and kidney. An increase in the production of NO due to the induction of inducible NO synthase (iNOS) has been proposed to be responsible for this multiple organ dysfunction⁷⁾ and selective iNOS inhibition has been shown to attenuate or prevent the syndrome.⁸⁾

In this study, we investigated whether *Sanguisorbae Radix*, a traditional crude drug which has been shown to strongly inhibit NO *in vitro*,⁹⁾ has a protective effect on renal dysfunction during endotoxic shock.

MATERIALS AND METHODS

Reagents Thioglycollate broth and 2-[4-(2-hydroxyethyl)-1-piperazinyl]ethanesulfonic acid (HEPES) were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). LPS, pepstatin A, chymostatin, aprotinin, phenylmethylsulfonyl fluoride, flavin adenine dinucleotide (FAD), tetrahydrobiopterin, dithiothreitol (DTT), β -nicotinamide-adenine di-

nucleotide phosphate (reduced, NADPH), lactate dehydrogenase (LDH), sodium pyruvate and aminoguanidine were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.).

Preparation of *Sanguisorbae Radix* Extract The roots of *Sanguisorbae Radix* (*Sanguisorba officinalis* L.), grown in China and supplied by Uchida Wakan-yaku Co., Ltd., Tokyo, Japan, were finely powdered and extracted with distilled water at 100 °C for 1 h (roots: water=1:10, w/v). After removal of the insoluble portion by filtration, the filtrate was concentrated under reduced pressure and then lyophilized to yield a brown residue. The yield of the extract was 17.4% by weight of the original material.

Animals and Treatments Male LWH: Wistar rats with a body weight of 125–130 g were purchased from Shizuoka Agricultural Cooperative Association for Laboratory Animals (Hamamatsu, Japan). They were kept in wire-bottomed cages and exposed to a conventional dark/light cycle. The room temperature (approximately 25 °C) and humidity (approximately 60%) were controlled automatically. Laboratory pellet chow (Clea Japan Inc., Tokyo, Japan; comprising 24.0% protein, 3.5% lipid and 60.5% carbohydrate) and water were given *ad libitum*. Following several days of adaptation, the animals were divided into four groups—a normal, a control, and two treatment groups—avoiding any inter-group difference in body weight. The normal and control groups were given water, while the others were given *Sanguisorbae Radix* extract orally, at a dose of 50 or 100 mg/kg body weight/d, for 30 consecutive days. For the LPS shock experiments, rats were given intravenous LPS, 5 mg/kg body weight. At 6 h after the LPS challenge, the rats were anesthetized by intraperitoneal administration of sodium pentobarbital (30 mg/kg body weight). In separate experiments, 2 groups of rats received a bolus injection plus a continuous infusion of vehicle (saline, 0.6 ml/kg/h) or aminoguanidine (5 mg/kg intravenous bolus loading dose, followed by a continuous infusion of 5 mg/kg/h in 0.6 ml/kg/h saline for 4 h) at 2 h after injection of LPS, according to the method described by Wu *et al.*¹⁰⁾ Blood samples were obtained by cardiac puncture, and the serum was separated immediately by cen-

* To whom correspondence should be addressed.

trifugation. The kidneys were subsequently removed from each rat following renal perfusion through the renal artery with ice-cold physiological saline. The tissues were quickly frozen and kept at -80°C until analysis. Six rats were used for each experimental group.

Determination of Urea Nitrogen and Creatinine (Cr) in Serum Serum urea nitrogen and Cr were determined using the commercial reagents BUN Kainos and CRE-EN Kainos (Kainos Laboratories, Tokyo, Japan).

Determination of Nitrite and Nitrate in Serum Nitrite and nitrate levels were measured primarily following the method of Misko *et al.*¹¹⁾ Briefly, serum was filtered through an Ultrafree-MC microcentrifuge filter unit (Millipore, Bedford, MA) for 1 h at 14000 rpm to remove hemoglobin released by cell lysis. As nitrite in serum is mostly oxidized to nitrate by reaction with the iron-heme center of hemoglobin, the resulting nitrate was first reduced to nitrite by incubation with nitrate reductase and measured by a microplate assay method based on the Griess reaction.¹²⁾

Measurement of iNOS Activity Using the methodology of both Vaziri *et al.*¹³⁾ and Suh *et al.*,¹⁴⁾ kidney homogenates (20% w/v) were prepared in 40 mM cold Tris-buffer (pH 8.0), containing 5 $\mu\text{g}/\text{ml}$ pepstatin A, 1 $\mu\text{g}/\text{ml}$ chymostatin, 5 $\mu\text{g}/\text{ml}$ aprotinin and 100 μM phenylmethylsulfonyl fluoride. The homogenates were centrifuged at $12000\times g$ for 5 min at 4°C to remove tissue debris. The supernatant was used for the determination of enzyme activity and protein content. iNOS activity was measured using a slight modification of the method of Suh *et al.*¹⁴⁾ The reaction mixture consisted of 10 μl supernatant 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. The mixture was incubated at 37°C for 180 min in 96-well plates. Residual NADPH was oxidized enzymatically with 10 units/ml LDH and 5 mM sodium pyruvate in a final volume of 130 μl , with incubation for a further 5 min at 37°C , and the Griess assay was performed as above.¹²⁾ Protein was determined by the micro-biuret method,¹⁵⁾ with bovine serum albumin as a standard.

Statistics Results are presented as means $\pm$ S.E. The data were analyzed for statistical significance using Dunnett's method. Differences at $p < 0.05$ were considered statistically significant.

RESULTS

Urea Nitrogen and Cr in Serum As shown in Table 1, the serum urea nitrogen and Cr levels in LPS-treated control rats were increased significantly in comparison with those in normal rats, reflecting uremia. In contrast, the urea nitrogen level in rats given Sanguisorbae Radix extract for 30 d decreased from 38.1 to 33.8 mg/dl at the 50-mg level (an 11% change, $p < 0.05$) and from 38.1 to 31.6 mg/dl at the 100-mg level (a 17% change, $p < 0.001$). Similarly, the Cr level in rats given Sanguisorbae Radix extract orally for 30 d was significantly decreased at both the 50- and 100-mg dose levels compared with that in the control rats. Moreover, the increased urea nitrogen and Cr levels in LPS-treated control rats were decreased by infusion of aminoguanidine.

Nitrite and Nitrate in Serum The serum nitrite and nitrate in the LPS-treated rats increased to 6.50 μM , as shown in Table 2. Administration of Sanguisorbae Radix extract at a

Table 1. Effect of Sanguisorbae Radix Extract on Urea Nitrogen and Cr Levels in Serum

Group	Urea nitrogen (mg/dl)	Cr (mg/dl)
Normal	21.3 $\pm$ 2.0	0.36 $\pm$ 0.01
LPS-treated		
Control	38.1 $\pm$ 2.9 ^{a)}	1.20 $\pm$ 0.08 ^{a)}
Sanguisorbae Radix extract (50 mg/kg/d)	33.8 $\pm$ 3.1 ^{a,b)}	0.78 $\pm$ 0.10 ^{a,d)}
Sanguisorbae Radix extract (100 mg/kg/d)	31.6 $\pm$ 2.3 ^{a,d)}	0.68 $\pm$ 0.08 ^{a,d)}
LPS-treated		
Control	37.8 $\pm$ 1.6 ^{a)}	1.18 $\pm$ 0.06 ^{a)}
Aminoguanidine (5 mg/kg plus 5 mg/kg/h)	32.4 $\pm$ 2.8 ^{a,c)}	0.66 $\pm$ 0.14 ^{a,d)}

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

Table 2. Effect of Sanguisorbae Radix Extract on Nitrite/Nitrate Level in Serum

Group	Nitrite/nitrate (μM)
Normal	1.78 $\pm$ 1.02
LPS-treated	
Control	6.50 $\pm$ 1.35 ^{b)}
Sanguisorbae Radix extract (50 mg/kg/d)	4.39 $\pm$ 1.82 ^{b,c)}
Sanguisorbae Radix extract (100 mg/kg/d)	3.72 $\pm$ 0.89 ^{a,c)}
LPS-treated	
Control	6.39 $\pm$ 1.24 ^{b)}
Aminoguanidine (5 mg/kg plus 5 mg/kg/h)	3.13 $\pm$ 1.28 ^{c)}

Statistical significance: a) $p < 0.01$, b) $p < 0.001$ vs. normal values, c) $p < 0.001$ vs. LPS-treated control values.

Table 3. Effect of Sanguisorbae Radix Extract on iNOS Activity in Kidney

Group	iNOS (pmol/mg protein/min)
Normal	1.94 $\pm$ 0.11
LPS-treated	
Control	3.67 $\pm$ 0.27 ^{b)}
Sanguisorbae Radix extract (50 mg/kg/d)	2.69 $\pm$ 0.10 ^{a,c)}
Sanguisorbae Radix extract (100 mg/kg/d)	2.58 $\pm$ 0.06 ^{a,c)}
LPS-treated	
Control	3.64 $\pm$ 0.29 ^{b)}
Aminoguanidine (5 mg/kg plus 5 mg/kg/h)	2.02 $\pm$ 0.18 ^{c)}

Statistical significance: a) $p < 0.01$, b) $p < 0.001$ vs. normal values, c) $p < 0.001$ vs. LPS-treated control values.

dose of 50 mg/kg of body weight/d for 30 d resulted in a significant reduction in the serum nitrite/nitrate levels from 6.50 to 4.39 μM . A further increase in the dose of Sanguisorbae Radix to 100 mg produced a further reduction in the nitrite/nitrate levels. Infusion of aminoguanidine, not saline, significantly reduced the increased nitrite and nitrate level caused by LPS. The level in rats given aminoguanidine was 49% of the control value.

iNOS Activity in Kidney As shown in Table 3, LPS-treated rats showed a significant increase in iNOS activity in their kidneys (from 1.94 to 3.67 pmol/mg protein/min). However, in the kidneys pretreated with 50 mg/kg body weight/d *Sanguisorbae Radix* extract, iNOS activity was suppressed significantly to 2.69 pmol/mg protein/min. This suppression became more marked as the dose of *Sanguisorbae Radix* extract increased, the iNOS activity (2.58 pmol/mg protein/min) in rats given 100 mg being comparable with that in the untreated control group (3.67 pmol/mg protein/min). A significant reduction in iNOS activity was also observed in LPS-treated rats given aminoguanidine.

DISCUSSION

It has been demonstrated that NO plays an important role in the kidney, both under normal and pathological conditions. NO is produced from L-arginine by the action of NO synthase. In the kidney, three isoforms of NOS, located in different regions of the kidney and expressing different functions, have been found. The neuronal isoform (nNOS) is found in macula densa cells and the epithelium of Bowman's capsule; epithelial NOS (eNOS) is present in the endothelium of the glomerular capillaries, the afferent and efferent arteriole, the intrarenal arteries and the medullary vasa recta, and iNOS is found in the proximal tubule and the glomerulus.¹⁶⁾ NO generated by these NOS isoforms has been shown to exert different effects on renal physiology and pathology. Both nNOS and eNOS release a low and constant amount of NO, which plays a major role in the modulation of renal vascular tone and sodium excretion. Under normal conditions, iNOS also generates a physiological level of NO, which may participate in the modulation of vascular tone by an indirect mechanism in mesangial cell relaxation. However, as iNOS is induced by certain cytokines and hypoxia, it generates NO in a large quantities and for a prolonged period.¹⁷⁾ Excessive NO has been shown to be strongly cytotoxic, injuring cells and tissues. In freshly isolated rat proximal tubules, it has been demonstrated that NO and its metabolic product, peroxynitrite (ONOO⁻) mediate tubular hypoxia-reperfusion injury.¹⁸⁾ Cattell *et al.*¹⁹⁾ also provided experimental evidence for the effects of NO in accelerated nephrotoxic nephritis using isolated rat glomeruli. In our study, LPS-treated rats showed a rapid decline in renal function, which was indicated by large increases in two renal function parameters, blood urea nitrogen and Cr. The serum nitrite/nitrate level, an indicator of NO formation, was also seen to be markedly increased in LPS-treated rats compared with that seen in normal rats. As a more direct indicator, we monitored the activity of iNOS in renal homogenate using the method of Suh *et al.*¹⁴⁾ LPS-treatment resulted in an approximately 1.9-fold increase in the activity of iNOS, suggesting the possible association of additional induction of iNOS with renal dysfunction *in situ*.

Various inhibitors of NO or NOS have been used in attempts to improve or attenuate the pathology involved in excessive generation of NO. However, conflicting results have been obtained. Using isolated renal proximal tubules, Yu *et al.*¹⁸⁾ reported that the NOS inhibitor, *N*-nitro-L-arginine methyl ester, protected the renal tubular epithelium against hypoxic injury. Weinberg *et al.*²⁰⁾ demonstrated that oral administration of *N*^G-monomethyl-L-arginine prevented the de-

velopment of glomerulonephritis and reduced the intensity of inflammatory arthritis in MRL-*lpr/lpr* mice. In contrast to these observed beneficial effects, NOS inhibitors have been shown to aggravate renal dysfunction in several *in vivo* models of acute renal failure.^{21,22)} It is speculated that these contradictory results were attributable to a lack of selective NOS inhibitors.

As excessive generation of NO in renal disease is mainly associated with the induction of iNOS, the therapeutic strategy has concentrated on developing effective iNOS inhibitors. During systematic studies of the traditional drugs used in the treatment of renal diseases related to free radical injury, we found that *in vitro* *Sanguisorbae Radix*, a traditional crude drug which contains a large amount of tannin as its major constituent, has a strong scavenging effect on NO induced by sodium nitroprusside, an NO donor.⁹⁾ We also recently observed that *Sanguisorbae Radix* extract effectively inhibited the activity of iNOS in activated macrophages induced by LPS (data not shown). These findings prompted us to see if *Sanguisorbae Radix* extract could improve impaired renal function related to excessive generation of NO *in vivo*. We, therefore, conducted the present experiment and found that *Sanguisorbae Radix* extract significantly improved the impairment of renal function caused by LPS. The raised levels of serum urea nitrogen and Cr were markedly reduced in the two groups treated with different doses of *Sanguisorbae Radix*. Reduced serum nitrite/nitrate levels and renal iNOS activity demonstrated a protective action against the renal dysfunction caused by LPS, although these effects were weaker than those produced by aminoguanidine, a selective iNOS inhibitor.

In summary, bacterial endotoxin causes renal dysfunction, which is associated with enhanced formation of NO and the subsequent cytotoxic effects. iNOS had a higher activity in rats treated with LPS. *Sanguisorbae Radix* extract attenuated the impairment of renal function caused by LPS. Although the complete mechanism has still to be determined, these data suggest that the inhibitory effect of *Sanguisorbae Radix* extract on NO or iNOS plays an important role in protecting the kidneys from endotoxic injury.

Acknowledgment This work was supported in part by a grant from the Japan Foundation for Aging and Health.

REFERENCES

- 1) Galle J., Wanner C., *Kidney Blood Pressure Res.*, **19**, 2—15 (1996).
- 2) Lahera V., Navarro-Cid J., Cachofeiro V., Garcia-Estan J., Ruilope L. M., *Am. J. Hypertension*, **10**, 129—140 (1997).
- 3) Fujihara C. K., De Nucci G., Zatz R., *J. Am. Soc. Nephrol.*, **5**, 1498—1507 (1995).
- 4) Noiri E., Peresleni T., Miller F., Goligorsky M. S., *J. Clin. Invest.*, **97**, 2377—2383 (1996).
- 5) Thorup C., Persson A. E., *Am. J. Physiol.*, **271**, F246—F252 (1996).
- 6) Wolkow P. P., *Inflammation Res.*, **47**, 152—166 (1998).
- 7) Thiemeermann C., Ruetten H., Wu C. C., Vane J. R., *Br. J. Pharmacol.*, **116**, 2845—2851 (1995).
- 8) Liaudet L., Rosselet A., Schaller M. D., Markert M., Perret C., Feihl F., *J. Infectious Diseases*, **177**, 127—132 (1998).
- 9) Yokozawa T., Chen C. P., Tanaka T., *Phytomedicine* (in press).
- 10) Wu C. C., Ruetten H., Thiemeermann C., *Eur. J. Pharmacol.*, **300**, 99—104 (1996).
- 11) Misko T. P., Schilling R. J., Salvemini D., Moore W. M., Currie M. G., *Anal. Biochem.*, **214**, 11—16 (1993).

- 12) Green L. C., Wagner D. A., Glogowski J., Skipper P. L., Wishnok J. S., Tannenbaum S. R., *Anal. Biochem.*, **126**, 131—138 (1982).
- 13) Vaziri N. D., Ni Z., Wang X. Q., Oveisi F., Zhou X. J., *Am. J. Physiol.*, **274**, F642—F649 (1998).
- 14) Suh N., Honda T., Finlay H. J., Barchowsky A., Williams C., Benoit N. E., Xie Q., Nathan C., Gribble G. W., Sporn M. B., *Cancer Res.*, **58**, 717—723 (1998).
- 15) Itzhaki R. F., Gill D. M., *Anal. Biochem.*, **9**, 401—410 (1964).
- 16) Kone B. C., Baylis C., *Am. J. Physiol.*, **272**, F561—F578 (1997).
- 17) Bachmann S., Mundel P., *Am. J. Kidney Diseases*, **24**, 112—129 (1994).
- 18) Yu L., Gengaro P. E., Niederberger M., Burke T. J., Schrier R. W., *Proc. Natl. Acad. Sci. U.S.A.*, **91**, 1691—1695 (1994).
- 19) Cattell V., Cook T., Moncada S., *Kidney Int.*, **38**, 1056—1060 (1990).
- 20) Weinberg J. B., Granger D. L., Pisetsky D. S., Seldin M. F., Misukonis M. A., Mason S. N., Pippen A. M., Ruiz P., Wood E. R., Gilkeson G. S., *J. Exp. Med.*, **179**, 651—660 (1994).
- 21) Mashiach E., Sela S., Winaver J., Shasha S. M., Kristal B., *Nephron*, **80**, 458—467 (1998).
- 22) Schwartz D., Blum M., Peer G., Wollman Y., Maree A., Serban I., Grosskopf I., Cabili S., Levo Y., Iaina A., *Am. J. Physiol.*, **267**, F374—F379 (1994).