Effects of Daio-botampi-to and its component drugs on cephaloridine-induced renal injury

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Abstract

Daio-botampi-to (大黄牡丹皮湯) has been proved to decrease the severity of renal injury induced by cephaloridine, in which proximal uriniferous tubules represent the main site of injury. Variations in the nitrite/nitrate ratio and the activity of radical scavenger enzymes also suggested the protective effect of Daio-botampi-to against oxygen stress. In addition, a cell-protective effect on a cultured renal epithelial cell line, LLC-PK_1, was observed. These effects are considered to result from combined actions of the component drugs of this preparation, i.e., Rhei Rhizoma, Moutan Cortex and Persicae Semen.

Key words Daio-botampi-to (大黄牡丹皮湯), cephaloridine, proximal uriniferous tubule, LLC-PK_1, Rhei Rhizoma, Moutan Cortex, Persicae Semen.

Introduction

The kidney is an organ which requires a great amount of oxygen to meet its high metabolic needs.[1] This means that the kidney is subject to great oxygen stress. In a previous study, we demonstrated the protective effect of the Oriental medical prescription Daio-botampi-to (大黄牡丹皮湯) against renal failure due to ischemia-reperfusion injury.[2] Canavese *et al.*[3] and Higuchi and Sanaka[4] have reported that in renal ischemia-reperfusion, the proximal uriniferous tubules are mainly injured because of their structural features. Focusing on this aspect, we investigated the effect of Daio-botampi-to in rats given cephaloridine, which is known to induce selective injury of the proximal tubules.[5-7] The effect of this prescription was also examined using a cultured proximal uriniferous tubule-derived cell line.

Materials and Methods

Prescription and crude drugs : The prescription's extract was made according to the same standard (formula composition, dosage of each crude drug and production techniques but without any excipient) as that of the commercial product from Kotaro Pharmaceutical Co., Ltd., Osaka, Japan. The crude drug extract was treated in the same manner as the prescription. A voucher specimen is deposited in the laboratory of Kotaro Pharmaceutical Co.

Medium and reagents : Dulbecco's modified Eagle medium/nutrient mixture F-12 (D-MEM/F-12) and fetal calf serum (FCS) were purchased from Life Technologies, Inc. (Grand Island, NY, USA) and Cell Culture Laboratories (Cleveland, OH, USA), respectively. A commercial kit (lactate dehydrogenase CII-Test Wako) for assaying lactate dehydrogenase (LDH) was obtained from Wako Pure Chemical Industries, Ltd., Osaka, Japan. Cephaloridine was purchased from Sigma Chemical Co., St. Louis, MO, USA.

Animal experiments : Male LWH : Wistar rats with a body weight of 150-160 g were purchased from Shizuoka Agricultural Cooperative Association for Laboratory Animals (Hamamatsu, Japan). They

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were kept in a wire-bottomed cage under a conventional lighting regimen with a dark night. The room temperature (about 25 °C) and humidity (about 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 5 groups, avoiding any intergroup difference in body weight gain. One group was given water, while the other was given Daio-botampi-to, Rhei Rhizoma, Moutan Cortex or Persicae Semen orally at a dose of 200 mg/kg body weight/day for 20 consecutive days. A single dose of cephaloridine (1 g/kg body weight) was administered intravenously to rats which had been given Daio-botampi-to or each component, or an equivalent volume of water orally for the preceding 20 days, and urine specimens were collected for 1-2 days after cephaloridine administration. Blood samples were obtained by cardiac puncture without anesthesia, and the serum was separated immediately by centrifugation. The kidneys were subsequently extirpated from each rat following renal perfusion through the renal artery with ice-cold physiological saline. Six rats were used for each experimental group.

Determination of blood and urine samples : Urea nitrogen, albumin and glucose were determined using commercial reagents ; BUN Kainos (Kainos Laboratories, Inc., Tokyo, Japan) and A/G B-Test Wako and Glucose B-Test Wako (both Wako Pure Chemical Industries Ltd., Osaka, Japan). Malondialdehyde (MDA) was determined using the method of Naito and Yamanaka,[8] and protein was assayed by the sulfosalicylic acid method.[9] Sodium (Na) and potassium (K) were measured with an electrolyte analyzer (AHS/Japan Corporation, Tokyo, Japan) using a hydrogen electrode.[10] Osmolarity was measured with an osmometer (OSA-21 ; Nikkiso Co. Ltd., Tokyo, Japan) using the cryoscopic method, and nitrite (NO_2^-) and nitrate (NO_3^-) were measured with a NOX measuring device, TCI-NOX 1000 (Tokyo Kasei Kogyo Co. Ltd., Tokyo).

Enzyme assays : The kidney was homogenized with a 4-fold volume of iced physiological saline and the activities of enzymes in the homogenate were determined. Superoxide dismutase (SOD) activity was assayed by the nitrous acid method,[11] and catalase activity was determined in terms of the decrease in the amount of hydrogen peroxide.[12] Glutathione peroxidase (GSH-Px) activity was determined by colorimetry of 2-nitro-5-thiobenzoic acid, a compound produced through the reaction of glutathione and 5,5'-dithiobis (2-nitrobenzoic acid).[13] Protein was determined by the method of Itzhaki and Gill, with bovine serum albumin as a standard.[14]

LDH and MDA leakage assay : LLC-PK_1 cells were maintained at 37°C in a humidified atmosphere of 5 % CO_2 in air in 96-well culture plates (Corning Glass Works, Corning, NY) with 5 % FCS-supplemented D-MEM/F-12 medium. After confluence had been reached, the cells were seeded in culture plates at 10^4 per well. Cephaloridine and/or Daio-botampi-to or its individual components were added to the culture 2 h later, and the plates were incubated for 48 h. Leakage of LDH into the culture medium was assayed as an index of cytotoxicity using a commercial kit from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). The extent of lipid peroxidation was estimated by measuring the concentration of MDA, as described by Yagi.[15] All assays were performed in 5 determinations.

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

Results

Blood components

Table I shows the serum components of untreated rats and rats given Daio-botampi-to or its three major components—Rhei Rhizoma, Moutan Cortex and Persicae Semen. The urea nitrogen levels in control rats with induced renal failure increased significantly to reach 36.1±4.1 mg/dl, reflecting uremia. Blood levels of glucose and MDA were also increased significantly in these rats, but in contrast, their albumin and Na levels were decreased significantly compared with normal rats. In comparison with the control group, no clear change in the level of urea nitrogen, albumin, glucose, Na or K was observed in the Daio-botampi-to group. However, there was a significant difference in the level of MDA between the

Table I Effect of Daio-botampi-to and its main components on blood components.

Group	Urea nitrogen (mg/dl)	Albumin (g/dl)	Glucose (mg/dl)	MDA (nmol/ml)	Na (mmol/l)	K (mmol/l)
Normal rats	14.7±0.4	4.02±0.06	107.7±2.5	2.57±0.11	136.9±1.3	4.46±0.09
Rats with induced renal failure						
Control	36.1±4.1[c]	3.45±0.08[c]	137.5±4.0[c]	4.02±0.32[c]	133.9±0.5[c]	4.33±0.12
Daio-botampi-to	29.6±5.4[c]	3.51±0.09[c]	135.6±8.0[c]	3.36±0.12[c,f]	134.7±0.4[c]	4.34±0.10
Rhei Rhizoma	31.0±3.2[c]	3.46±0.05[c]	149.4±6.5[c,d]	3.24±0.22[c,f]	134.4±0.7[c]	4.16±0.18[b]
Moutan Cortex	30.5±3.3[c]	3.38±0.04[c]	157.8±6.9[c,f]	2.98±0.24[a,f]	133.7±0.2[c]	4.12±0.10[b]
Persicae Semen	25.5±2.3[c,f]	3.64±0.06[c,e]	159.0±7.2[c,f]	3.00±0.16[a,f]	134.2±0.5[c]	3.77±0.15[c,f]

Statistical significance : [a]$p<0.05$, [b]$p<0.01$, [c]$p<0.001$ *vs.* normal rats, [d]$p<0.05$, [e]$p<0.01$, [f]$p<0.001$ *vs.* control rats with renal failure.

control and prescription-treated groups. A decrease of MDA was also observed in the other three treated groups (decreases of 19 %, 26 % and 25 % for Rhei Rhizoma, Moutan Cortex and Persicae Semen, respectively). In addition, oral administration of Persicae Semen reduced significantly the blood levels of urea nitrogen and K, and increased significantly the levels of albumin and glucose.

Urine components

The results of urinalysis are summarized in Table II. Urine output of normal rats was 21.0 ml/day ; in control rats with renal failure, it increased significantly to approximately 1.82 times the normal value. The urinary excretion of glucose, protein, Na and K was also increased by 23.28, 5.96, 1.36 and 1.50 times the level in normal rats, respectively, while osmolarity and NO_2^-/NO_3^- ratio were decreased by 42 % and 45 %, respectively. Administration of Rhei Rhizoma reduced urine output from 38.2 to 23.0 ml/day (a 40 % decrease, $p<0.001$). The osmolarity in rats given Rhei Rhizoma and Moutan Cortex was increased to 1409 and 1161 mOsm/l, compared with 985 mOsm/l in control rats. A reduction of glucose excretion was clearly observed in all four treated groups, among which there were obvious differences in rats with induced renal failure. In terms of protein excretion, Persicae Semen caused a decrease from 75.7 to 47.9 mg/day (a 37 % change, $p<0.001$). In the Daio-botampi-to and Rhei Rhizoma groups, Na and K were reduced to levels even lower than those in the control group. Daio-botampi-to, Rhei Rhizoma and Moutan Cortex significantly increased NO_2^-/NO_3^-, although there was no obvious variation in the Persicae Semen-treated group.

Enzyme activities

In comparison with normah rats, enzyme activities were significantly decreased in rats with induced renal failure that were not given the prescription or any of its three components, the values being 41 % lower for SOD activity, 55 % lower for catalase activ-

Table II Effect of Daio-botampi-to and its main components on urinalysis.

Group	Urine volume (ml/day)	Osmolarity (mOsm/l)	Glucose (mg/day)	Protein (mg/day)	Na (nmol/l)	K (nmol/l)	NO_2^-/NO_3^- ($\times10^{-6}$M/l)
Normal rats	21.0±3.1	1688±61	36± 4	12.7±3.0	2.68±0.13	1.09±0.07	497±49
Rats with induced renal failure							
Control	38.2±3.4[c]	985±65[c]	838±77[c]	75.7±7.3[c]	3.65±0.24[c]	1.64±0.05[c]	271±25[c]
Daio-botampi-to	32.9±3.6[c]	1052±62[c]	644±41[c,f]	71.7±7.1[c]	3.17±0.18[b,e]	1.30±0.09[b,f]	390±32[c,f]
Rhei Rhizoma	23.0±2.6[f]	1409±99[c,f]	673±55[c,e]	68.7±5.6[c]	3.22±0.20[b,d]	1.41±0.09[c,f]	420±37[a,f]
Moutan Cortex	36.8±2.9[c]	1161±71[c,e]	645±74[c,e]	79.4±4.9[c]	3.75±0.21[c]	1.60±0.06[c]	380±28[c,f]
Persicae Semen	41.7±3.1[c]	791±55[c,e]	680±57[c,e]	47.9±4.4[c,f]	3.66±0.15[c]	1.64±0.06[c]	280±29[c]

Statistical significance : [a]$p<0.05$, [b]$p<0.01$, [c]$p<0.001$ *vs.* normal rats, [d]$p<0.05$, [e]$p<0.01$, [f]$p<0.001$ *vs.* control rats with renal failure.

Table III Effects of Daio-botampi-to and its main components on oxygen species-scavenging enzymes in kidney.

Group	SOD (U/mg protein)	Catalase (U/mg protein)	GSH-Px (U/mg protein)
Normal rats	27.33±0.87	285.2±5.9	155.3±4.8
Rats with induced renal failure			
Control	16.05±0.85[a]	128.7±9.9[a]	101.4±5.2[a]
Daio-botampi-to	21.31±1.31[a,c]	165.3±9.5[a,c]	108.9±5.0[a]
Rhei Rhizoma	20.32±0.73[a,c]	165.5±5.1[a,c]	115.2±7.0[a,b]
Moutan Cortex	17.59±0.51[a]	122.2±7.7[a]	101.7±4.3[a]
Persicae Semen	16.76±0.90[a]	128.8±8.3[a]	106.8±5.1[a]

Statistical significance : [a]$p<0.001$ *vs.* normal rats, [b]$p<0.01$, [c]$p<0.001$ *vs.* control rats with renal failure.

ity and 35 % lower for GSH-Px activity, as shown in Table III. In contrast, the activities of SOD and catalase were higher in rats given Daio-botampi-to, although there was no obvious variation in GSH-Px activity. A similar trend was found in rats given Rhei Rhizoma. In addition, Rhei Rhizoma increased the activity of GSH-Px significantly, although the percentage increase was still smaller than those of SOD or catalase, whereas the other two components had no effect, as shown in Table III.

LDH leakage from cultured cells

The cells cultured under routine conditions released limitable LDH (115.9±3.8 mIU/ml), and this efflux was effectively prevented by administering

Table IV Effect of Daio-botampi-to and its main components on LDH leakage from LLC-PK$_1$ cells.

Group	Concentration (μg/ml)	LDH activity (mIU/ml)	
		Cephaloridine(-)	Cephaloridine(+)
Control	—	115.9±3.8	172.5±4.9
Daio-botampi-to	2.5	111.0±5.8	167.0±3.8
	12.5	109.8±3.3	158.9±6.3[c]
	25	93.2±4.6[c]	144.1±4.8[c]
	50	76.6±6.6[c]	136.7±3.9[c]
	125	70.2±3.9[c]	134.5±3.7[c]
Rhei Rhizoma	2.5	111.3±6.3	171.7±4.6
	12.5	108.4±4.7	159.2±3.4[b]
	25	90.8±3.1[c]	145.1±3.1[c]
	50	77.1±5.2[c]	136.4±3.1[c]
	125	76.7±5.2[c]	134.6±5.9[c]
Moutan Cortex	2.5	114.7±5.4	168.2±5.5
	12.5	110.3±7.5	162.4±7.1
	25	104.4±6.3[a]	152.5±3.3[c]
	50	94.5±7.1[c]	141.8±4.2[c]
	125	91.9±3.3[c]	136.2±2.5[c]
Persicae Semen	2.5	115.0±5.9	168.7±4.5
	12.5	114.1±5.1	162.1±5.6
	25	111.7±4.4	155.2±6.5[c]
	50	107.6±5.6	151.5±6.9[c]
	125	102.7±5.9[a]	141.6±6.4[c]

Statistical significance : [a]$p<0.05$, [b]$p<0.01$, [c]$p<0.001$ *vs.* control values.

Table V Effect of Daio-botampi-to and its main components on MDA leakage from LLC-PK_1 cells.

Group	Concentration (μg/ml)	MDA (nmol/well)	
		Cephaloridine(-)	Cephaloridine(+)
Control	—	0.116±0.004	0.162±0.007
Daio-botampi-to	2.5	0.113±0.006	0.157±0.010
	12.5	0.107±0.009	0.152±0.013
	25	0.104±0.010	0.144±0.017
	50	0.089±0.009^c	0.140±0.007^a
	125	0.086±0.009^c	0.138±0.004^b
Rhei Rhizoma	2.5	0.111±0.010	0.150±0.007
	12.5	0.103±0.010	0.144±0.009
	25	0.096±0.006^b	0.139±0.008^a
	50	0.091±0.006^c	0.136±0.010^b
	125	0.077±0.007^c	0.122±0.008^c
Moutan Cortex	2.5	0.109±0.008	0.148±0.013
	12.5	0.108±0.007	0.144±0.012
	25	0.098±0.012^a	0.142±0.011
	50	0.091±0.008^b	0.138±0.012^a
	125	0.085±0.006^c	0.134±0.009^b
Persicae Semen	2.5	0.113±0.011	0.160±0.010
	12.5	0.109±0.012	0.153±0.009
	25	0.103±0.009	0.151±0.007
	50	0.099±0.008	0.149±0.011
	125	0.098±0.010^a	0.144±0.008

Statistical significance : $^a p<0.05$, $^b p<0.01$, $^c p<0.001$ *vs.* control values.

various concentrations of Daio-botampi-to and its three components, as shown in Table IV. Upon coculture with different concentrations of Daio-botampi-to, Rhei Rhizoma and Moutan Cortex, this leakage was significantly suppressed at 25 to 125 μg/ml, respectively. It was observed that exposure to cephaloridine (0.1 μM) caused a large amount of LDH leakage, being nearly 1.5-fold that observed in the routine blank (172.5±4.9 mIU/ml). When the cells were cultured under identical conditions but with different doses of the prescription and its three components, this leakage was substantially inhibited in a concentration-dependent manner. Pretreatment of the cells with Daio-botampi-to or Rhei Rhizoma limited this leakage markedly, only 12.5μg/ml appearing in the medium. Higher concentrations of Moutan Cortex and Persicae Semen (double those of both Daio-botampi-to and Rhei Rhizoma) were required to inhibit LDH release significantly.

MDA leakage from cultured cells

MDA (0.116 nmol/well) was detected in the culture medium of control cells incubated under routine conditions, and the amount of this lipid peroxidant formed declined markedly when the cells were cultured in the presence of Daio-botampi-to, Rhei Rhizoma and Moutan Cortex, as shown in Table V. Their inhibition of MDA formation was concentration-dependent. Exposure to cephaloridine increased the MDA level in the control cell medium sharply, to about 1.4 times the intact control value. Of the four samples tested, 25 μg/ml Rhei Rhizoma, twice the concentration of Daio-botampi-to and Moutan Cortex, reduced MDA production significantly. However, Persicae Semen had no such effect on MDA release, as shown in Table V.

Discussion

Rats given cephaloridine showed increased urinary excretion of Na and glucose and dysfunction of

the proximal uriniferous tubules, as demonstrated by an increase in urinary volume and urinary protein and a decrease in urinary osmotic pressure. In contrast, rats given oral doses of Daio-botampi-to prior to intravenous cephaloridine showed significantly lower urinary levels of Na, K and glucose ; the improvement in the urinary excretion of glucose was particularly evident. The urinary volume and osmotic pressure tended to be normalized. These urinary findings indicate that Daio-botampi-to ameliorates renal injury. This effect was also reflected in the blood urea nitrogen level.

With regard to the mechanism of cephaloridine-induced renal injury, Suzuki and Sudo[16,19] have proposed that peroxidation of lipids in the renal cell membrane is caused by free radicals from cephaloridine itself or oxygen radicals generated through the process of cephaloridine metabolism (oxidation), resulting in damage to the kidney. In this connection, we determined the urinary NO_2^-/NO_3^- ratio as an index of the amount of nitric oxide (NO) in the body. Although the levels of both NO_2^- and NO_3^- reflect the amount of NO, two different routes of NO_2^- generation, i.e., one from NO through N_2O_2 and N_3O_3 and the other from a combination of NO and Fe^{2+} of Hb, are generally considered to be safe metabolic pathways.[20] In contrast, Beckman *et al.*[21] and Radi *et al.*[22,23] have shown that peroxynitrite, which is produced through reaction of NO and O_2^-, or ·OH derived from peroxynitrite, causes damage to the tissue. In addition, since NO_3^- is produced from ·OH and NO_2, the ratio of NO_2^- to NO_3^- can serve as an index of whether or not oxygen stress is present. In the present study, whereas the NO_2^-/NO_3^- ratio in rats given cephaloridine alone was lower than normal, the ratio was significantly increased in rats given Daio-botampi-to prior to cephaloridine administration. The activity of SOD and catalase, which are O_2^- scavengers, was significantly increased in the latter group, suggesting that the protective effect of Daio-botampi-to against oxygen stress led to amelioration of renal injury.

Daio-botampi-to is an Oriental medical prescription consisting of Rhei Rhizoma, Moutan Cortex, Benincasae Semen, Natrium Sulfuricum and Persicae Semen, exerting antipyretic (Rhei Rhizoma, Moutan Cortex, Benincasae Semen), cathartic (Rhei Rhizoma, Natrium Sulfuricum) and laxative (Persicae Semen) actions, activating blood and body fluid energy (Rhei Rhizoma, Moutan Cortex, Persicae Semen) and promoting pus discharge (Benincasae Semen).[24] Among traditional Chinese medicines, those which activate blood and body fluid energy are usually used for treating disturbances of the vascular system, which are often seen in renal injury. Taking this into consideration, we examined the effects of Rhei Rhizoma, Moutan Cortex and Persicae Semen, and found that Rhei Rhizoma exerted an effect similar to that of Daio-botampi-to on the NO_2^-/NO_3^- ratio, the activity of radical scavenger enzymes, and the urinary excretion of glucose, Na and K. The urinary volume and osmotic pressure were restored to near-normal levels, indicating that Rhei Rhizoma had a more potent effect than Daio-botampi-to. Moutan Cortex also caused an increase in the NO_2^-/NO_3^- ratio and urinary osmotic pressure, and a decrease in the urinary excretion of glucose. Although conversely Persicae Semen caused a decrease in urinary osmotic pressure, it also tended to normalize the urinary excretion of glucose and protein. Thus, these findings suggest that Rhei Rhizoma, Moutan Cortex and Per sicae Semen contribute to the beneficial effect of Daio-botampi-to through their own individual mechanisms of action.

In cephaloridine-induced renal injury, the proximal uriniferous tubules are chiefly injured. Physiological, biochemical and histopathological changes related to such injury have been investigated by Silverblatt *et al.*,[5] Tune *et al.*[6] and Tune and Fravert,[7] leading to a better understanding of the pathogenic mechanism of cephaloridine-induced renal injury. In this connection, in an *in vitro* system using a swine kidney-derived, proximal uriniferous tubule-like cultured epithelial cell line, LLC-PK_1, we determined the effect of cephaloridine in terms of leakage of the lysosomal enzyme LDH into the culture medium due to cell membrane damage. In the presence of 0.1 μM cephaloridine, the level of LDH leakage into the medium was about 1.5 times higher than that in the absence of cephaloridine, reflecting cell membrane damage. However, when Daio-botampi-to was added to the medium at concentrations varying from 12.5 to 125 μg/ml, the LDH leakage was suppressed in pro-

portion to Daio-botampi-to concentration, indicating that this prescription suppressed cephaloridine-induced renal injury. A similar effect was found with Rhei Rhizoma, Moutan Cortex or Persicae Semen. The cell-protective effect of Daio-botampi-to, Rhei Rhizoma or Moutan Cortex was also demonstrated without cephaloridine-induced renal injury. These results therefore suggest the prophylactic effect of this medical prescription and its component drugs against renal injury. On the other hand, Suzuki and Sudo[16-19] have reported that cephaloridine causes the production of oxygen radicals and lipid peroxidation in rat kidney-derived microsomal membranes. In the present study, we also observed a marked increase in MDA leakage into the culture medium in the presence of cephaloridine. However, when Daio-botampi-to, Rhei Rhizoma or Moutan Cortex was added to the medium in combination with cephaloridine, the MDA leakage was significantly suppressed, demonstrating their cell-protective effect. Considering the previously reported finding that Daio-botampi-to lessens cell damage under condition of hypoxia-reoxygenation,[2] the beneficial effect of this prescription on proximal uriniferous tubule function seems to be attributable to inhibition of lipid peroxidation via free radicals.

和文抄録

cephaloridineによる腎での障害部位は近位尿細管であるが，大黄牡丹皮湯に腎障害を軽減する作用を有していることが示され，NO_2^-/NO_3^-，ラジカル消去酵素活性の動態から，酸素ストレスに対し大黄牡丹皮湯が防御的に作動していることが示唆された。また培養腎上皮細胞株 LLC-PK_1 における保護作用も認められ，これら作用は構成生薬の複合によるものと考えられた。

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