Identification of Effective Component from a Traditional Herbal Medicine and the Inhibitory Effects on Experimental Glomerular Lesion in Mice

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(Received 26 June 1991/Accepted 31 October 1991)

ABSTRACT. Effective constituents for renal disease were partially purified from a herbal prescription. The efficacy on renal diseases was examined using an experimental model system of glomerular lesion in mice induced by *Agkistrodon acutus* venom (Ac_1-P). The extract of boiling water of the herbal prescription (P-3) was first fractionated by ether into an acidic fraction and a mixture of basic and neutral fractions. The acidic fraction was proved to be more effective and then further examined by thin layer chromatography and spectrophotometry. One of the main components was confirmed to be caffeic acid which had inhibitory effect on renal failure in mice by Ac_1-P. This effect was considered to be caused by caffeic acid inhibiting the proteolytic enzyme activity of Ac_1-P. Caffeic acid should thus have prophylactic or inhibitory effect on glomerular disease in which proteolytic enzymes may have pathogenic roles.—KEY WORDS: *Agkistrodon acutus* venom, caffeic acid, glomerular lesion, herbal medicine, renal pathology.

J. Vet. Med. Sci. 54(1): 111–118, 1992

For the treatment of renal disease, herbage has long been used throughout the world, and traditional prescriptions are available for man and domestic animals. Some traditional herbal prescriptions are also being used in Taiwan to treat renal diseases, though in most cases, they are without scientific basis and the effective components are not known. The efficacy of such prescriptions is due to the interaction of components in the herbs. To guarantee reproducibility of efficacy and for general application, the effective components should be determined.

Various experimental animal models have been used to study the pathogenesis, developmental mechanisms and therapy of renal diseases [1, 4, 6, 10, 12, 16, 17, 21, 22]. From the early 1900s, snake venom has been known to cause injury to glomeruli in various animals [2, 3, 5, 7, 9, 13]. The authors also have established an experimental model system in which glomerular injury is produced in mice following a single intravenous dose of purified 100-pace snake (*Agkistrodon acutus*) venom [15]. Glomerular injury was observed as mild to severe pathologic changes within short duration.

A prescription of herbal medicine, P-3, was previously shown to be effective for treating renal disease induced by the *Agkistrodon acutus* venom (Ac_1-P) in mice [18], though the effective constituents and mechanism involved are still not clear.

This study was conducted to determine the effective components of P-3 consisting of twelve herbs for renal disease. Efficacy was assessed on the basis of inhibitory effects on renal failure.

MATERIALS AND METHODS

Snake venom: Lyophilized venom (The Japan Snake Institute, Gunma, Japan) from 100-pace snake (*Agkistrodon acutus*) was purified by the method of Nikai *et al.* [11]. It was subsequently used to induce renal lesions in mice.

Mice: Male mice used were of the albino ddY strain, 4 weeks old, with an average body weight of approximately 17 g.

Herbal medicine: P-3, the same as in our previous reports [18] was used and comprised of the following twelve herbs: *Achyranthes obtusifolia, Clerodendrum camitosum, Desmodium styracifolium,* Leaves of *Eriobotrya japonica, Glechoma hederacea, Hypericum japonicum, Ludwigia octovalvis, Pogonatherum crinitum, Serissa japonica, Solanum surattense sensu act.,* Hair of *Zea mays* and Seed of *Nasturtium indicum*.

The procedure for fractional extraction of herbal medicine is shown in Fig. 1. Powdered P-3 was extracted twice with boiling water [18] for 30 min

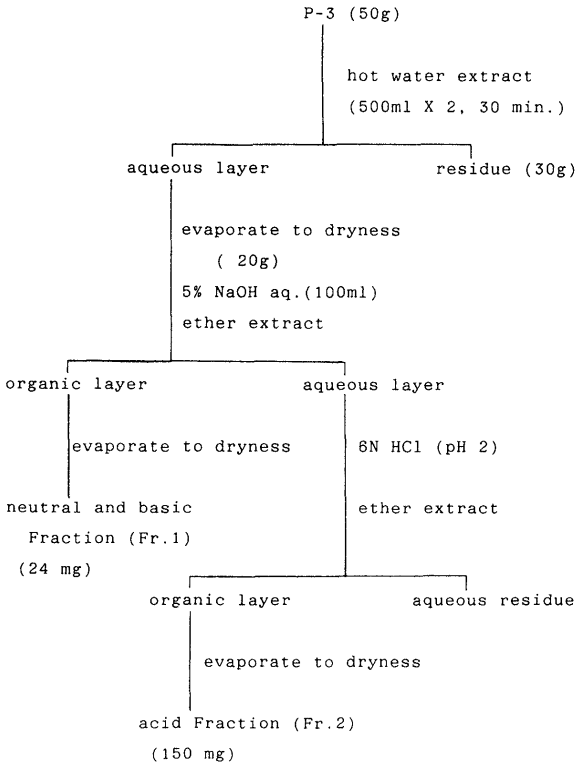

Fig. 1. Chart of fractionation of P-3.

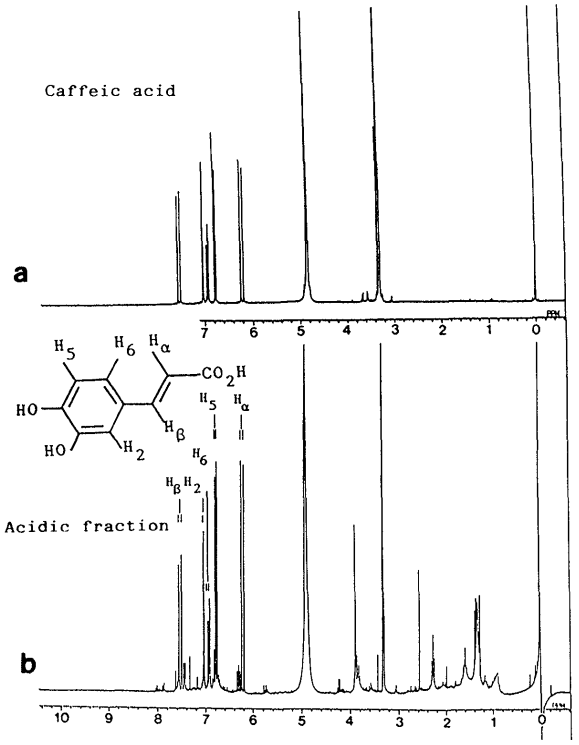

Fig. 2. 270 MHz ^{1}H-NMR spectrum in CD_3OD. NMR spectrum of acidic fraction of P-3 (b) showed that five proton signals (Hα, H_5, H_6, H_2, Hβ) at olefinic region agreed with those of standard caffeic acid (a) and indicated the presence of caffeic acid (b) as a major component.

after the treatment with 5% sodium hydroxide and extraction by ether. The extract obtained was considered to be a mixture of basic and neutral fractions (Fr. 1). The residual aqueous alkaline layer was acidified with hydrochloric acid followed by additional 5 times extraction with ether (Fr. 2). The organic solvent was removed under reduced pressure at 35°C. The final extracts obtained as Fr. 1 and Fr. 2 were 0.125 and 0.75% of the boiling water extract of P-3, respectively. Fr. 2 showed positive reaction in ferric chloride test. In the infrared (absorption) spectrum (IR), Fr. 2 showed absorption from 3,000 to 3,400 cm^{-1} (hydroxyl group), 1,700 (carbonyl) and 1,600 and 1,500 (aromatic ring), indicating the presence of phenolic carboxylates.

The ^{1}H-NMR spectrum of Fr. 2 is shown in the Fig. 2b. In addition to the strong solvent signals at 3.31 and 4.88 (methanol), presence of 5 protons in caffeic acid (Fig. 2a) is evident and assignment of Hα and Hβ are shown in the Fig. 2b. From the spectrum pattern, presence of caffeic acid and other components is indicated. Thin layer chromatography (TLC) of Fr. 2 also showed the presence of caffeic acid, which was confirmed using a reference

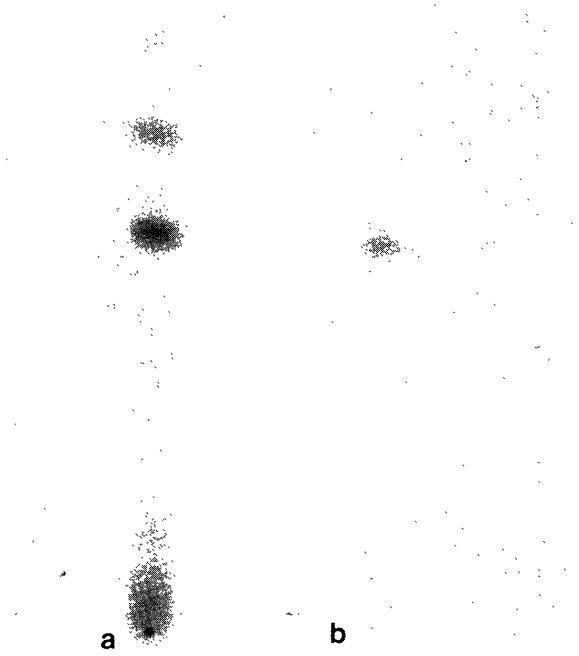

Fig. 3. Thin layer chromatography of Fr. 2 (a) with a developing solvent (toluene 7, ethyl acetate 4, formic acid 1) produced a band corresponding to caffeic acid (b).

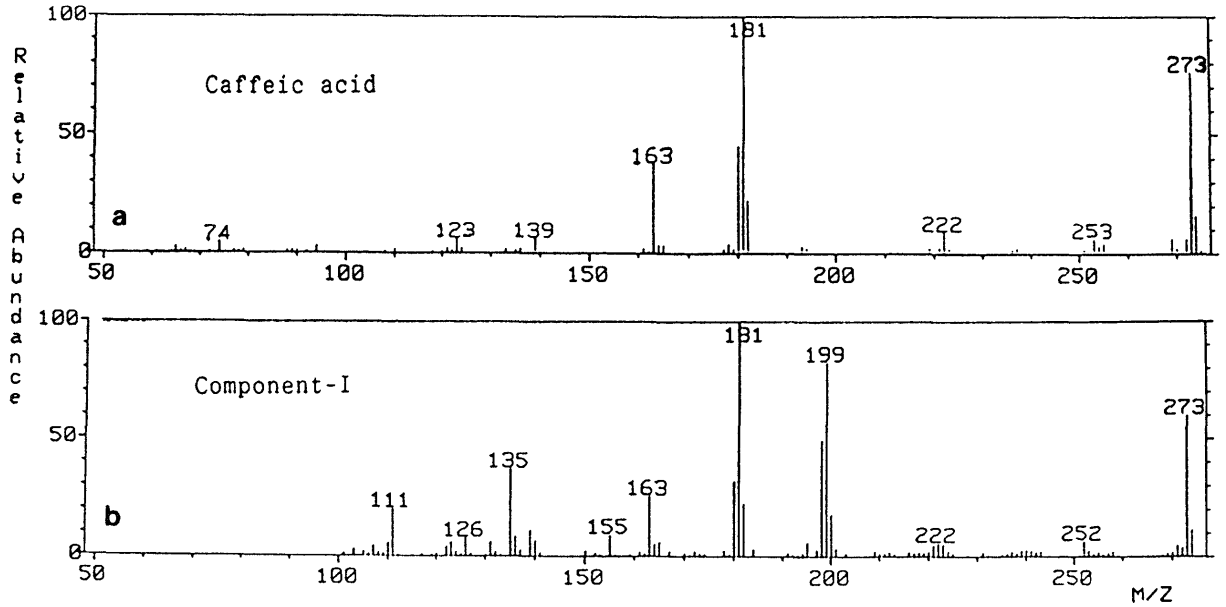

Fig. 4. FAB-MS spectrum of component-I in acidic fraction of P-3 (b) and caffeic acid (a). Spectrum of caffeic acid showed the ion peaks at m/z 163 ($M+H-H_2O$), 181 ($M+H$), and 273 ($M+H+C_3H_8O_3$), corresponding to those of component-I which shows additional ion peaks of m/z 199 ($M+H+H_2O$) and 291 ($M+H+C_3H_8O_3+H_2O$).

compound (Wako, mol wt. 180.16) (Fig. 3). Preparative TLC of Fr. 2 with a developing solvent (toluene 7, ethyl acetate 4, formic acid 1) afforded a small amount of white powder (component-I). The FAB-MS spectrum of this material showed an $[M+H]^+$ base peak at m/z 181, fragment peak at 163 ($M+H-H_2O$), and an additional compound with glycerin of matrix at 273 ($M+H+C_3H_8O_3$), identical with that of caffeic acid (Fig. 4).

Dosage of extract or caffeic acid: In our previous report [18], the crude extract was administered to each mouse at the concentration of 10 mg/ 0.1 m*l* and showed the maximum inhibitory effect on experimental glomerular diseases. The dosage of the material (Frs. 1 and 2 and caffeic acid) was qualified in the preliminary experiments and the most effective dosage was so deduced and adopted in this study. However, the activity of Fr. 2 was estimated by the most effective dose to have decreased to about 15% of that of extract of boiling water by purification.

Experiemntal design: The fractions and caffeic acid were dissolved in saline so as to contain 5 mg and 1 mg per 1 m*l*, respectively. Two experiments were performed twice, one for Frs. 1 and 2 and the other for caffeic acid. Each of mice in the treated groups was injected intraperitoneally with the solution of an extract or caffeic acid at a dose of 0.1 m*l*, while those in the control groups received only saline of 0.1 m*l*. For comparison of efficacy of two fractions, 100 mice were divided into 3 groups, two treatment groups and one control group (Table 1). The survivors at 1 week after Ac_1-P inoculation were sacrificed and blood urea nitrogen (BUN) was measured. For the efficacy of caffeic acid, a total of 254 mice were divided into 6 groups (Table 2), 4 groups served as treated groups with caffeic acid or crude extract of P-3 and 2 as controls. The mice were sacrificed 48 hr or 1 week after Ac_1-P inoculation. Survival rate, body and kindey weights and BUN were determined at the times of sacrifice. Hemorrhage in stomach, lungs and kidneys and hematuria were assessed in the mice that were sacrificed at 48 hr or had died within 48 hr. Kidney specimens were stained hematoxylin and eosin (HE) and periodic acid-Schiff (PAS) and were examined for pathologic changes under a light microscope for survivors after 48 hr and 1 week. Microangiography was performed as previously described [19].

Tissue preparation for light microscopy: The kidneys were fixed with 10% neutral-buffered formalin. Paraffin sections were stained with HE and PAS.

Numerical treatments: For statistical analysis, nonparametric data were analyzed by the Mann-Whitney U-test, normal data by the Student's *t*-test

Table 1. Effects of the extracted fractions of P-3 on survival and BUN values of mice 1 week after the injection with Ac_1-P

Groups	No. of Mice tested	Dead	Survived	Survival rate[a)]	BUN[b)] (mg/d*l*)
Fraction 1	20	10	10	50.0 (%)	37.0±10.6
Fraction 2	40	13	27	67.5*	27.8± 3.7*
Control	40	22	18	45.0	35.2± 9.2

Mice in two treated groups received intraperitoneally each fraction (0.1 m*l*, 0.5 mg) every 2 days before venom Ac_1-P (0.2 m*l*, i.v.) injection.
*P<0.05, significance from the value of control.
a) Analyzed by χ^2 test.
b) Blood urea nitrogen, representing the mean±standard deviation and analyzed by U-test.

Table 2. Survival rate of control and treated groups 48 hr and 1 week after the injection with Ac_1-P

	No. of Mice tested	Dead	Survived	Survival rate[a)]	BUN[b)]
(48 hr)				(%)	(mg/d*l*)
P-3 (crude)	31	5	26	83.9*	33.8±29.4*
Caffeic Acid	33	8	25	75.8	39.0±39.3*
Control	33	15	18	54.5	78.3±70.5
(1 week)					
P-3 (crude)	47	10	37	78.7*	25.7± 8.4**
Caffeic Acid	55	15	40	72.7*	27.6± 9.1*
Control	55	27	28	50.9	35.5±12.8

P-3 (0.1 m*l*, 10 mg) or caffeic acid (0.1 m*l*, 0.1 mg) was given intraperitoneally every 2 days before venom Ac_1-P (0.2 m*l*, i.v.) injection.
**P<0.01, *P<0.05, significance from the value of control.
a) Analyzed by χ^2 test.
b) Blood Urea Nitrogen, represents the mean±standard deviation and analyzed by U-test.

and proportional data by the χ^2-test.

RESULTS

1) *Effects of the two fractions*

Survival rate and BUN one week after inoculation of Ac_1-P are shown in Table 1. In the acidic fraction group, survival rate was higher and BUN significantly lower than those of the control, whereas in the basic and neutral fraction group no signfiicant differences could be found, thus showing the acidic fraction to have greater effect than the basic and neutral fraction.

2) *Efficacy of caffeic acid*

General changes: Survival rate was higher and BUN lower in the treated groups than those of the control 48 hr and 1 week after the inoculation with Ac_1-P, though survival rate was not significantly so at 48 hr in the caffeic acid group (Table 2). Body and kidney weights in the treated group of the survivors at 1 week were higher (Table 3), which possibly indicates the better physiological condition of treated mice than the control mice.

Pathologic findings: On dissection, the incidence of hemorrhage in the stomach, lungs, and kidneys along with hematuria was less in the treated mice which were sacrificed at or died within 48 hr than that of the control (Table 4). Although the incidence of the hemorrhage was not significantly less between the treated groups, the degree of the hemorrhage was severe in the caffeic mice than in P-3 mice. Anemic kidneys with reduced size and granular surface were much fewer in the treated groups at 1 week.

At 48 hr after Ac_1-P, predominant glomerular findings were slight increase in cells and/or matrix in the mesangium in the treated mice and cystic formation of glomerular capillary tufts with fibrin thrombi in the control (Table 5, Figs. 5a and 5b). At 1 week, intact glomeruli and the mesangial changes could be seen more frequently in the treated mice than in the control. Thrombosed cystic lesions were replaced by cell proliferation, followed by sclerosis.

Table 3. Body and kidney weights of mice sacrificed 1 week after the injection with Ac_1-P

Groups	No. of Mice tested	B.W. before[a)]	B.W. after[a)]	K.W.[b)]	K.W./B.W. (%)
P-3 (crude)	35	17.5±0.8	22.8±3.0	0.30±0.05*	1.30±0.18*
Caffeic Acid	33	17.4±0.7	24.4±2.4*	0.31±0.07*	1.25±0.18
Control	23	17.3±0.8	21.9±4.3	0.27±0.07	1.22±0.17

P-3 (0.1 m*l*, 10 mg) or caffeic acid (0.1 m*l*, 0.1 mg) was given intraperitoneally every 2 days before venom Ac_1-P (0.2 m*l*, i.v.) injection.
*P<0.05, significance from the value of control analyzed by U-test.
a) B.W. before and after, represent body weight in gram on 2 days before and 7 days after the injection with Ac_1-P, respectively.
b) Kidney weight in gram. Values are indicated as the Mean±S.D.

Table 4. Hemorrhage in three organs and hematuria of control and treated groups sacrificed 48 hr or died within 48 hr after the injection with Ac_1-P

Groups	No. of Mice tested	Hemorrhage			Hematuria (%)
		Stomach (%)	Lungs (%)	Kidneys (%)	
P-3 (crude)	31	7(22.6)**	8(25.8)**	8(25.8)**	10(32.3)**
Caffeic Acid	33	11(33.3)	12(36.4)	13(39.4)*	15(45.5)*
Control	33	18(54.5)	20(60.6)	21(63.6)	24(72.7)

P-3 (0.1 m*l*, 10 mg) or caffeic acid (0.1 m*l*, 0.1 mg) was given intraperitoneally every 2 days before venom Ac_1-P (0.2 m*l*, i.v.) injection.
**P<0.01, *P<0.05, significance from the value of control analyzed by χ^2 test.

Table 5. Glomerular changes of mice sacrificed 48 hr and 1 week after the injection with Ac_1-P

Groups	No. of mice examined	Mean No. of glomeruli observed	Intact	Increase in mesangial cells and/or matrix[a)]	Cystic lesion			Crescents	
					Thrombosed	Proliferative	Sclerotic	Early or Incomplete	Complete
(48 hr)			(%)	(%)	(%)	(%)	(%)	(%)	(%)
Herb (P-3)	22	179	8.3	60.4**	31.4**	0	0	0.8	0.1
Caffeic Acid	22	174	8.1	59.3*	32.6*	0	0	1.1	0.1
Control	15	175	7.4	46.7	45.9	0	0	3.5	0.3
(1 week)									
Herb (P-3)	27	180	8.4*	64.6**	0.5	19.6*	7.0*	0.7	17.3*
Caffeic Acid	27	175	7.9*	61.2*	0.2	22.0*	8.8*	0.9	20.1*
Control	22	167	6.8	50.1	0.3	29.6	13.2	0.7	31.2

P-3 (0.1 m*l*, 10 mg) or caffeic acid (0.1 m*l*, 0.1 mg) was given intraperitoneally every 2 days before venom Ac_1-P (0.2 m*l*, i.v.) injection. *P<0.05, **P<0.01, significance from the value of control analyzed by *t*-test after the arcsin transformation. a) Glomeruli with cystic lesion are excluded.

Crescent formation was noted in a greater number in the control group (Table 5, Figs. 6a and 6b); that is, pathologic lesions in the mice treated with caffeic acid and crude extract were mild but severe in the control.

Microangiography: Tortuosity of arcuate arteries and attenuation and pruning with decrease in interlobular arteries were observed in the control mice and to a lesser extent in the treated mice (Figs. 7a, 7b and 7c).

In pathology and microangiography, the degree of changes in mice treated with caffeic acid lay between control and P-3 mice, although there was no significant difference between the two treated groups.

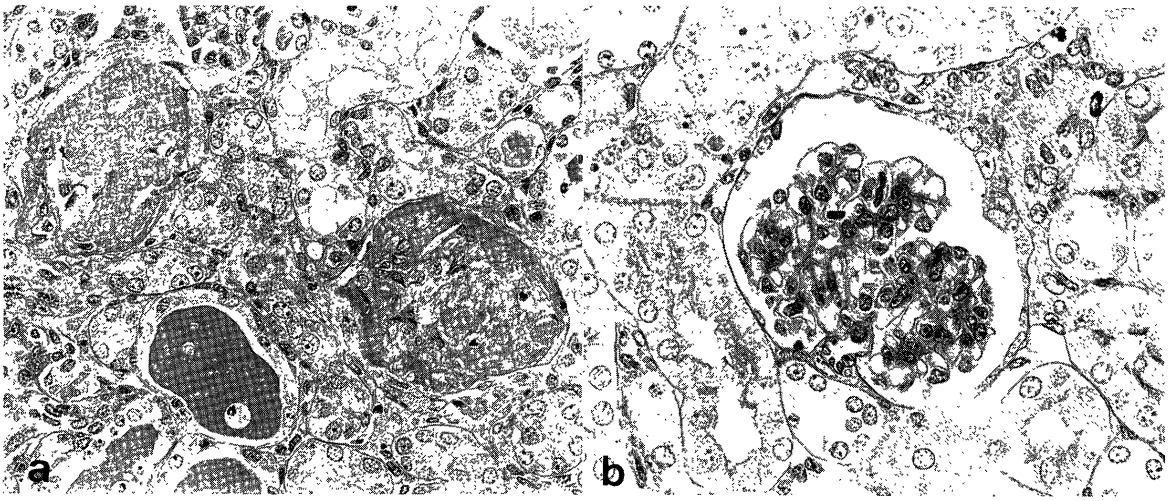

Fig. 5. Glomerular changes 48 hr after inoculation.
a: Control. Glomeruli show the beginning of cellular ingrowth into two occluding fibrin thrombi which developed in the cystic lesions. Some tubules contain PAS-positive homogenous contents. PAS stain; × 300.
b: Caffeic acid. Mesangial cell and matrix are slightly increased in the mesangium. PAS stain; × 400.

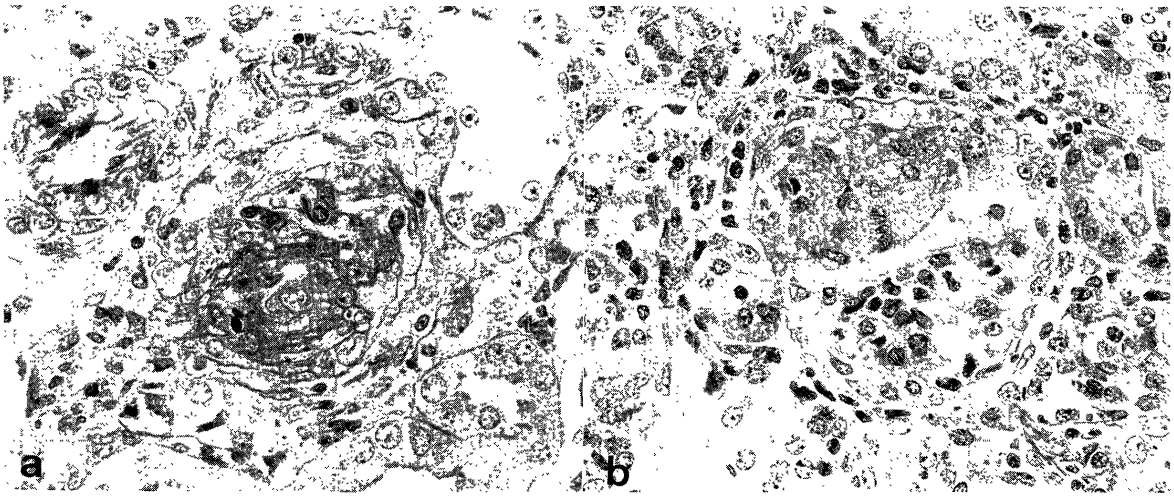

Fig. 6. Glomerular changes 1 week after inoculation.
a: Control. The glomerulus shows the early segmental sclerosis and a global crescent. PAS stain; × 480.
b: Caffeic acid. A half of the glomerulus shows a slight increase of cells and matrix in the mesangium and another half cellular ingrowth into the thrombus. PAS stain; × 480.

DISCUSSION

To find medicine for treating the disease effectively, the purification of effective component(s) from previously known herbal medicine may be of some help. The efficacy of traditional herbal medicine for treating renal lesions induced experimentally in mice by snake venom was examined [18]. It was not clear, however, whether the effect of P-3 was due to cumulative and complex effect of herbs or substances contained or to mainly single effect of a herb or a single substance. If the effect is exerted by a combination of components, their detection should be possible. The results of analysis and experiments indicate that the acidic fraction followed by caffeic acid is the components that most likely exert beneficial effect. Some phenolic substances have been shown effective for treating renal lesions. Gallotannin (galloylglucoses) from *Paeniae radix* showed decreasing activity of BUN in rat serum [12, 17] and a tetramer of caffeic acid (magnesium lithospermate B) from *Salviae miltiorrizae radix* improved uremic symptoms induced by an adenine diet in rats [21, 22]. However, the efficacy of a

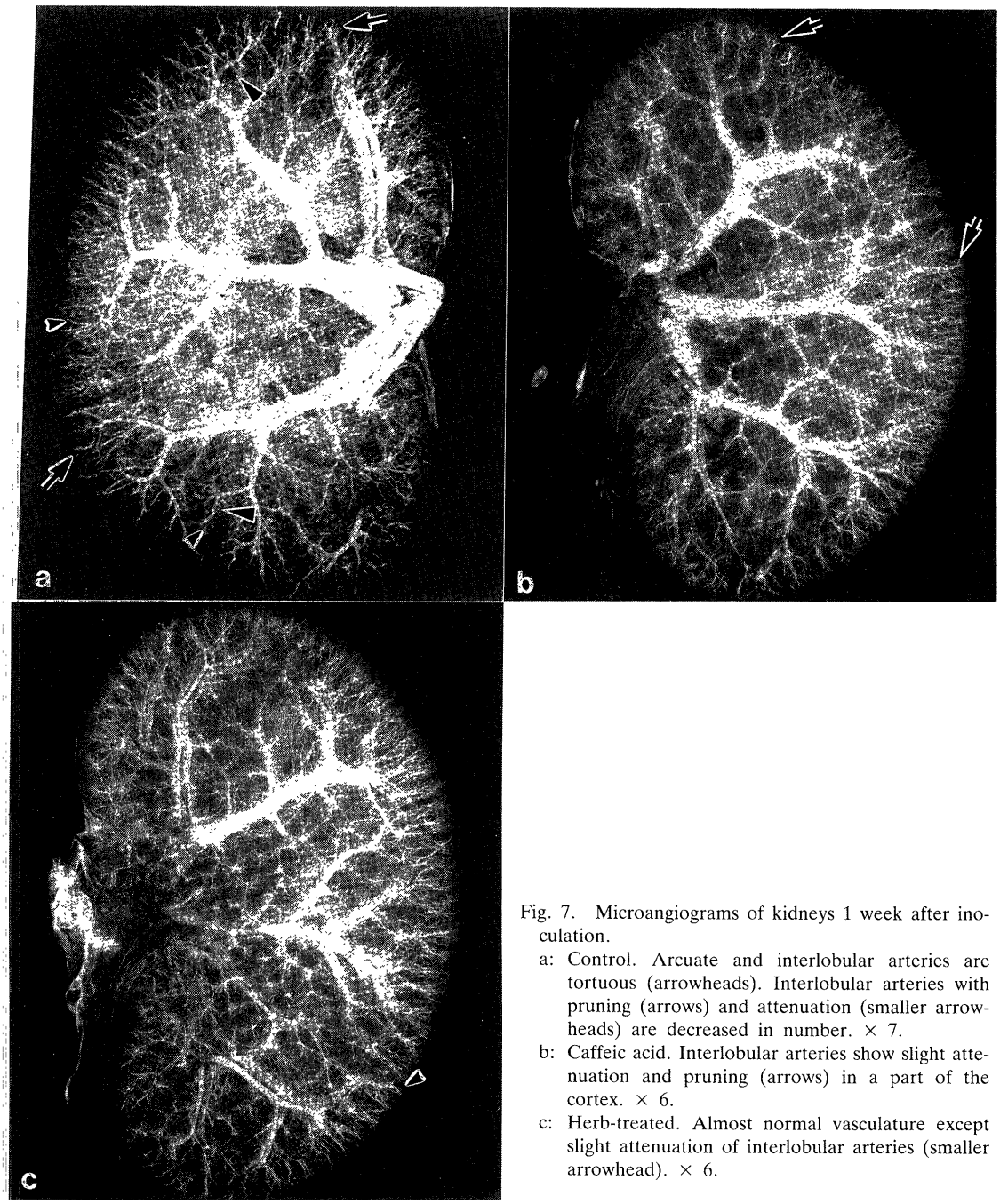

Fig. 7. Microangiograms of kidneys 1 week after inoculation.
a: Control. Arcuate and interlobular arteries are tortuous (arrowheads). Interlobular arteries with pruning (arrows) and attenuation (smaller arrowheads) are decreased in number. × 7.
b: Caffeic acid. Interlobular arteries show slight attenuation and pruning (arrows) in a part of the cortex. × 6.
c: Herb-treated. Almost normal vasculature except slight attenuation of interlobular arteries (smaller arrowhead). × 6.

simple substance such as caffeic acid toward renal diseases is unknown. Examination of the effects of caffeic acid in a prescription containing many herbs by an animal model system may provide important information.

Caffeic acid was found in the present study to be an effective component of P-3 on the basis of its physiological and pathologic effects. Caffeic acid and magnesium lithospermate B [19, 20] have common structural features. The inhibition of prostaglandin synthase from arachidonic acid through antioxidation by caffeic acid derivatives has been reported [8, 14, 20]. Caffeic acid may thus possibly suppress renal lesions together with the consequent antiinflammatory action.

The present results suggest the prophylactic or inhibitory effect of caffeic acid or P-3 on glomerular disease in which proteolytic enzymes may be essen-

tial pathogenically. However, the efficacy of caffeic acid appears less than that of the crude extract, in consideration of the pathologic and microangiographic changes. Although no diuretic action on the part of caffeic acid could be detected, P-3 caused an increase in urine volume in rats (unpublished data). There would thus appear to be other effective component(s) in the extract of P-3. Decreased activity of Fr. 2 after purification may also be indicative of the presence of additive effects with other components. Other components of this herbal medicine are presently being sought.

ACKNOWLEDGEMENTS. We wish to thank Mr. Manabu Miyata for technical assistance in the microangiography and Messrs. Tsuneji Noguchi and Ken'ichi Uematsu for their preparing photographs.

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