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Original article

Renal protective effects of aspalathin and nothofagin from rooibos (*Aspalathus linearis*) in a mouse model of sepsis

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ABSTRACT

Background: Aspalathin (Aspt) and nothofagin (Not) were reported to have antioxidant activity and are the two major active dihydrochalcones in green rooibos. This study was conducted to determine whether Asp and Not can modulate renal functional damage in a mouse model of sepsis and to elucidate the underlying mechanisms.
Methods: The potential of Aspt and Not treatment to reduce renal damage induced by cecal ligation and puncture (CLP) surgery in mice was measured by assessing blood urea nitrogen (BUN), serum creatinine, total urine protein, levels of lactate dehydrogenase (LDH), nitric oxide (NO), tumour necrosis factor (TNF)-α, interleukin (IL)-6, and myeloperoxidase (MPO), lipid peroxidation, total glutathione, glutathione peroxidase activity, catalase activity, and superoxide dismutase activity.
Results: Treatment with Aspt and Not decreased plasma levels of BUN, creatinine, urine protein, and LDH in mice with CLP-induced renal damage. Moreover, Aspt and Not inhibited nuclear factor (NF)-κB activation and reduced the induction of NO synthase and excessive production of nitric acid. Aspt and Not treatment also reduced the plasma levels of NO, TNF-α, IL-6, and MPO and reduced lethality due to CLP-induced sepsis, increased lipid peroxidation, and markedly enhanced the antioxidant defence system by restoring the levels of superoxide dismutase, glutathione peroxidase, and catalase in the kidney tissues.
Conclusion: Our results suggest that Aspt and Not protect mice against sepsis-triggered renal injury.
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Introduction

Sepsis is defined as a systemic inflammatory response syndrome caused by infection and is a common cause of morbidity and mortality, despite recent advances in antibiotic therapy and intensive care [1]. In the past 30 years, sepsis has become one of the major causes of hospital admission and medical expenses [2]. Although cytokine activation is part of the host defence response to infection, excessive production and secretion of cytokines can cause widespread tissue injury and organ failure [3]. Septic conditions activate inducible nitric oxide synthase (iNOS) and increase the plasma concentration of nitric oxide (NO), which ultimately lead to cytotoxicity [4,5]. Sepsis is also known to enhance the synthesis of reactive oxygen species (ROS) such as superoxide anions and hydrogen peroxide [4,5]. Excessive production of ROS can cause significant oxidative stress by decreasing endogenous antioxidant defences and lipid peroxidation. The rate of organ failure due to sepsis can be decreased by reducing inflammatory cytokines and inhibiting iNOS activity [4,6]. Because interventions that reduce the production or effect of ROS have been shown to have beneficial effects on sepsis [7,8], agents that can decrease cytokine production and ROS may also prevent or lessen the pathological cascade of inflammation caused by sepsis.

Teas and herbal infusions are natural beverages that contain compounds of interest to health science fields because of their potential *in vivo* biological properties [9,10]. Infusions of the leaves of rooibos (*Aspalathus linearis*), a plant endemic to the Western Cape province of South Africa, are caffeine-free, low-tannin

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beverages that are a source of uncommon glycosylated polyphenol compounds [11]. It is known that rooibos tea contains abundant flavonoids, particularly aspalathin (Aspt) and nothofagin (Not) [12]. The antioxidant activity of natural rooibos infusions and extracts was reported to depend on the total polyphenol content of the sample, which is determined by how the plant was processed [11]. Rooibos tea contains sodium, potassium, magnesium, calcium, and trace elements such as zinc. Although some of the biological activities and pharmacological functions of Aspt and Not have been reported, the protective effects Aspt and Not against renal damage have not been examined. In the current study, we investigated the renal protective effects of Aspt and Not in an animal sepsis model.

Materials and methods

Reagents

Aspalathin (Aspt) was obtained from Sigma (St. Louis, MO, USA) and nothofagin (Not) was obtained from Chem Faces (Wuhan, China).

Animals and cecal ligation and puncture

Male C57BL/6 mice (6–7 weeks old, weight 27 ± 1.3 g) were obtained from Orient Bio Co. (Sungnam, Republic of Korea) and allowed a 12-d acclimatization period. The mice were housed under controlled temperature (20–25 °C) and humidity conditions (40–45% relative humidity), with a 12-h light:12-h dark cycle. They were fed a normal rodent pellet diet and had *ad libitum* access to water during acclimatization. To induce sepsis, the mice were first anesthetised with Zoletil (tiletamine and zolazepam, 1:1 mixture, 30 mg/kg) and rompum (xylazine, 10 mg/kg). Sepsis was induced by cecal ligation and puncture (CLP) as previously described [13,14]. As controls, sham-operated animals were used; in these mice, the cecum was exposed, but not ligated or punctured, and then returned to the abdominal cavity. Animals were randomly divided into 11 treatment groups (n = 10 each): sham-operated control; Aspt- or Not-only (1.00 mg/kg body weight) in 0.5% DMSO; CLP surgery only; and CLP + Aspt or Not (0.08, 0.17, 0.33, or 1.00 mg/kg body weight). To determine the effects of Aspt or Not on CLP-induced lethality, animals were randomly divided into 5 treatment groups (n = 20 each): sham-operated control; 0.5% DMSO; CLP surgery only; and CLP + Aspt or Not (1.00 mg/kg body weight) in 0.5% DMSO. Aspt or Not was intravenously injected at 12 h after CLP and again at 50 h after CLP. Blood and organ samples were collected 4 days after the second injection of Aspt or Not for functional assays. This protocol was approved by the Animal Care Committee prior to conducting the study (IRB No. KNU 2016-54).

Sample preparation

Four days after the second injection of Aspt or Not, the mice were anesthetised as described above and sacrificed. Blood samples were collected from the posterior vena cava and allowed to clot. Serum was separated by centrifugation at 4000 rpm for 10 min, stored at −80 °C until analysis, and was used to assess plasma BUN and creatinine levels. Kidney samples were immediately removed and weighed. The kidneys were then minced with scissors and homogenized in 0.1 M phosphate-buffered saline (pH 7.4); the tissue was fractionated under refrigeration by centrifugation at 10,000 ×g for 10 min. The homogenate was stored at −80 °C until analysis by various biochemical assays. Protein concentrations were determined using the Bradford assay.

Evaluation of nephrotoxicity and lactate dehydrogenase

Renal dysfunction was assessed by measuring changes in the levels of BUN and creatinine, as well as of protein in the urine. BUN, creatinine, and lactate dehydrogenase (LDH), another important marker of tissue injury, were measured using commercial assay kits (Pointe Scientific, Lincoln Park, MI, USA). Urine samples were collected from each animal using a metabolic cage at 12 h after CLP surgery and the supernatant was obtained. Urinary protein concentrations were determined by the Bradford assay using bovine serum albumin as the protein standard.

Plasma nitrite/nitrate determination

Nitrite and nitrate concentrations in the plasma were determined using Griess reagents and vanadium solution (VCl_3) as previously described [15]. Briefly, 100 μL of VCl_3 was added to 100 μL of sample, immediately followed by addition of Griess reagents (0.1% N-1-naphthylethylenediamine dihydrochloride and 1% sulfanilamide in 5% phosphoric acid). After 30 min of colour development, absorbance was determined by measuring the optical density (OD) at 540 nm using a microplate reader (Tecan GmbH, Männedorf, Switzerland). Concentrations were determined by comparing the absorption values with those of a standard curve prepared using sodium nitrite.

ELISA for tumour necrosis factor (TNF)-α, interleukin (IL)-6

The plasma concentrations of interleukin (IL)-6 and tumour necrosis factor (TNF)-α were determined using enzyme-linked immunosorbent assay (ELISA) kits (R&D Systems, Minneapolis, MN, USA). Values were measured using an ELISA plate reader (Tecan).

Renal myeloperoxidase (MPO) activity

Renal MPO activity was evaluated as a quantitative indicator of neutrophil influx into the kidney; MPO activity was measured using ELISA kits (Abcam, Cambridge, UK).

Evaluation of oxidative stress markers

Lipid peroxidation was measured as the formation of thiobarbituric acid reactive substances (TBARSs). The level of malondialdehyde (MDA) in kidney tissue was measured spectrophotometrically using an OxiSelect TBARS assay kit (Cell Biolabs, San Diego, CA, USA). MDA values were expressed in units of nM/mg protein. Total glutathione (GSH) contents in the kidney tissue were measured as described previously [16]. A tissue homogenate was prepared, and then samples were added to metaphosphoric acid and allowed to stand for 5 min to precipitate proteins. Phosphate buffer and 5,5′-dithiobis-2-nitro-benzoic acid were added for colour development. GSH was determined by measuring the absorbance at 415 nm and absolute concentrations were calculated using a GSH standard (Sigma Aldrich). Values of total GSH were expressed as nM/mg protein. Superoxide dismutase (SOD) activity was measured using an SOD assay kit (Fluka, Munich, Germany). Values of SOD were expressed as U/mg protein. Glutathione peroxidase (GSH-Px) activity was determined using the cellular activity assay kit CGP-1 (Sigma Aldrich). Values of GSH-Px were expressed in units of U/mg protein. Catalase activity (CAT) was determined by a CAT assay kit (Sigma Aldrich) using the decomposition rate of the substrate H_2O_2 as determined at 240 nm. Total CAT values were expressed in units of U/mg protein.

Western blots of renal tissue

Kidney samples were homogenized in radioimmunoprecipitation buffer containing protease inhibitors; equal amounts of

protein were separated by SDS-PAGE (10%) and then electroblotted overnight onto an Immobilon membrane (Millipore, Billerica, MA, USA). The membranes were blocked for 1 h with 5% low-fat milk-powder TBS (50 mM Tris-HCl, pH 7.5, 150 mM NaCl) containing 0.05% Tween 20 and then incubated with primary antibodies for iNOS, inhibitory kappa B (IκB), nuclear factor (NF)-κB, and β-actin at 4 °C overnight. The membranes were incubated with horseradish-peroxidase-conjugated secondary antibody, and enhanced chemiluminescence detection was performed according to the manufacturer's instructions. Densitometry analysis was performed using the ImageJ Gel Analysis tool (NIH, Bethesda, MD, USA).

Cell culture

Primary human umbilical vein endothelial cells were obtained from Cambrex Bio Science (Charles City, IA, USA) and maintained as previously described [13,17]. These cells were used at passages 3–5.

Preparation of nuclear extracts and ELISA for NF-κB p65

After endothelial cells were isolated from kidney samples using CD-31 (n = 5), nuclear extracts were prepared on ice as previously described [18]. Briefly, the cells were harvested and washed with 1 mL of buffer A (10 mM HEPES, pH 7.9, 1.5 mM $MgCl_2$, 19 mM KCl) for 5 min at 600 ×g. Subsequently, the cells were resuspended in buffer A, centrifuged at 600 ×g for 3 min, resuspended in 30 μL of buffer B (20 mM HEPES, pH 7.9, 25% glycerol, 0.42 M NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA), rotated for 30 min at 4 °C, and centrifuged at 13,000 ×g for 20 min. The supernatant was used as the nuclear extract. The nuclear extracts were analysed for protein content using the Bradford assay. The activity of NF-κB p65 was quantified in accordance with the manufacturer's instructions using a commercially available ELISA kit (Cell Signaling Technology, Danvers, MA, USA).

Statistical analysis

All experiments were performed independently at least three times. Values are expressed as the means ± standard deviation (SD). The statistical significance of differences between test groups was evaluated using SPSS for Windows, version 16.0 (SPSS, Chicago, IL, USA). Statistical relevance was determined by one-way analysis of variance and Tukey's *post-hoc* test. A values of p less than 0.05 were considered to indicate significance.

Results

Effects of Aspt or Not on CLP-induced renal tissue injury

The effects of CLP surgery on nephrotoxic markers are shown in Table 1; the plasma levels of BUN and creatinine and protein levels in the urine were significantly higher on the fourth day after the second injection of Aspt or Not than in the sham-operated group. Sham operation or administration of Aspt or Not only to mice did not result in any obvious changes in the plasma levels of BUN and creatinine or urine protein levels. The increase in the levels of serum BUN and creatinine and urine protein after surgery was inhibited by single administration of Aspt or Not (12 h after CLP, data not shown). Thus, we administered two equal doses of Aspt or Not, one at 12 h after CLP and the other at 50 h after CLP. We found that Aspt or Not decreased BUN, creatinine, and protein levels in the urine (Table 1). Another important marker of tissue injury, LDH, was reduced by Aspt and Not in CLP-operated mice (Table 1).

Effects of Aspt or Not on plasma nitrite and nitrate production after CLP surgery

The effects of Aspt or Not treatment on inflammatory responses in the kidney tissue were investigated *in vivo* by measuring plasma nitrite and nitrate levels (stable end products of NO). In sham-operated and Aspt- or Not-only mice, the levels of plasma NO did not significantly change (Table 2). However, CLP surgery caused an approximately 7.4-fold increase in mouse plasma NO production compared to that in control mice (Table 2). Treatment with Aspt or Not post surgery increased NO levels to 38% or 39%, respectively, which were lower than those in the CLP group (Table 2).

Effects of Aspt or Not on plasma TNF-α, IL-6, and MPO levels

The effects of Aspt or Not on CLP-induced inflammatory responses were investigated by measuring the plasma levels of TNF-α and IL-6. CLP surgery significantly increased plasma TNF-α and IL-6 levels; treatment with Aspt or Not post surgery inhibited these increases (Table 2). The plasma concentrations of TNF-α and IL-6 were lower by 63% or 61%, respectively, in the CLP + Aspt or Not (1.00 mg/kg) group than in the CLP group. Next, we determined the effects of Aspt or Not treatment on neutrophil infiltration after CLP surgery. Kidney tissues were collected, homogenized, and centrifuged, and the supernatants were assayed for MPO levels by ELISA.

Table 1
Effects of Aspt and Not treatment on plasma levels of BUN, creatinine, urine level of protein, and LDH in CLP-operated mice[a].

	BUN (mg/dL)	Creatinine (mg/dL)	Urine protein (mg/12 hour)	LDH (U/dL)
Sham	18.5 ± 0.5	0.127 ± 0.015	1.9 ± 0.11	279 ± 25.2
Aspt (1.00 mg/kg)	18.2 ± 0.7	0.115 ± 0.014	2.0 ± 0.22	289 ± 24.1
Not (1.00 mg/kg)	18.3 ± 0.5	0.130 ± 0.012	2.1 ± 0.18	282 ± 19.5
CLP	80.2 ± 5.3[#]	0.475 ± 0.024[#]	13.5 ± 0.58[#]	3610 ± 315.2[#]
CLP + Aspt (0.08 mg/kg)	79.6 ± 4.3	0.482 ± 0.037	12.9 ± 1.11	3580 ± 223.7
CLP + Aspt (0.17 mg/kg)	62.1 ± 3.5[*]	0.351 ± 0.031[*]	9.2 ± 0.62[*]	2850 ± 230.7[*]
CLP + Aspt (0.33 mg/kg)	45.3 ± 2.3[*]	0.285 ± 0.022[*]	6.5 ± 0.37[*]	2250 ± 156.2[*]
CLP + Aspt (1.00 mg/kg)	32.5 ± 3.1[*]	0.253 ± 0.015[*]	4.2 ± 0.33[*]	1370 ± 115.2[*]
CLP + Not (0.08 mg/kg)	81.1 ± 4.2	0.478 ± 0.026	13.1 ± 0.76	3520 ± 250.3
CLP + Not (0.17 mg/kg)	63.7 ± 3.6[*]	0.375 ± 0.031[*]	8.9 ± 0.42[*]	2980 ± 150.4[*]
CLP + Not (0.33 mg/kg)	40.5 ± 3.1[*]	0.275 ± 0.025[*]	6.1 ± 0.37[*]	2360 ± 195.8[*]
CLP + Not (1.00 mg/kg)	31.2 ± 1.8[*]	0.242 ± 0.027[*]	3.9 ± 0.31[*]	1580 ± 123.2[*]

Sham, sham-operated mice; Aspt or Not, mice treated with Aspt or Not (1.00 mg/kg body weight) at 12 and 50 h; CLP, CLP-operated mice; Aspt or Not + CLP, mice treated with Aspt or Not at 12 and 50 h after CLP surgery. Sample was collected 4 days after the second injection of Aspt or Not.
[a] Each value represents the mean ± SD (n = 10).
[#] $p < 0.01$ as compared to sham.
[*] $p < 0.05$ as compared to CLP.

Table 2
Effects of Aspt and Not treatment on NO, TNF-α, IL-6 levels and renal MPO activity in CLP-operated mice[a].

	NO (μM)	TNF-α (pg/mL)	IL-6 (pg/mL)	MPO (U/g tissue)
Sham	30.65 ± 3.05	132.25 ± 11.39	0.75 ± 0.07	0.62 ± 0.05
Aspt (1.00 mg/kg)	32.32 ± 2.65	128.89 ± 11.68	0.72 ± 0.06	0.69 ± 0.06
Not (1.00 mg/kg)	31.28 ± 2.96	130.58 ± 14.58	0.81 ± 0.05	0.66 ± 0.08
CLP	226.68 ± 21.32$^{\#}$	559.65 ± 42.25$^{\#}$	78.28 ± 4.29$^{\#}$	3.65 ± 0.41$^{\#}$
CLP + Aspt (0.08 mg/kg)	218.23 ± 14.58	562.68 ± 45.32	79.32 ± 8.12	3.47 ± 0.32
CLP + Aspt (0.17 mg/kg)	185.27 ± 17.65*	398.45 ± 32.36*	65.32 ± 5.11*	2.92 ± 0.231
CLP + Aspt (0.33 mg/kg)	132.35 ± 13.32*	289.64 ± 24.21*	47.57 ± 4.31*	2.08 ± 0.22*
CLP + Aspt (1.00 mg/kg)	85.35 ± 5.67*	206.65 ± 19.65*	23.58 ± 3.21*	1.63 ± 0.12*
CLP + Not (0.08 mg/kg)	220.29 ± 15.65	559.97 ± 45.32	80.32 ± 6.65	3.56 ± 0.31
CLP + Not (0.17 mg/kg)	195.62 ± 14.49*	384.36 ± 32.68*	67.32 ± 4.61*	2.82 ± 0.23*
CLP + Not (0.33 mg/kg)	148.38 ± 10.26*	301.35 ± 27.64*	42.38 ± 3.78*	2.28 ± 0.21*
CLP + Not (1.00 mg/kg)	87.96 ± 7.65*	215.45 ± 19.58*	28.53 ± 2.63*	1.47 ± 0.17*

Sham, sham-operated mice; Aspt or Not, mice treated with Aspt or Not (1.00 mg/kg body weight) at 12 and 50 h; CLP, CLP-operated mice; Aspt or Not + CLP, mice treated with Aspt or Not at 12 and 50 h after CLP surgery. Sample was collected 4 days after the second injection of Aspt or Not.
[a] Each value represents the mean ± SD (n = 10).
[#] $p < 0.01$ as compared to sham.
[*] $p < 0.05$ as compared to CLP.

MPO activity can act as an indicator of renal infiltration by neutrophils. We observed a marked increase in the MPO level after CLP surgery (Table 2), which was associated with nephritis. Treatment with Aspt or Not post surgery resulted in a significantly lower MPO concentration in renal tissues than that in CLP-operated mice.

Effects of Aspt or Not on kidney tissue MDA

MDA concentration is an indicator of lipid peroxidation levels. In the kidney tissues of CLP-operated mice, a significant increase in MDA levels was observed (Table 3). Treatment with Aspt or Not post surgery led to significantly lower MDA levels than those in the control group.

Effects of Aspt or Not on total GSH and activities of antioxidant enzymes in renal tissues

To test the effect of Aspt or Not on CLP-induced oxidative stress, we analysed the activities of the antioxidant GSH and oxidative stress-associated enzymes SOD, GSH-Px, and CAT. Total GSH levels and the activities of SOD, GSH-Px, and CAT were similar in the Aspt- or Not-only and sham-operated groups. In contrast, total GSH levels and renal activities of all three enzymes were reduced in CLP mice. However, treatment with Aspt or Not post surgery increased total GSH and renal enzyme activities (Table 3).

Effects of Aspt or Not on levels of the renal proteins iNOS, IκB, and NF-κB and on cellular toxicity

To explore the mechanisms responsible for mediating the anti-inflammatory effects of Aspt and Not, we measured iNOS, IκB, and NF-κB protein levels in the kidney tissues of mice. iNOS protein levels were low in the kidney homogenates obtained from the control groups but were significantly elevated in CLP-operated mice. Treatment with Aspt or Not post surgery significantly reduced this increase in iNOS (Fig. 1). Next, we investigated whether Aspt and Not could inhibit CLP-induced degradation of IκB and prevent the translocation of the subunit of NF-κB p65 from the cytosol to the nucleus. Degradation of IκB was significantly lower in the Aspt or Not + CLP group than in the CLP-only group. Additionally, an increase in the level of p65 in the cytosol fraction was detected in the CLP + Aspt or Not group (Fig. 1A and B). To confirm that Aspt and Not prevent translocation of NF-κB p65 from the cytosol to the nucleus, nuclear extracts were isolated from kidney endothelial cells and NF-κB p65 activity in the nuclear extracts was measured by ELISA. The data showed that CLP increased the activity of NF-κB p65 in the nuclear extracts, which was significantly reduced in the Aspt and Not groups (Fig. 1C).

Effects of Aspt or Not in CLP-induced septic lethality

To evaluate whether the renal protective responses identified after Aspt or Not treatment influenced the survival rate of mice

Table 3
Effects of Aspt and Not treatment on MDA level and the activities of renal antioxidant enzymes in CLP-operated mice[a].

	MDA (nM/mg protein)	GSH (nM/mg protein)	SOD (U/mg protein)	GSH-Px (U/mg protein)	CAT (U/mg protein)
Sham	178.35 ± 15.36	27.56 ± 2.32	1.25 ± 0.02	2.25 ± 0.21	4.25 ± 0.31
Aspt (1.00 mg/kg)	182.35 ± 18.64	26.69 ± 1.52	1.23 ± 0.03	2.36 ± 0.23	4.39 ± 0.32
Not (1.00 mg/kg)	179.82 ± 17.69	28.12 ± 1.75	1.18 ± 0.04	2.31 ± 0.25	4.33 ± 0.40
CLP	331.52 ± 24.36$^{\#}$	17.32 ± 1.52$^{\#}$	0.69 ± 0.04$^{\#}$	1.41 ± 0.13$^{\#}$	2.89 ± 0.23$^{\#}$
CLP + Aspt (0.08 mg/kg)	332.31 ± 25.61	17.21 ± 1.71	0.71 ± 0.05	1.39 ± 0.12	2.92 ± 0.15
CLP + Aspt (0.17 mg/kg)	282.37 ± 20.21*	20.35 ± 1.05*	0.85 ± 0.08*	1.59 ± 0.14*	3.45 ± 0.29*
CLP + Aspt (0.33 mg/kg)	235.65 ± 15.69*	22.12 ± 2.52*	0.91 ± 0.06*	1.79 ± 0.17*	3.75 ± 0.31*
CLP + Aspt (1.00 mg/kg)	215.36 ± 18.65*	25.35 ± 2.25*	0.98 ± 0.07*	2.01 ± 0.13*	4.02 ± 0.35*
CLP + Not (0.08 mg/kg)	321.85 ± 27.95	17.23 ± 1.53	0.72 ± 0.05	1.38 ± 0.12	2.87 ± 0.23
CLP + Not (0.17 mg/kg)	268.68 ± 22.95*	19.95 ± 1.71*	0.83 ± 0.06*	1.71 ± 0.16*	3.44 ± 0.29*
CLP + Not (0.33 mg/kg)	225.97 ± 17.14*	21.37 ± 2.25*	0.88 ± 0.06*	1.82 ± 0.12*	3.87 ± 0.23*
CLP + Not (1.00 mg/kg)	211.65 ± 12.35*	24.25 ± 1.69*	0.97 ± 0.07*	2.09 ± 0.11*	4.09 ± 0.27*

Sham, sham-operated mice; Aspt or Not, mice treated with Aspt or Not (1.00 mg/kg body weight) at 12 and 50 h; CLP, CLP-operated mice; Aspt or Not + CLP, mice treated with Aspt or Not at 12 and 50 h after CLP surgery. Sample was collected 4 days after the second injection of Aspt or Not.
[a] Each value represents the mean ± SD (n = 10).
[#] $p < 0.01$ as compared to sham.
[*] $p < 0.05$ as compared to CLP.

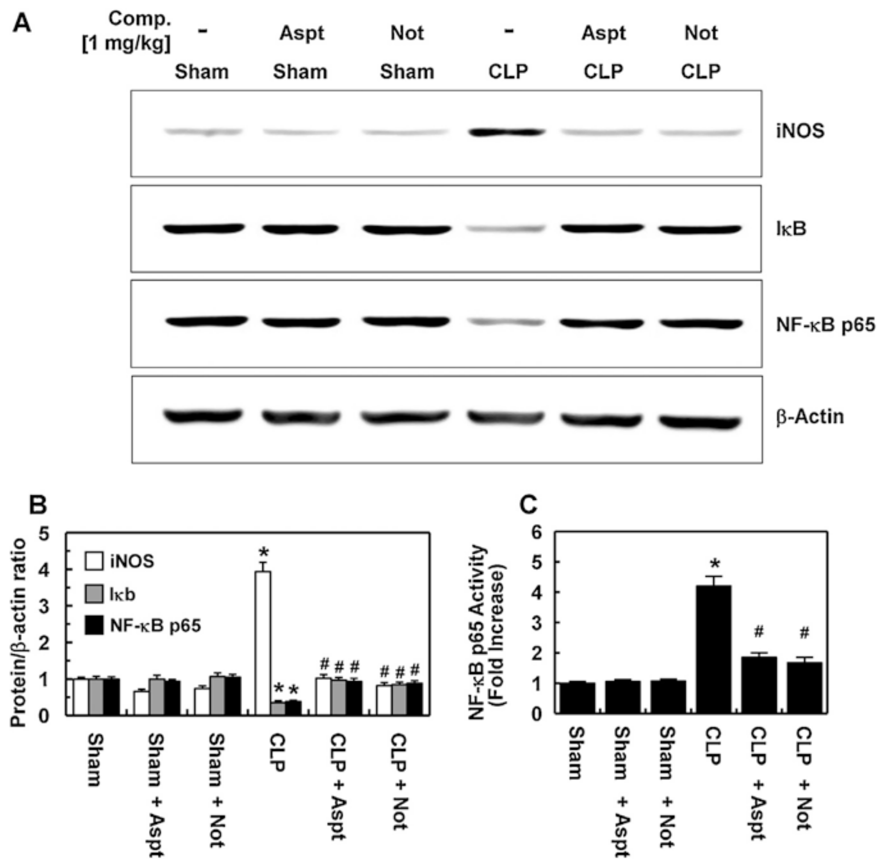

Fig. 1. Effects of Aspt and Not treatment on renal iNOS, IκB, and NF-κB expression in CLP-operated mice. (A) Sham-operated mice; Sham + **Aspt** or Not, mice treated with **Aspt** or Not (1.00 mg/kg body weight) at 12 and 50 h after sham operation. CLP-operated mice; CLP + **Aspt** or Not, mice treated with **Aspt** or Not (1.00 mg/kg body weight) at 12 and 50 h after CLP surgery (from left line). Western blots of iNOS, IκB, NF-κB (cytosol), and β-actin. The image is representative of results obtained from three different experiments. (B) The graphs show the densitometric intensities of iNOS, IκB, and NF-κB normalized to β-actin. n = 3 blots. (C) The activity of NF-κB p65 in the nuclear extracts isolated from kidney endothelial cells was measured by ELISA. The results shown are the mean ± SD from three separate experiments on different days in triplicate wells. * $p < 0.01$ *versus* Sham only or # $p < 0.01$ *versus* CLP only.

with CLP-induced sepsis, we administered two equal doses of Aspt or Not (1.00 mg/kg): one at 12 h after CLP and the other at 50 h after CLP. Aspt and Not treatment increased the rate of survival of mice with sepsis (50% and 55%, respectively) according to a Kaplan-Meier survival analysis ($p < 0.0001$, Fig. 2). Additionally, 0.5% DMSO did not affect mouse mortality. The marked improvement in the survival rate achieved by these treatments suggested that Aspt and Not can be used as therapies for severe vascular inflammatory diseases, such as sepsis and septic shock.

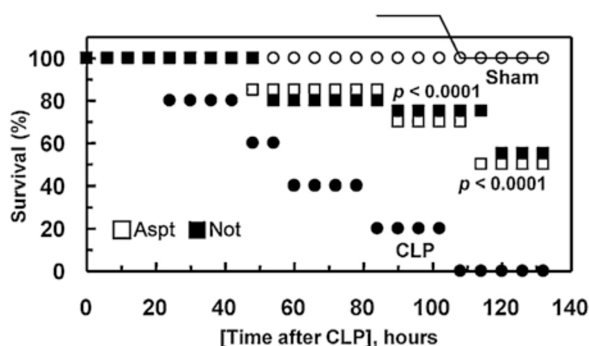

Fig. 2. Effects of Aspt and Not on CLP-induced septic lethality. Male C57BL/6 mice (n = 20) were administered **Aspt** (*iv* □) or Not (*iv* ■) at 1.00 mg/kg at 12 h and 50 h after CLP. Animal survival was monitored every 12 h for 132 h after CLP. Control CLP mice (●) and sham-operated mice (○) were administered sterile saline (n = 20). Kaplan-Meier survival analysis was used to determine the overall survival rates *versus* CLP treated mice.

Discussion

Studies of human sepsis are limited by the complexity of the pathological processes, heterogeneity of the affected population, lack of well-established diagnostic markers, and methodological and ethical restrictions [19]. Thus, animal models of sepsis have been developed which are both affordable and valuable research tools [20]. Several experimental sepsis models reproduce the range of clinical changes and alterations in laboratory-testable parameters. Infection-based sepsis models vary with regards to the experimental species involved, method of infection, and type of microorganism used. Infection methods are classified as non-surgical (lipopolysaccharide application, LPS model) and surgical (CLP model) [21].

LPS can be introduced intravenously, intraperitoneally, or intratracheally to establish a simple sepsis model. However, this model does not accurately reproduce the full range of characteristics of clinical sepsis. For example, cardiovascular changes in the animals are not consistent with those observed in humans. Furthermore, cytokine responses in the animals appear sooner and are stronger than those in humans; however, they are of shorter duration [22,23]. Overall, the clinical course and development of disease in LPS models is much more dynamic than that observed in humans [22,23]. Although LPS-induced sepsis is an unsuitable

model of human sepsis, it can be used to study the pathophysiological processes in endotoxemia and as a model of endotoxic shock [24,25]. The most widely used sepsis model is based on CLP surgery, which is the most compatible with clinical outcomes [22,26,27]. The immune response in animals with CLP-induced sepsis is similar to that in clinical sepsis. The profiles and dynamics of cytokine production at two immunologically different stages, the pro-inflammatory and compensatory anti-inflammatory phase, in animals were reported to be consistent with those in humans [25,28]. The principal advantage of the CLP-induced sepsis model is the similarity of the development of clinical sepsis, which is characterized by an early hyperdynamic state followed by a pronounced hypodynamic state and hypermetabolism [22,26,27]. However, it is difficult to control the amount of vascular leakage in the CLP mouse model, and thus a wide range of septic manifestations may occur. Furthermore, the intestinal flora of different species is not uniform [25,28]. Although it produces a complex series of pathological symptoms, the CLP procedure is relatively simple and shows consistent results [25,28]. The overall reliability of CLP-induced sepsis support our choice of this model in the present study.

The aim of the present study was to evaluate the potential effects of Aspt and Not, an active compound isolated from the leaves of rooibos (*A. linearis*), on renal damage in mice with acute CLP-induced sepsis. Our data demonstrated that post-surgical treatment with Aspt and Not significantly ameliorated CLP-induced deterioration in renal function. Furthermore, Aspt and Not reduced the levels of TNF-α, IL-6, NO, and MPO which were elevated after CLP; additionally, Aspt and Not treatment reduced elevated iNOS levels after CLP surgery. These ameliorative effects were accompanied by increased activities of antioxidant enzymes and reduced levels of lipid peroxidation products in the renal tissues. Additionally, Aspt and Not inhibited CLP-induced degradation of IκB and prevented translocation of the subunit of NF-κB p65 from the cytosol to the nucleus. Thus, the underlying molecular mechanism of Aspt and Not with respect to their renal protective effects appeared to involve the suppression of inflammatory cytokines (TNF-α, IL-6), upregulation of the activities of antioxidant enzymes, reduction of lipid peroxidation levels, inhibition of IκB degradation, and suppression of NF-κB activation. Therefore, the results of the present study suggest that Aspt and Not have beneficial effects that can be used in therapies to prevent acute renal injury due to sepsis.

We found that excretion of urinary proteins and the levels of plasma BUN and creatinine increased after CLP surgery; these findings are consistent with those of previous reports [29–31]. Our study also showed that changes in kidney function after CLP surgery were ameliorated by Aspt and Not treatment, as significant reductions in BUN, creatinine, and urine protein levels were detected.

NO is an important proinflammatory molecule released during inflammatory responses. In pathological conditions, iNOS is induced and then NO is synthesized, which can affect many parts of the inflammatory cascade [8]. There is substantial evidence that CLP-mediated renal inflammatory damage occurs because of increased iNOS activity and consequent abnormal NO levels [4–6]. Our results revealed increases in NO production in the blood and iNOS expression in the kidney tissue of CLP-operated mice; these changes were significantly reduced by treatment with Aspt and Not.

TNF-α and IL-6 are involved in CLP-induced tissue damage and are regarded as major regulators of severe inflammatory diseases such as sepsis or septic shock [3,28]. In this study, we found that Aspt and Not treatment reduced the levels of TNF-α and IL-6. Because increased release of cytokines, particularly TNF-α and IL-6, appears to be an essential aspect of pathogenesis in the inflammation process, the inhibitory effects of Aspt and Not on CLP-induced TNF-α and IL-6 production may be a crucial step in the anti-inflammatory action of Aspt and Not. The nuclear transcription factor NF-κB amplifies and regulates many genes, including multiple cytokines and iNOS, in response to inflammatory stimuli. When activated by such stimuli, NF-κB dissociates from IκB and translocates to the nucleus, leading to gene transcription [32]. NF-κB is a promising target for treating a variety of diseases because it plays a diverse role in the expression of inflammatory genes. In this study, Aspt and Not blocked CLP-induced activation of NF-κB by inhibiting the degradation of IκB. These observations indicate that interference with NF-κB can explain, as least in part, the inhibitory effects of Aspt and Not on iNOS, TNF-α, and IL-6 levels.

The antioxidant enzymes SOD, CAT, and GSH-Px are considered to be the primary defences against oxidative damage in tissues [33]. Septic conditions were found to impair the balance between free radical scavenging and production by cellular antioxidant systems [34,35]. Our data showed decreased levels of these three enzymes in the kidney tissue of CLP-operated mice; the activities of these enzymes were significantly raised by Aspt and Not treatment. These results indicate the potential therapeutic value of Aspt and Not in oxidative stress-associated kidney diseases. The major lipid peroxidation product, MDA, is a good indicator of oxidative stress; a negative correlation has been reported between the MDA level and activities of endogenous antioxidant enzymes [36–38]. Our data showed that renal MDA levels were increased in CLP-operated mice and that Aspt and Not treatment significantly reduced this increase. As described above, Aspt and Not may promote the activities and levels of SOD, CAT, and GSH-Px in the kidney tissues of CLP mice. Therefore, our data indicate that Aspt and Not could provide renal protection against CLP-induced oxidative injury by inhibiting lipid peroxidation as well as promoting the activities and expression of endogenous antioxidant enzymes.

In conclusion, we observed a renal protective effect by Aspt and Not against CLP-induced kidney injury and septic lethality. The ameliorative effects of Aspt and Not were associated with down-regulation of TNF-α and IL-6 production reduction of iNOS expression and lowering of NO production by blocking the NF-κB pathway. These effects were accompanied by enhanced antioxidant defence and decreased lipid peroxidation in the kidney and plasma *in vivo*. Overall, our results suggest that Aspt and Not should be considered for therapeutic use for treating renal inflammatory damage and sepsis-induced oxidative stress.

Conflicts of interest

None

Competing financial interests

The authors declare no competing financial interests.

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