Protective effect of Sanguisorbae Radix against peroxynitrite-mediated renal injury

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Abstract

3-Nitrotyrosine, an oxidative product of protein that is produced *via* peroxynitrite ($ONOO^-$) nitration, was detected by HPLC analysis in plasma obtained from rats injected with lipopolysaccharide (LPS) and subjected to renal ischemia followed by reperfusion (LPS+ischemia-reperfusion), but not in rats subjected to sham-treatment. Rats pretreated with Sanguisorbae Radix extract orally for 30 days before LPS+ischemia-reperfusion, had lower 3-nitrotyrosine levels than rats without the pretreatment. Plasma levels of urea nitrogen and creatinine, indicators of renal dysfunction, were markedly lower in the animals pretreated with Sanguisorbae Radix extract than in those without the pretreatment. In addition, DNA fragmentation in renal tissues was significantly inhibited by administration of Sanguisorbae Radix prior to LPS+ischemia-reperfusion. These results suggest that Sanguisorbae Radix extract ameliorates oxidative damage caused by $ONOO^-$.

Key words Sanguisorbae Radix, peroxynitrite, 3-nitrotyrosine, DNA fragmentation, renal function, rat.

Introduction

Nitric oxide (NO) reacts rapidly with superoxide (O_2^-) to form a potentially more toxic agent, peroxynitrite ($ONOO^-$). $ONOO^-$ is highly reactive, oxidizes a large number of bio-molecules and initiates of a wide range of toxic reactions, including tyrosine nitration, lipid peroxidation, direct inhibition of mitochondrial respiratory chain enzymes, inhibition of membrane sodium/potassium ATPase activity, inactivation of membrane sodium channels, and other oxidative modifications of proteins.[1 6] It has been suggested that $ONOO^-$ may contribute to the toxic effect of NO and, therefore, may be a major factor in oxidative tissue injury.

Sanguisorbae Radix, an oriental drug used rarely in Japan, is used in China for hemostasis and to treat hematemesis, hemoptysis, melena and hypermenorrhea. It is also used externally to treat dermatitis, eczema and incision wounds. The pharmacological data available for this drug are incomplete; there are only a few reports about its anti-burn, hemostatic and antiemetic effects in rabbits and pigeons, and on its antibacterial effects against *Staphylococcus aureus* and *Pseudomonas aeruginosa.*[7] In a screening test *in vitro,* we found that Sanguisorbae Radix extract had high activity as a NO scavenger.[8] We also found that this crude preparation inhibited inducible NO synthase (iNOS) activity, reduced NO generation and ameliorated renal damage in an endotoxin-challenged rat model.[9] Moreover, Sanguisorbae Radix extract ameliorated renal dysfunction and reduced DNA fragmentation in a renal ischemia-reperfusion model, which was closely associated with excessive generation of reactive oxygen species including O_2^-.[10]

In view of these properties of Sanguisorbae Radix extract in inhibiting NO and O_2^-, and the potential

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role of $ONOO^-$ in oxidative tissue injury, we were interested in whether Sanguisorbae Radix extract has scavenging activity or an inhibitory effect against $ONOO^-$ *in vivo*. Such activities might explain how Sanguisorbae Radix extract protects against renal dysfunction in cases of ischemia-reperfusion injury or lipopolysaccharide (LPS)-shock, and would suggest that Sanguisorbae Radix extract is a potential therapeutic agent for pathological conditions involving NO and $ONOO^-$. In the present study, we examined the effect of Sanguisorbae Radix extract using a rat model of renal injury.

Materials and Methods

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). The insoluble portion was removed by filtration, then the filtrate was concentrated under reduced pressure and lyophilized, yielding a brown residue. The residue represented 17.4 % by weight of the original material and was composed mainly of tannin (about 46.3 %), based on measurements made according to the method of the Japanese Industrial Standard.[11]

Animals and treatments : Male LWH : Wistar rats weighing 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 12 h light/12 h dark cycle. The room's temperature and humidity were maintained automatically at about 25°C and 60 %. 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 eight groups of animals, avoiding any intergroup difference in body weight. Two groups were given Sanguisorbae Radix extract, while the other six groups were given water. Sanguisorbae Radix extract was dissolved in water, and given to rats orally every day as drinking water. The dose was adjusted to 100 or 200 mg/kg body weight/day by regulating its concentration in response to water consumption. After 30 consecutive days of administration, the rats were either injected with LPS (from *Escherichia coli* serotype 055 : B5, Sigma Chemical Co.), subjected to renal ischemia-reperfusion, or both (LPS+ischemia-reperfusion). Control animals underwent sham treatments. The LPS-treated rats received 5 mg/kg body weight of LPS injected intravenously. Ischemia-reperfusion was achieved by occluding bilateral renal artery using a nontraumatic vascular clamp for 60 min and then releasing for 350 min. In those subjected to both (LPS+ischemia-reperfusion), ischemia-reperfusion was carried out as the same as above, but at 50 min after ischemia started, 5 mg/kg body weight of LPS was injected intravenously. In separate experiments, two groups of rats received a bolus injection plus a continuous infusion of vehicle (saline, 0.6 ml/kg body weight/h) or aminoguanidine (a 5 mg/kg body weight intravenous bolus loading dose, followed by a continuous infusion of 5 mg/kg body weight/h in 0.6 ml/kg body weight/h saline) for 4 h, commencing 2 h after the injection of LPS, according to the method of Wu *et al.*[12] Six hours after the LPS challenge, the rats were anesthetized by intraperitoneal administration of sodium pentobarbital (30 mg/kg body weight). Blood samples were obtained by cardiac puncture, and the plasma was separated immediately by centrifugation. The kidneys were removed from each rat following perfusion through the renal artery with ice-cold physiological saline. The tissues were quickly frozen and kept at -80°C until analysis.

Measurement of 3-nitrotyrosine in plasma : The plasma concentration of 3-nitrotyrosine was determined by HPLC, following the methods of both Eiserich *et al.*[13] and Skinner *et al.*[14] with modification. Briefly, the blood samples were centrifuged for 15 min at 14,000 rpm, and then the plasma was incubated with proteinase K (1 unit/10 mg protein) for 18 h at 55°C. The samples were centrifuged again for 15 min at 14,000 rpm and then passed through a 10,000 Da molecular mass cut-off filter. Eighty μl of the samples were loaded onto a reversed-phase column (Nucleosil 5 μ C-18, 250 × 46 mm) at 25°C and eluted with 50 mM KH_2PO_4 -H_3PO_4 (pH 3.01) in 10% methanol (v/v) at a flow rate of 1 ml/min. The proteins were detected using an

ultraviolet detector set at 274 nm (sensitivity 0.01). The detection limit of this technique is 0.2 μM. The identity of the major peak was confirmed by adding authentic 3-L-nitrotyrosine (Sigma Chemical Co.) to additional samples. The authentic 3-L-nitrotyrosine co-eluted with the peak in the samples, under three different elution systems.

Measurement of urea nitrogen and creatinine in plasma: Plasma urea nitrogen and creatinine were determined using the commercial reagents BUN Kainos and CRE-EN Kainos (Kainos Laboratories, Tokyo, Japan).

Analysis of DNA fragmentation : Each kidney was homogenized and lysed in a cold lysis buffer (50 mM Tris-HCl, 10 mM disodium EDTA, and 0.5 % Triton X-100, pH 8.0) for 10 min at 4°C, following the method of Katoh *et al.*[15] The DNA was extracted twice with half volumes of phenol/chloroform and incubated at 55°C for 10 min. After centrifugation at 14,000 rpm for 20 min, the upper layer was incubated with 2 μl proteinase K (20 mg/ml) at 37°C for 60 min followed by incubation with 2 μl ribonuclease A (20 mg/ml) at 37°C for 60 min. The DNA was precipitated by adding 0.1 volume of 10 M ammonium acetate and 2.5 volumes of 100% ethanol and maintained at -20°C overnight. DNA was collected by centrifugation at 15,000× g for 20 min, air-dried, and resuspended in TE buffer (10 mM Tris-HCl, 5 mM EDTA, pH 7.4). The resulting DNA preparations were electrophoresed through a 2 % agarose gel containing ethidium bromide. Equal quantities of DNA (based on optical density measurements at 260 nm) were loaded in each lane, and a 1-kb multimer was used as a molecular mass standard. DNA fragmentation was visualized and photographed under ultraviolet illumination. The photograph was scanned using a Bio-Rad densitometer (model GS-670) with Molecular Analyst software package (version 1.1). DNA fragmentation level (%) was calculated from the ladder area ratio to that of the total area in each lane.

Statistics: The results are presented as the means ± S.E. of 6 rats. The data were analyzed for statistical significance using Dunnett's method. Differences at $p<0.05$ were considered statistically significant.

Results

Plasma concentrations of 3-nitrotyrosine

3-Nitrotyrosine was not detected in the plasma from both normal (sham treatment) and ischemia-reperfusion groups, whereas rats treated with LPS, or LPS + ischemia-reperfusion, had high levels of 3-nitrotyrosine (482.9 and 699.6 pmol/ml, respectively; Table I). Administration of Sanguisorbae Radix extract to rats for 30 consecutive days at 100 mg/kg body weight/day significantly reduced the formation of 3-nitrotyrosine to 329.3 pmol/ml. Increasing the dosage resulted in a further decrease in 3-nitrotyrosine formation; 3-nitrotyrosine was non-detectable in the 200 mg/kg body weight/day group. The formation of 3-nitrotyrosine induced by LPS + ischemia-reperfusion was not affected by an infusion of saline, but was significantly reduced by an infusion of aminoguanidine.

Urea nitrogen and creatinine in plasma

The plasma urea nitrogen concentration in the LPS-treated or ischemia-reperfusion rats increased significantly in comparison with normal rats, indicating impairment of renal function (Table II). The combination of LPS+ischemia-reperfusion aggravated renal failure; the urea nitrogen level further in-

Table I Effect of Sanguisorbae Radix extract on 3-nitrotyrosine levels in plasma.

Group	3-Nitrotyrosine (pmol/ml)
Sham treatment	N.D.
LPS	482.9±18.9
Ischemia-reperfusion	N.D.
LPS+ischemia-reperfusion	
No pretreatment	699.6±67.1
Sanguisorbae Radix extract (100 mg/kg/d)	329.3±30.6[a]
Sanguisorbae Radix extract (200 mg/kg/d)	N.D.
LPS+ischemia-reperfusion	
Saline	708.8±38.9
Aminoguanidine (5 mg/kg plus 5 mg/kg/h)	259.6±14.5[a]

Statistical significance: [a]$p<0.001$ *vs.* LPS + ischemia-reperfusion without pretreatment. N.D., not detectable.

Table II Effect of Sanguisorbae Radix extract on urea nitrogen and creatinine levels in plasma.

Group	Urea nitrogen (mg/dl)	Creatinine (mg/dl)
Sham treatment	17.6±1.5	0.33±0.09
LPS	43.9±1.8[a]	0.71±0.05[a]
Ischemia-reperfusion	57.9±2.6[a]	1.47±0.21[a]
LPS+ischemia-reperfusion		
No pretreatment	86.7±2.0[a]	2.03±0.10[a]
Sanguisorbae Radix extract (100 mg/kg/d)	77.9±4.0[a,b]	1.77±0.12[a,b]
Sanguisorbae Radix extract (200 mg/kg/d)	68.2±1.9[a,c]	1.24±0.05[a,c]
LPS+ischemia-reperfusion		
Saline	86.5±1.7[a]	2.03±0.14[a]
Aminoguanidine (5 mg/kg plus 5 mg/kg/h)	72.1±3.5[a,c]	1.52±0.12[a,c]

Statistical significance: [a]$p<0.001$ *vs.* normal values, [b]$p<0.01$, [c]$p<0.001$ *vs.* LPS+ischemia-reperfusion without pretreatment.

creased to 86.7 mg/dl. Renal dysfunction induced by these treatments was also confirmed by the variation in plasma creatinine level. The creatinine level in normal rats was 0.33 mg/dl, whereas in LPS-treated and ischemia-reperfusion rats it was 0.71 and 1.47 mg/dl, respectively. The creatinine level rose to 2.03 mg/dl in LPS+ischemia-reperfusion rats. Administration of Sanguisorbae Radix extract to the rats orally for 30 days prior to the treatment significantly ameliorated renal dysfunction. At Sanguisorbae Radix extract dosages of both 100 and 200 mg, urea nitrogen and creatinine levels were significantly lower than in the control group; urea nitrogen decreased from 86.7 to 77.9 and 68.2 mg/dl, and creatinine levels fell from 2.03 to 1.77 and 1.24 mg/dl, respectively. Moreover, infusion of aminoguanidine, but not saline, also markedly reduced the elevation in urea nitrogen and creatinine levels caused by LPS+ischemia-reperfusion treatment.

DNA fragmentation

DNA fragmentation in the injured kidney tissues was observed by gel electrophoresis, as shown in Fig. 1. DNA from rats subjected to ischemia-reperfusion and in rats subjected to LPS+ischemia-reperfusion displayed a DNA ladder fragmentation pattern that is characteristic of apoptosis, with intervals of about 180-200 bp. DNA ladder formation was absent in samples from sham-operation control rats. LPS-treatment did not produce DNA laddering, but a 'smear' pattern of DNA fragmentation was observed. Administration of

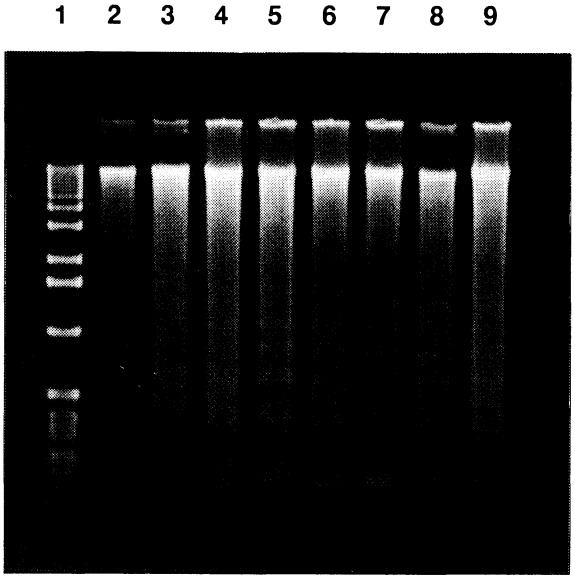

Fig. 1 Agarose gel electrophoresis of DNA. Lane 1: 1-kb marker DNA; lane 2: normal; lane 3: LPS; lane 4: ischemic and reperfused; lane 5: LPS plus ischemic and reperfused control (water drink); lane 6: LPS plus ischemic and reperfused control (saline infusion); lane 7: ischemic and reperfused aminoguanidine-treated; lane 8: LPS plus ischemic and reperfused Sanguisorbae Radix extract-treated (100 mg/kg/d); lane 9: LPS plus ischemic and reperfused Sanguisorbae Radix extract-treated (200 mg/kg/d).

Table III Effect of Sanguisorbae Radix extract on DNA fragmentation.

Group	Fragmentation level (%)
Sham treatment	–
LPS	3.9±0.5
Ischemia-reperfusion	15.1±1.7
LPS+ischemia-reperfusion	
No pretreatment	25.2±2.2
Sanguisorbae Radix extract (100 mg/kg/d)	14.7±1.9[a]
Sanguisorbae Radix extract (200 mg/kg/d)	13.3±1.6[a]
LPS+ischemia-reperfusion	
Saline	24.7±1.7
Aminoguanidine (5 mg/kg plus 5 mg/kg/h)	18.4±2.5[a]

Statistical significance: [a]$p < 0.001$ *vs.* LPS+ischemia-reperfusion without pretreatment.

Sanguisorbae Radix extract prevented the DNA laddering caused by LPS+ischemia-reperfusion, at both 100 and 200 mg. These observations were supported by the results of our semiquantative densitometry analysis of the DNA fragmentation level. The DNA fragmentation level increased significantly in both the ischemia-reperfusion and LPS+ischemia-reperfusion groups, and decreased from 25.2 % of the control value to 14.7 % and 13.3 % for rats given 100 or 200 mg/kg body weight/day Sanguisorbae Radix extract. In addition, aminoguanidine, but not saline, reduced DNA fragmentation, but this effect was weak (Table III).

Discussion

$ONOO^-$ is a new focus of interest in studies of free radicals, particularly those related to the detrimental effect of NO. The potential pathophysiological effects of $ONOO^-$ include activation of poly (ADP-ribose) synthetase, inhibition of mitochondrial respiration, and activation of caspase-3, as well as alteration of the lipid-aggregatory properties of surfactant protein A, which may play an important role in tissue damage and organ dysfunction.[16,17] Although $ONOO^-$ is generated in a large number of pathophysiological conditions, few *in vivo* models are available that produce enough $ONOO^-$ for evaluating its cytotoxicity and its contribution to NO cytotoxicity as well as to tissue injury. As $ONOO^-$ is generated *via* a reaction between NO and O_2^-, we decided to treat the rats with LPS and ischemia-reperfusion, the former providing abundant NO and the latter causing elevated O_2^-.

It is important to know whether $ONOO^-$ is elevated in this model. Until now, it has been very difficult to detect $ONOO^-$ directly in biological fluids and tissues because it is unstable and decomposes rapidly. However, $ONOO^-$ can oxidize the tyrosine in protein into nitrotyrosine, which is stable and detectable. Nitrotyrosine has been considered as evidence for *in vivo* formation of $ONOO^-$.[18,19] In the present experiment, we observed a high level of nitrotyrosine in plasma from LPS+ischemia-reperfusion rats. In comparison, the nitrotyrosine level induced by LPS-treatment alone was lower, and nitrotyrosine was not detected in rats subjected to a sham-operation and ischemia-reperfusion. We could not conclude, on these grounds, that nitrotyrosine is not formed in ischemia-reperfusion injury because several studies have demonstrated that $ONOO^-$ is an important mediator in ischemia-reperfusion injury,[20,21] and the method we used, HPLC with UV detection, has limited sensitivity. However, our results suggest that $ONOO^-$ formation increases in the presence of excessive generation of NO and O_2^-.

To evaluate the effect of elevated $ONOO^-$ on impaired renal function, we measured urea nitrogen and creatinine in plasma. Treatment of the rats with LPS+ischemia-reperfusion resulted in significantly higher urea nitrogen and creatinine levels than either LPS-treatment or ischemia-reperfusion alone, which suggested that $ONOO^-$ aggravates the impairment of renal function.

We have previously reported that Sanguisorbae Radix extract ameliorates renal dysfunction in an LPS-challenged rat model, and that this effect involves in the inhibition of NO generation and iNOS activation.[9] On the other hand, we found, in a recent preliminary *in vitro* experiment, that this extract inhibited $ONOO^-$ directly. Hence, it is possible that Sanguisorbae Radix extract ameliorated renal dysfunction by inhibiting excessive generation of NO and/or $ONOO^-$, or by scavenging O_2^-. Using the LPS+is-

chemia-reperfusion model, we have clarified this effect. Our results show that administration of Sanguisorbae Radix extract significantly ameliorated increases in plasma urea nitrogen and creatinine, which corresponded with a significant decrease in the $ONOO^-$ level. This suggested that inhibition of $ONOO^-$ may be attributable to the improvement of renal dysfunction. However, a protective action against the renal dysfunction was weaker than that of $ONOO^-$ level, which implied that some other factors 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.

$ONOO^-$ is a highly cytotoxic oxidant, which oxidizes proteins and inhibits cellular respiration. The resulting changes in mitochondrial function lead to activation of caspase, and subsequently induce DNA fragmentation and enterocyte apoptosis.[16] We have previously reported that ischemia-reperfusion can induce DNA ladder formation.[10] In the present study, rats subjected to LPS+ischemia-reperfusion had more distinct DNA ladders than rats subjected to ischemia-reperfusion alone, suggesting that $ONOO^-$ is a strong cytotoxic agent. The ladders we observed had intervals of about 180-bp, which is characteristic of apoptosis. LPS-treatment did not result in ladder formation, and instead a 'smear' pattern was observed, which indicates that necrosis was a major pathway of cell death in endotoxin shock. Pretreatment with Sanguisorbae Radix extract significantly ameliorated DNA laddering. At doses of 100 and 200 mg/kg body weight/day Sanguisorbae Radix extract, the DNA ladders were indistinct. A semiquantative method showed that the DNA fragmentation level was markedly decreased in the Sanguisorbae Radix extract-treated groups. On the other hand, infusion of saline did not affect DNA fragmentation, whereas aminoguanidine (a specific iNOS inhibitor[22]) treatment reduced it significantly. The effect of aminoguanidine was weaker than that of 200 mg/kg body weight/day Sanguisorbae Radix extract ($p < 0.001$). This effect of aminoguanidine may be due to inhibition of NO and to blocking of $ONOO^-$ formation. These results suggested that $ONOO^-$ mediates, at least partially, cell death, and that Sanguisorbae Radix extract protects renal cells against such injury.

In summary, $ONOO^-$ is formed in the presence of excessive generation of NO and O_2^-, aggravates the impairment of renal function, and mediates cell death by apoptosis. This toxic effect can be ameliorated by pretreatment with Sanguisorbae Radix extract. Sanguisorbae Radix extract may be a potential therapeutic agent for the protection of renal cells and tissue against free radical injury. Although the complete mechanism by which Sanguisorbae Radix extract provides its protective effect remains to be clarified, inhibition of the pathways responsible for formation of NO, O_2^- and $ONOO^-$ is expected to be important.

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和文抄録

パーオキシナイトライトは蛋白中のチロシンをニトロ化して3-ニトロチロシンを生成するが，この3-ニトロチロシンをHPLCで測定した結果，リポポリサッカライドと虚血-再灌流を施したラット血漿で検出され，偽処理した場合には検出されなかった。一方，リポポリサッカライドと虚血-再灌流を施す前に30日間地楡エキスを経口投与したラットでは，非投与群より低い3-ニトロチロシン値を示し，腎機能の指標の血漿尿素窒素，クレアチニンレベルも著しく低下していた。また腎組織中のDNA断片化も抑制され，地楡エキスがパーオキシナイトライトによる酸化的損傷を軽減することが推測された。

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