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Protective effect of baicalein against endotoxic shock in rats in vivo and in vitro

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ABSTRACT

Dried roots of *Scutellaria baicalensis* Georgi (*Huang qin*) are widely used in traditional Chinese medicine. Baicalein is a major bioactive flavonoid component of *H. qin* that shows a wide range of biological activities, including antioxidant and anti-inflammatory actions. We evaluated therapeutic effects and possible mechanisms of action of baicalein on circulatory failure and vascular dysfunction during sepsis induced by lipopolysaccharide (LPS; 10 mg/kg, i.v.) in anesthetized rats. Treatment of the rats with baicalein (20 mg/kg, i.v.) significantly attenuated the deleterious hemodynamic changes of hypotension and tachycardia caused by LPS and significantly inhibited the elevation of plasma tumor necrosis factor α (TNF-α). Baicalein also decreased levels of inducible nitric oxide synthase (iNOS) and the overproduction of NO and superoxide anions caused by LPS. It also increased the survival rate of ICR mice (25–30 g) challenged by LPS (60 mg/kg). Moreover, infiltration of neutrophils into the liver and lungs of rats 6 h after treatment with LPS was also reduced by baicalein. To investigate the mechanism of action of baicalein on sepsis, RAW 264.7 cells were used as a model. Baicalein inhibited iNOS protein production, and suppressed LPS-induced degradation of IκBα, the formation of a nuclear factor kappa B (NF-κB)-DNA complex and NF-κB-dependent reporter gene expression. Thus, the therapeutic effects of baicalein were associated with reductions in TNF-α and superoxide anion levels during sepsis. The inhibitory effects of baicalein on iNOS production may be mediated by inhibition of the activation of NF-κB. Baicalein may thus prove a potential agent against endotoxemia.

1. Introduction

Sepsis is generally considered a systemic inflammatory disorder. It can be defined as a progressive failure of the circulation, characterized clinically by systemic hypotension, hyporeactiveness to vasoconstrictors and subsequent organ perfusion and functional changes followed by multiple organ failure [1]. Septic shock is a serious clinical problem with high mortality [1–4]. According to Bone et al. [1], the progression from septicemia to septic shock involves several steps. First, local inflammation may provoke the release of proinflammatory mediators, such as tumor necrosis factor (TNF) and interleukins, to reduce tissue injury. Then, proinflammatory mediators cause the migration of leukocytes, lymphocytes and platelets to infected areas. Systemic pathological changes include endothelial damage, increased microvascular permeability, platelet

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aggregation, local blood flow reduction and ischemia-reperfusion injury. Excessive inflammatory response may result in tissue injury. Finally, the cumulative inflammatory response leads to multiple organ injury [5–7].

Many pathophysiological cascades of Gram-negative shock are triggered by the outer membrane component of bacterial cell walls, i.e., lipopolysaccharide (LPS). This endotoxin can induce experimental endotoxemia, the use of which has become a valuable and reproducible experimental model for septicemia and which has been widely studied in laboratory animals. Most effects of LPS act via endogenous mediators, such as cytokines [8]. Among these cytokines, tumor necrosis factor-α (TNF-α) seems to be particularly important for endotoxic effects [9], because it has been shown to induce most characteristics of endotoxic shock, to stimulate the production of antibodies against TNF-α, which attenuate lethality and improve hemodynamic functions caused by sepsis or endotoxin [10,11]. Interleukin-10 (IL-10) inhibits LPS-induced production of TNF-α by mononuclear cells in vitro and in vivo [12,13], and reduces morbidity of endotoxemic mice [14]. Induction of IL-10 can therefore be considered as being part of endogenous host-protective mediation during endotoxemia.

The excessive generation of nitric oxide (NO) has been suggested to lead to LPS-induced hypotension, vascular hyporeactivity and death, indicating that the overproduction of NO plays an important role in septic shock [15]. LPS-dependent induction of inducible NO synthase (iNOS) is responsible for the overproduction of NO during circulatory shock [16]. This enzyme is expressed in many types of cells, such as neutrophils, macrophages, endothelial cells, vascular smooth muscle cells, mesangial cells and chondrocytes. The triggers for iNOS include LPS, interferon γ and many kinds of proinflammatory cytokines (e.g., TNF-α) [17,18].

LPS activates macrophages, causing the generation of free radicals, including the superoxide anion ($O_2^{\bullet -}$), hydrogen peroxide (H_2O_2) and the hydroxyl radical ($^{\bullet}OH$), leading to oxidative damage in many tissues, such as the liver [19]. NO can react with $O_2^{\bullet -}$ to form the peroxynitrite anion ($ONOO^-$), which oxidizes sulfhydryl groups and generates $^{\bullet}OH$ [20]. This precipitates circulatory failure, which is associated with a substantial increase in mortality [21]. The inhibition of NOS activity by a selective inhibitor of iNOS activity can attenuate the hepatic, renal and pancreatic dysfunction associated with LPS-induced endotoxemia in rats [22]. In addition, overproduction of NO and NO-dependent free radicals seems to be related to the development of multiple organ failure in cases of endotoxic shock.

Huang qin (*Radix scutellariae*), the dry roots of *Scutellaria baicalensis* Georgi (Lamiaceae), is officially listed in the Chinese Pharmacopoeia and is used traditionally against chest discomfort, nausea, acute dysentery, jaundice and carbuncles. Baicalein (5,6,7-trihydroxy-2-phenyl-4H-1-benzopyran-4-one) is a major bioactive flavone constituent of *H. qin*, and possesses a wide range of biological activities, including antiviral [23], antioxidant [24], anti-inflammatory [25], antithrombotic [26] and anticancer activities [27]. Baicalein also suppresses the LPS-induced production of NO in RAW 264.7 mouse macrophages [28]. However, there is no information on the effects of baicalein on LPS-induced shock.

We studied whether baicalein could protect against vascular dysfunction and injury caused by LPS-induced shock in rats, and investigated its possible mechanisms.

2. Materials and methods

2.1. Animal preparation

Male Wistar-Kyoto rats (220–280 g) were purchased from the National Laboratory Animal Breeding and Research Center of National Science Council, Taiwan. All animals were housed at an ambient temperature of 23 ± 1 °C and humidity of 55 ± 5%. The rats were anesthetized by intraperitoneal injections of urethane (1.2 g/kg) and pentobarbital (5 mg/kg, i.v.). The trachea was cannulated to facilitate respiration. The left femoral artery was cannulated with a polyethylene-50 (PE-50) catheter and connected to a pressure transducer (P231D, Statham, Oxnard, CA, USA) for the measurement of mean arterial pressure (MAP) and heart rate (HR), which were displayed on a Gould model TA5000 polygraph recorder (Gould, Valley View, OH, USA). The left femoral vein was cannulated for the administration of drugs. After the completion of surgery, all cardiovascular parameters were allowed to stabilize for 30–60 min.

2.2. Drug administration

Animals were randomly assigned into three groups: (I) the control group ($n = 6$) treated with the vehicle, dimethylsulfoxide (DMSO; 0.1%, i.v., 0.25–0.3 ml); (II) the LPS group (animal models of endotoxemia, $n = 6$) treated with *Escherichia coli* lipopolysaccharide 3129 at 10 mg/kg (i.v.) [29] and (III) the LPS + baicalein (LPS/baicalein) group ($n = 6$) treated with baicalein (5–30 mg/kg, i.v.) 30 min after LPS administration.

After recording the baseline hemodynamic parameters, animals were injected with vehicle or LPS and were monitored for 6 h. Before (at time 0) and every hour after vehicle or LPS administration, 0.5 ml of blood was withdrawn to measure any changes in TNF-α and nitrate concentrations. All blood withdrawn was immediately replaced by an injection of an equal volume of saline (i.v.) to maintain the animal's blood volume. Blood samples were centrifuged for 5 min at 12,000 × *g*. Plasma samples were stored at −70 °C until analysis. At the end of the 6 h experiment, thoracic aortas of each group were dissected out for the measurement of vascular responses, superoxide analysis and iNOS protein expression assays.

2.3. Organ bath experiments

The blood vessels were cleared of adhering periadventitial fat and cut into sections (3–4 mm long). The segments were mounted in 20 ml organ baths filled with 95% O_2/5% CO_2 oxygenated Kreb's solution (pH 7.4) at 37 °C, consisting of (in mM): NaCl 118, KCl 4.7, $NaHCO_3$ 25, KH_2PO_4 1.2, $MgSO_4$ 1.2, $CaCl_2$ 2.5, glucose 11. Isometric force was measured using Grass FT03 type transducers (Grass instruments, Quincy, MA, USA) and recorded on a MacLab Recording and Analysis System (ADInstrument, Castle Hill, Australia). In the segments, 2 g passive tension was applied, and the preparations

were equilibrated for 60–90 min. The presence of functional endothelium was confirmed when the vessels, which were pre-contracted with norepinephrine (NE, 0.1 μM), by a minimum of 80% relaxation with acetylcholine (ACh, 1 μM). The Kreb's solution in the organ baths was changed every 15 min, two to three times.

After the tested intact segments were proven stable at the baseline tension level, the next steps were performed. Concentration–response curves for NE were performed by adding varying concentrations of NE (10 nM to 10 μM) to the organ bath; any changes in tension were monitored using the force displacement transducer, and recorded on a computer. Kreb's solution was used to wash the preparations twice every 15 min for the next step. Concentration–response curves for ACh were carried out by adding NE (1 μM) to obtain maximum contraction, then adding ACh cumulatively from 10 nM to 10 μM and recording relaxation. The Kreb's solution was changed twice every 15 min for the next step. Concentration–response curves for L-arginine were performed by adding NE (1 μM) to obtain maximum contraction, then adding L-arginine (10 nM to 10 μM) cumulatively and recording relaxation.

2.4. Cytokines detection by ELISA

Blood samples (0.5 ml) were collected at 0, 0.5, 1 and 2 h after the injection of LPS for measurement of the TNF-α level in plasma by an enzyme-linked immunoadsorbent assay (mouse TNF-α ELISA Kit, Genzyme Co., Cambridge, MA, USA), as described [29].

Frozen tissue (lung) samples were weighed and placed in homogenization buffer (4 °C) at a ratio of 100 mg per milliliter of buffer. Buffer contained a protease-inhibitor cocktail including 1 mmol/l phenylmethylsulfonglfluoride (PMSF), 1 mg/l pepstatin A, 1 mg/l aprotinin and 1 mg/l leupeptin in phosphate-buffered saline solution, pH 7.2, containing 5 g/l Triton X-100. Samples were homogenized and centrifuged at 18,000 rpm, 4 °C. Tissue supernatants were analyzed for TNF-α and IL-10 using ELISA kit (R&D Systems, Inc., Minneapolis, MN) according to the manufacturer's instructions.

2.5. Plasma nitrite/nitrate determination

Aliquots of 30 μl thawed plasma were deproteinated with 100 μl 95% alcohol for 30 min (4 °C). Serum samples were then centrifuged for 6 min at 12,000 × *g*. The supernatant (6 μl) was injected into a collection chamber containing 5% VCl_3. In this strong reducing environment, both nitrate and nitrite are converted to NO. A constant stream of helium gas was used to carry the output into a NO analyzer (Sievers 280NOA; Sievers Instruments Inc., Boulder, CO, USA), where the NO reacts with ozone (O_3), resulting in the emission of light. Light emission is proportional to the NO formed. Standard amounts of sodium nitrate were used for calibration (Sigma–Aldrich, St. Louis, MO, USA).

2.6. Histopathological studies

The vital organs, including the lung and liver, were harvested at 8 h for histopathological studies, as previously described [30]. These organ tissues were fixed in buffered formaldehyde (10% in phosphate-buffered saline) for more than 8 h. The fixed organs were dehydrated in graded ethanol and embedded in paraffin (Tissue-processor, Japan). Four-micrometer sections were cut (sliding microtome, Leica Jung SM 2000) and removed paraffin by xylene. Then, the tissue sections were stained with the hematoxylin and eosin reagent for light microscopy. This histologic alteration was quantitatively analyzed as an index of the severity of tissue injury. The index was determined by counting the numbers of polymorphonuclear neutrophil (PMN) in 10 randomly selected high-power fields and by the histologic changes (e.g., interstitial edema and/or congestion) evaluated by a pathologist in a blinded fashion.

2.7. Western blotting

Six hours after the injection of 0.1% DMSO or LPS, the experimental animals were euthanized. The thoracic aortas were obtained and frozen at −80 °C before assay. The tissue was ground in a mortar containing liquid nitrogen. The powdered tissue was then suspended in 1 ml of lysis buffer (50 mM HEPES, 5 mM EDTA, 50 mM NaCl, pH 7.5) containing protease inhibitors (10 μg/ml of aprotinin, 1 mmol/l phenylmethylsulfonylfluoride and 10 μg/ml of leupeptin) and agitated at 4 °C for 1 h. After centrifugation for 30 min at 10,000 × *g* (4 °C), the protein concentration in the supernatant was determined using a BCA protein assay kit (Pierce, Rockford, IL, USA).

Samples containing equal amounts of protein were loaded onto 10% sodium dodecyl sulfate-polyacrylamide gels, subjected to electrophoresis, and subsequently blotted onto nitrocellulose membrane (Millipore, Bedford, USA). Membranes were blocked with Tris-buffered saline buffer (TBS), pH 7.4, containing 0.1% Tween-20 and 5% skim milk, and then incubated overnight at 4 °C with various primary antibodies in TBS containing 0.1% Tween-20. The antibodies included mouse anti-iNOS (1:1000 dilution, Stressgen Biotechnologies Co., Victoria, BC, Canada), anti-IκBα polyclonal antibodies (1:1000 dilution, Cell Signaling Technology, MA, USA), mouse anti-β-actin (1:2000 dilution, Sigma–Aldrich, St. Louis, MO, USA). The membranes were incubated with horseradish peroxidase (HRP)-conjugated secondary antibodies (1:1000 dilutions, Cell Signaling).

The blots were detected with an enhanced chemiluminescence kit (Pierce, Rockford, IL, USA), and the membrane exposed to X-ray film (Kodak, Rochester, NY, USA) for 5 min. The density of the respective bands was quantified by densitometric scanning of the blots using Image-Pro software (Media Cybemetrics, Inc.).

2.8. Aortic superoxide anion detection by chemiluminescence

Superoxide anions were detected as described [31]. The thoracic aorta was cut into segments (3–4 mm long) and incubated in 95% O_2/5% CO_2 oxygenated modified Kreb's/HEPES solution (37 °C) for 30 min. Then the aorta sections were put into a 96-well plate in which every well was filled with 200 μl modified Kreb's/HEPES solution, and placed in a luminescence measurement system (Microplate Luminometer LB 96 V, Berthold,

Germany). This performed autoinjection of 250 μM lucigenin (final volume of 250 μl) into the vessels for interacting with superoxide. Counts were obtained at 15 min intervals at room temperature.

After recording was complete, the vessel segment was dried in a 95 °C oven for 24 h. The results were expressed as relative units of luminescence (RUL) per 15 min per milligram dry weight vessel (i.e., RUL/15 min per milligram).

2.9. Survival rate

The choice of species for studies of sepsis is dictated by many factors, among which are local availability, the familiarity of the laboratory with the peculiarities of a particular species and cost. Being inexpensive to purchase and maintain, small mammals are desirable for many studies, particularly when a large number of conditions (e.g., graded doses of an investigation therapeutic agent) are being evaluated. Studies using survival as a primary end point typically require fairly large sample sizes. Accordingly, smaller (less costly) species, such as mice, are commonly used for survival studies [32].

ICR strain mice (25–30 g) were purchased from the National Laboratory Animal Breeding and Research Center of National Science Council, Taiwan. All animals were housed at an ambient temperature of 23 ± 1 °C and humidity of 55 ± 5%. Animals ($n = 36$) were divided into three groups: group 1, vehicle (0.1% DMSO) was injected only; group 2, LPS at a lethal dose of 60 mg/kg was injected [32]; group 3, baicalein (20 mg/kg, i.p.) was injected at 2 and 6 h after LPS injection. Baicalein was administered twice to increase bioavailability and efficacy. Survival of the mice was monitored for 24 h.

2.10. Cell culture

Mouse macrophage RAW 264.7 cells were obtained from the American Type Culture Collection (Manassas, VA). Cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Gibco Life Technologies, Grand Island, NY, USA) with 10% heat-inactivated fetal bovine serum (FBS, Gibco Life Technologies), 100 U/ml of penicillin and 100 μg/ml streptomycin, and maintained at 37 °C in a humidified incubator under 5% CO_2 in air. Various concentrations of baicalein dissolved in DMSO were added together with LPS (2 μg/ml). The final DMSO concentration in the cells was ≤0.1%.

2.11. Cell viability

Macrophage viability was analyzed using a variation of the colorimetric 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, which is based on the ability of live cells to reduce MTT to a blue formazan product [33]. RAW 264.7 cells were cultured in 24-well plates. After the cells reached 80% confluence, they were treated with different concentrations of baicalein, 0.1% DMSO and LPS (2 μg/ml) for 24 h. At the end of treatment, 1 ml of DMEM with 0.5 mg/ml MTT was added into each well to react for 2–3 h. Finally, 1 ml of DMSO was added to each well to lyse the cells; absorbance at 550 nm wavelength was recorded using an ELISA reader (ELx 800, Bio-Tek Instrument, Winooski, VA, USA).

2.12. Electrophoretic mobility shift assays

The electrophoretic mobility shift assay (EMSA) uses a non-radioactive (biotin label) gel shift assay to investigate protein–DNA interactions. Nuclear extracts of the RAW 264.7 cells were prepared using a nuclear extraction kit (Panomics, Inc., Redwood City, CA, USA) according to the manufacturer's protocol. Protein concentrations were determined using the BCA protein assay kit (Pierce). The EMSA was performed using a commercially available kit (Panomics). Briefly, the double-stranded 5′-biotin-labeled NFκB oligonucleotide probe (consensus sequence 5′-AGTTGAGGGGACTTTCCCAGGC-3′; Panomics) was incubated with the nuclear extract in 5× binding buffer, poly d(I–C), for 30 min at 15–20 °C. Following addition of 1 μl loading dye, the mixture was resolved by gel electrophoresis (6.0% polyacrylamide) at 120 V for 55 min. After electrophoresis, the DNA–protein complex was transferred to a nylon membrane (Millipore) at 300 mA for 45 min, and then cross-linked for 3 min with a UV cross-linker. The membrane-bound DNA–protein complex was treated with a streptavidin–HRP conjugate according to the manufacturer's protocol and exposure of the blot to X-ray film (Kodak).

2.13. Transient transfection and luciferase assays

RAW 264.7 cells were seeded in 12-well plates. When the cells reached full confluence, the medium was replaced with serum-free DMEM. Then the cells were transfected with a pNFκB-Luc plasmid reporter gene and a pGFP control plasmid (for transfection efficiency) using LipofectAMINE™ reagent (Invitrogen life technologies). After 24 h of transfection, the medium was replaced with serum-free DMEM, and the cells were incubated with LPS (2 μg/ml) in the presence or absence of different concentrations of baicalein for 24 h. Each well was washed twice with phosphate-buffered saline (PBS, pH 7.4) and harvested in 100 μl of passive lysis buffer (Promega).

Luciferase activity was determined using a luciferase reporter assay kit (Promega) with 20 μl of cell lysates used in each assay.

2.14. Statistical analysis

Data are expressed as means ± S.E.s. One-way ANOVA was performed for the statistical analysis of data; when group comparisons showed a significant difference, the Student–Newman Keuls test was used. $P < 0.05$ was accepted as statistically significant. Comparisons among mortality rates of the groups were made using Fisher's exact test.

3. Results

3.1. Effects of baicalein on mean arterial pressure and heart rate in rats with endotoxemia

To determine a suitable dose of baicalein, a dose–response study was carried out, and the results are shown in Fig. 1A. In the control group, the mean arterial pressure (MAP) was

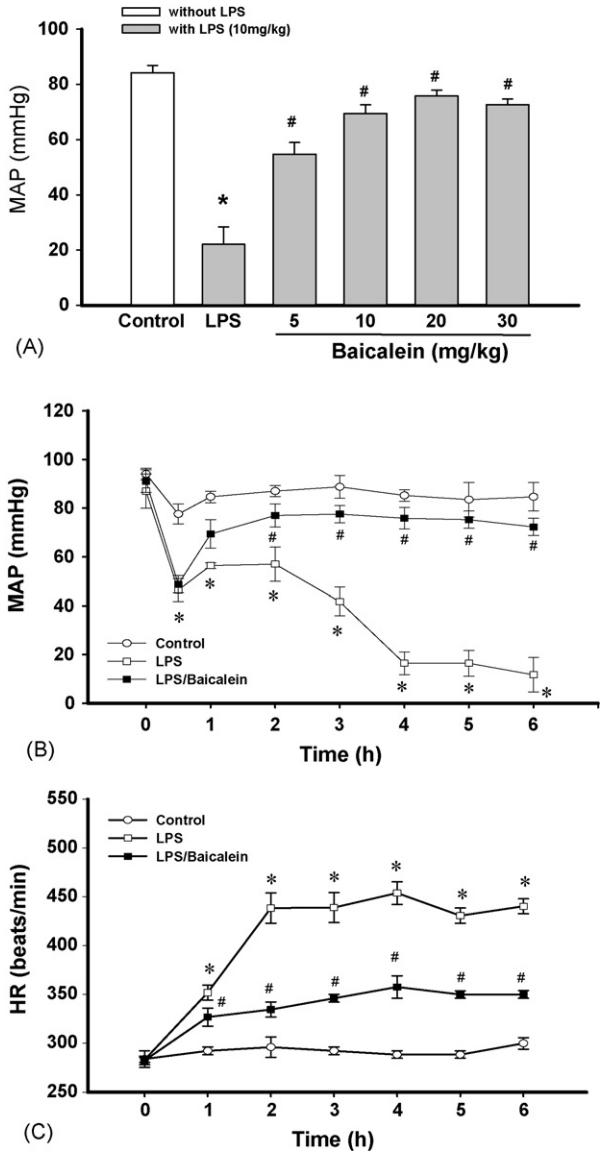

Fig. 1 – Effects of treatment with baicalein (5–30 mg/kg, i.v.) on the mean arterial pressure (MAP) (A) of rats treated with LPS (10 mg/kg, i.v.) for 6 h. The effects of baicalein (20 mg/kg, i.v.) on the time course of changes in MAP (B) and heart rate (HR) (C) after LPS administration for 6 h. Data are shown as means ± S.E.s ($n = 6$). $^{*}P < 0.05$: LPS vs. control, $^{\#}P < 0.05$: LPS/baicalein vs. LPS.

84 ± 3 mmHg. After 6 h of administration of LPS (10 mg/kg), MAP reduced to 22 ± 5 mmHg. When we used a series of doses of baicalein (5–30 mg/kg) 30 min after the administration of LPS, MAP was maintained at significantly higher levels at 6 h than in the LPS-treated controls. Baicalein at 20 mg/kg was the optimum dose for a cardiovascular protective effect.

Fig. 1B and C illustrate the time course of changes in MAP and HR. The baseline MAPs of the three groups ranged from 87 ± 7 to 94 ± 2 mmHg and did not show significant differences between groups. LPS caused a rapid decrease in MAP, from 88 ± 4 to 46 ± 5 mmHg within 30 min; the MAP gradually returned to 56 ± 7 mmHg at 2 h, and finally reached 12 ± 7 mmHg at the end of the experiment (6 h). The MAP of animals treated with baicalein (20 mg/kg) at 2–6 h after LPS administration was significantly greater than that of the LPS group (Fig. 1B).

The mean baseline values of HR in the three groups ranged from 284 ± 8 to 286 ± 7 beats/min, and there were no significant differences between groups. In the LPS group, HR progressively increased and stayed at a significantly high level. However, treatment with baicalein (20 mg/kg) significantly reduced HR at 1 h after LPS administration, and prevented LPS-induced tachycardia (Fig. 1C).

3.2. *Effects of baicalein on NE-induced vasoconstriction, ACh- and L-arginine-induced vasorelaxation in vitro*

To test vascular reactivity ex vivo, aortas were isolated after 6 h either from LPS or baicalein groups, and the vascular reactivity was examined as described under experimental procedures. In vascular rings obtained from LPS group, there was a significant reduction of the concentration–response contractions by NE compared with those obtained from control group. Baicalein posttreatment significantly attenuated the hyporeactivity observed in LPS group (Fig. 2A). Meanwhile, the ACh-induced endothelium-dependent relaxation of pre-constricted aortic rings from LPS group significantly reduced. Baicalein significantly attenuated the impairments of endothelium-dependent relaxation of rings insulted by LPS (Fig. 2B). To confirm that the diminished contraction to NE in aortas from LPS group was due to the induction of iNOS in the vessel, aorta was contracted with NE, and when maximum contraction was reached, L-arginine, a subtract of NOS, was added to bath cumulatively. Supplying high levels of L-arginine can elicit the vasorelaxation caused by iNOS-dependent NO release [34]. The L-arginine-induced vasorelaxation of rings from LPS group was significantly enhanced and it was partially recovered by baicalein (Fig. 2C).

3.3. *Effects of baicalein on cytokines levels in plasma and lung*

The basal plasma levels of TNF-α were not significantly different between the three experimental groups. At 30 min after injection of LPS, the plasma level of TNF-α elevated dramatically, reached a maximum at 1 h, and declined gradually thereafter. Plasma TNF-α levels of the LPS group were significantly higher than controls at 30 min, 1 and 2 h. Treatment with baicalein (20 mg/kg) significantly decreased the LPS-induced increase of plasma TNF-α level at 2 h compared with the LPS group (Fig. 3A).

Lung TNF-α content became significantly higher 2 h after administration of LPS as compared to the control animals (43.47 ± 0.047 pg/mg protein versus 2.75 ± 0.125 pg/mg protein, $P < 0.05$), while baicalein significantly inhibited the LPS-induced increase of TNF-α (27.95 ± 2.56 pg/mg protein, $P < 0.05$) (Fig. 3B).

Lung IL-10 content became significantly higher 2 h after administration of LPS as compared to the control animals (4.71 ± 0.89 pg/mg protein versus 1.05 ± 0.26 pg/mg protein, $P < 0.05$), while baicalein significantly inhibited the

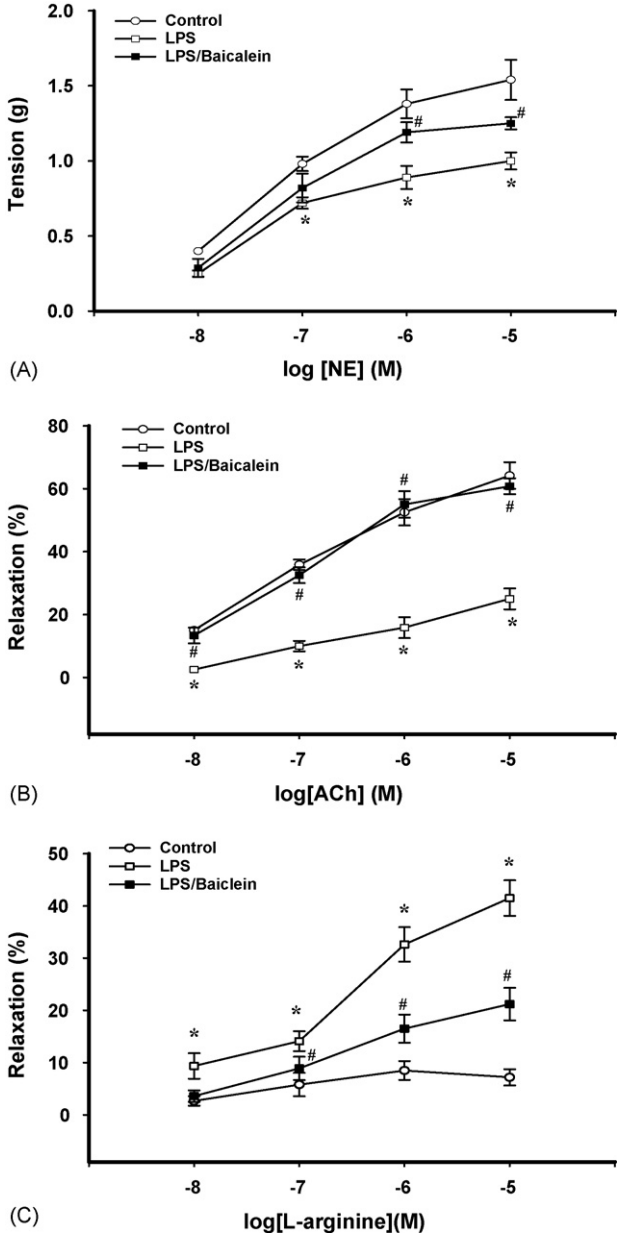

Fig. 2 – Effects of treatment with baicalein (20 mg/kg) on the concentration–response curves of norepinephrine (NE) (A), acetylcholine (ACh) (B) and L-arginine (C) in aortic rings from LPS-treated rats. Data are shown as means ± S.E.s ($n = 6$). $^{*}P < 0.05$: LPS vs. control, $^{\#}P < 0.05$: LPS/baicalein vs. LPS.

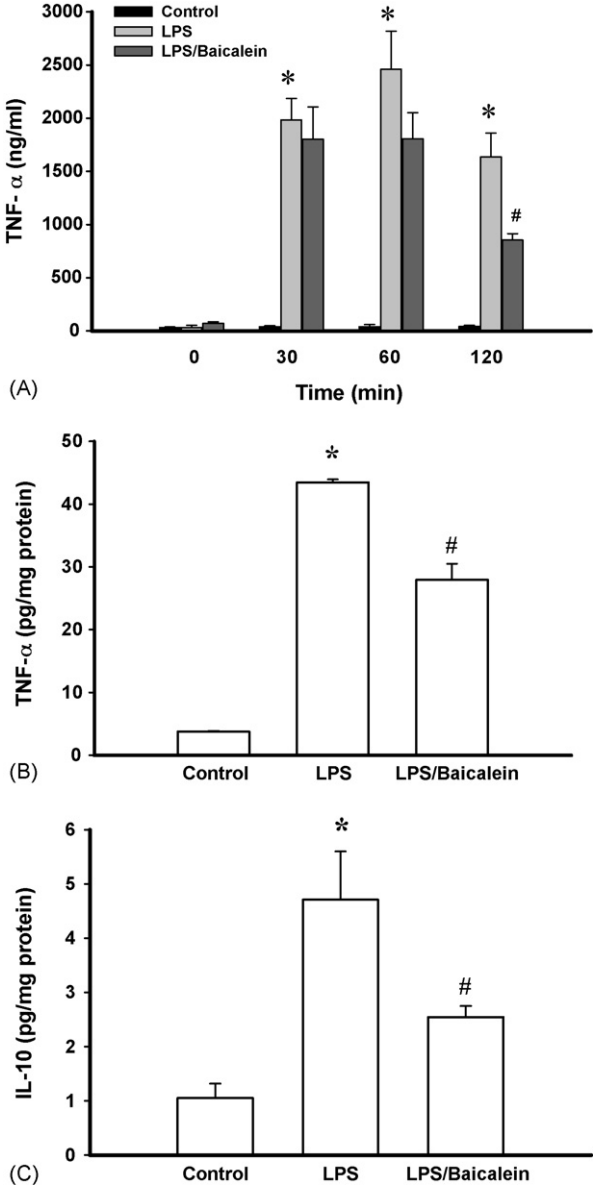

Fig. 3 – Effects of treatment with baicalein at 20 mg/kg on plasma TNF-α levels (A), TNF-α levels (B) and IL-10 levels in lung tissues (C) from rats treated with LPS for 6 h. Data are shown as means ± S.E.s ($n = 6$). $^{*}P < 0.05$: LPS vs. control, $^{\#}P < 0.05$: LPS/baicalein vs. LPS.

LPS-induced increase of IL-10 (2.54 ± 0.21 pg/mg protein, $P < 0.05$) (Fig. 3C).

3.4. *Effects of baicalein on superoxide anion formation*

The contents of superoxide anions in thoracic aorta tissues after 6 h LPS treatment were significantly higher than in control aorta (812.5 ± 87.5 RUL versus 218.75 ± 12.5 RUL, $P < 0.05$). Treatment with baicalein (20 mg/kg) significantly decreased contents compared with the LPS group (475 ± 68.75 RUL versus 812.5 ± 87.5 RUL, $P < 0.05$) (Fig. 4A).

3.5. *Effects of baicalein on plasma nitrite/nitrate content*

In the LPS group, plasma nitrite/nitrate content at 3–6 h was significantly higher than in the control group ($P < 0.05$). Treatment with baicalein (20 mg/kg) significantly suppressed the increased NO level induced by LPS (Fig. 4B).

3.6. *Effects of baicalein on the expression of iNOS protein in the aorta*

As shown in Fig. 5, iNOS protein expression was low in aorta homogenates obtained from the control rats, whereas a

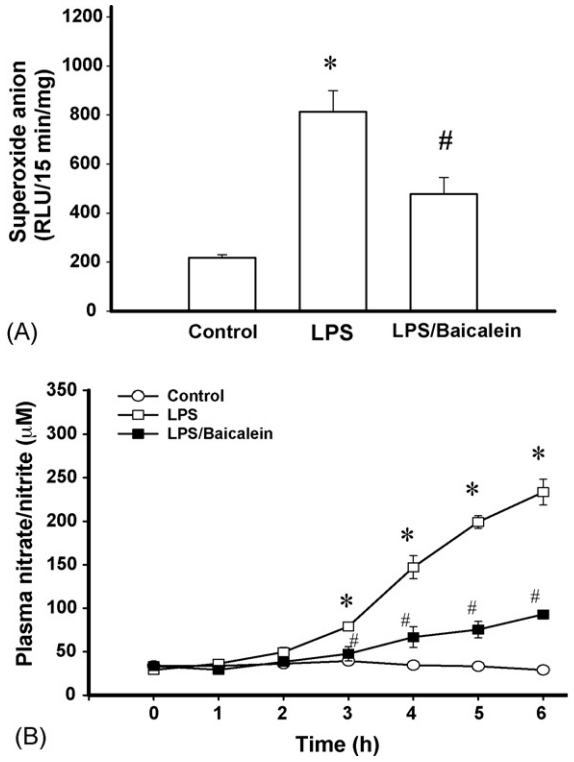

Fig. 4 – Effects of treatment with baicalein at 20 mg/kg on superoxide anion formation in aortic tissues (A) and plasma nitrite/nitrate values (B) from rats treated with LPS for 6 h. Data are shown as means ± S.E.s ($n = 6$). $^{*}P < 0.05$: LPS vs. control, $^{\#}P < 0.05$: LPS/baicalein vs. LPS.

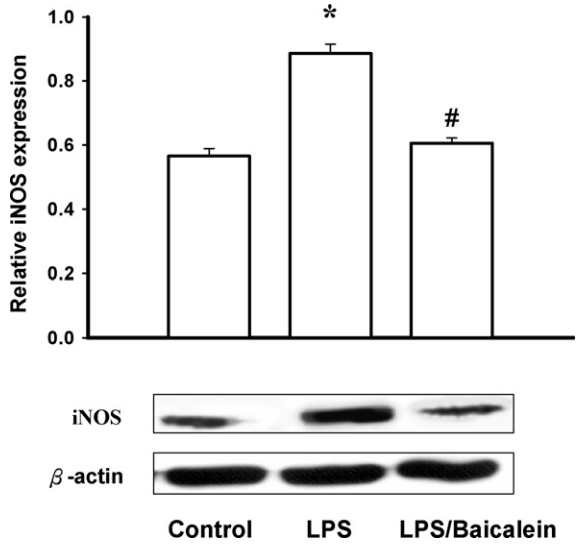

Fig. 5 – Effects of treatment with baicalein at 20 mg/kg on iNOS protein levels in the aorta from rats treated with LPS for 6 h. Depicted is a typical display of iNOS protein production (lower panel) and the statistical analysis of the changes of iNOS protein (upper panel). Data are shown as means ± S.E.s ($n = 6$). $^{*}P < 0.05$: LPS vs. control, $^{\#}P < 0.05$: LPS/baicalein vs. LPS.

significant induction of iNOS protein was observed in rats treated with LPS for 6 h. Treatment of rats with baicalein (20 mg/kg) significantly reduced the induction of iNOS in rats challenged with LPS.

3.7. *PMN infiltration*

In the control group, light microscopy did not show any infiltration or sequestration of PMN in the liver or the lung (Fig. 6A and D). In contrast, 6 h after LPS injection, there was an overt infiltration of PMN in the lung and liver (Fig. 6B and E). In LPS rats treated with baicalein, the PMN infiltrations in livers and lungs were significantly reduced (Fig. 6C and F).

3.8. *Effects of baicalein on the survival of mice treated with LPS*

To assess the in vivo anti-inflammatory effect of baicalein, we examined the effect of baicalein on mortality in mouse model of sepsis. ICR mice have been used in LPS-induced sepsis model and the amount of LPS used in mouse model of sepsis was determined according to previous reports [32].

The 24 h survival rate after administration of a lethal dose (60 mg/kg, i.p.) of LPS to ICR mice was 33%. In contrast, the survival rate of mice treated with baicalein (20 mg/kg) twice, 2 and 6 h after LPS increased to 84% (Fig. 7). Thus, baicalein significantly increased the survival rate of mice injected with LPS.

3.9. *Effects of baicalein on cultured cell viability*

To exclude any possible interference on cell viability, the effects of baicalein (1 and 5 μM) were tested on RAW 264.7 cells. The survival of cells was not influenced by LPS administration at 2 μg/ml. Cotreatment with baicalein and LPS for 24 h did not affect cell viability (Fig. 8).

3.10. *Effects of baicalein on iNOS expression in RAW 264.7 cells*

The expression of iNOS in RAW 264.7 cells was undetectable in control conditions. In contrast, it increased dramatically after treatment with LPS for 24 h. However, when cells were cotreated with baicalein (1 μM) plus LPS, baicalein significantly blunted the expression of iNOS compared with LPS alone (Fig. 9).

3.11. *Effects of baicalein on LPS-induced IκBα degradation in RAW 264.7 cells*

It has been reported that the degradation of IκBα occurs 4 min after the treatment of LPS in RAW cells and this effect persists for about 4 h [35]. We examined the effects of LPS (2 μg/ml) on IκBα degradation in whole cell homogenates 45 min after treatment. Immunoblotting analyses showed that the levels of IκBα in cultures treated with LPS alone and with LPS plus baicalein (5 μM) were 10% and 84%, respectively, when compared with untreated cultures (Fig. 10A).

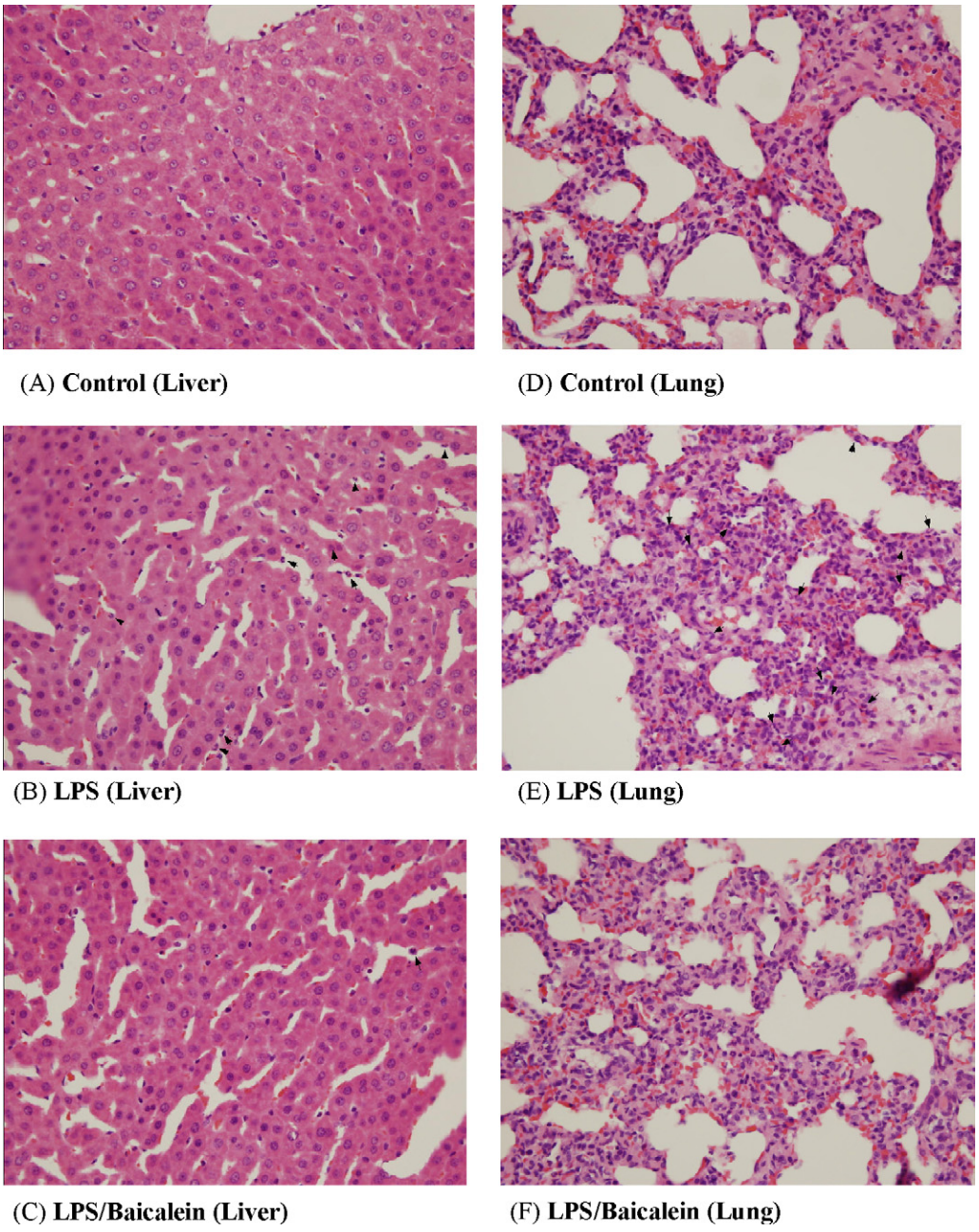

Fig. 6 – Histopathological studies by light microscope showing morphologically normal liver and lung tissues from rat in the control group (A and D), infiltration of PMN (arrows) in liver and lung from LPS-treated group (B and E) and only minimal infiltration by PMN (arrows) in liver and lung from LPS-treated rats treated with baicalein (C and F). Tissue sections were stained with hematoxylin and eosin and view by light microscopy (400×).

3.12. *Effects of baicalein on LPS-induced NF-κB activation in RAW 264.7 cells*

Electrophoretic mobility shift assays (Fig. 10B) confirmed that LPS increases NF-κB nuclear translocation. Densitometric analysis revealed that treatment with LPS for 1 h resulted in a two-fold increase in the amount of NF-κB in the nuclear extracts. Treatment with baicalein (1 and 5 μM) blunted the nuclear NF-κB levels challenged by LPS treatment.

Reporter gene analyses were carried out using a luciferase plasmid containing the NF-κB promoter to test whether the inhibition of iNOS induction by baicalein was associated with the suppression of NF-κB activation. Treatment with LPS increased the NF-κB reporter activity by about 1.5-fold (Fig. 10C). Baicalein (1 and 5 μM) cotreatment of the cells significantly blocked the NF-κB reporter activity induced by LPS.

4. Discussion

In the present study, we demonstrated that post-treatment with baicalein possesses the therapeutic effect on LPS-induced

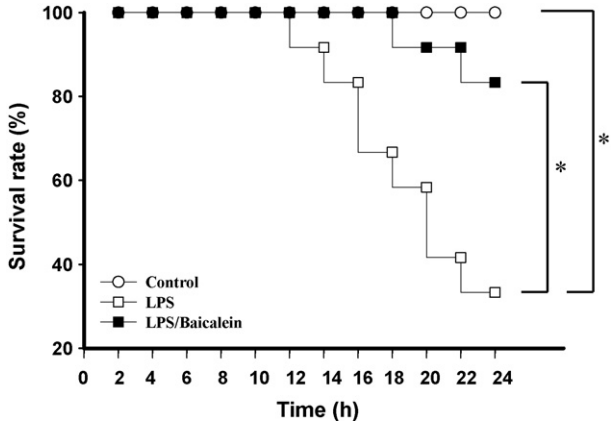

Fig. 7 – Effects of baicalein on the survival rate of LPS-treated mice. Each group consisted of 12 animals. Vehicle (DMSO) was injected into the control group. LPS (60 mg/kg) was injected (i.p.) into the LPS-treated group. Baicalein (20 mg/kg, i.p.) was injected at 2 and 6 h after LPS injection in LPS/baicalein group. $^{*}P < 0.05$ control and LPS/baicalein vs. LPS group.

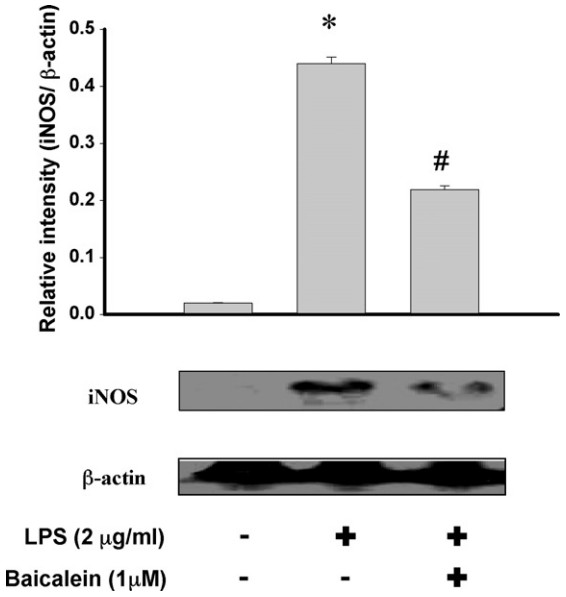

Fig. 9 – Effects of baicalein on iNOS protein expression in RAW 264.7 cells activated by LPS. Protein expression was analyzed by Western blotting. Depicted are a typical display of iNOS protein expression (lower panel) and the statistical analysis of the changes of iNOS protein (upper panel). Data are shown as means ± S.E.s ($n = 6$). $^{*}P < 0.05$: LPS vs. control, $^{\#}P < 0.05$: LPS/baicalein vs. LPS.

septic shock in rats. Baicalein improved circulatory function evidenced by preventing hypotension as well as bradycardia, and preserving vascular contracture ability as well as endothelial function in late stage. It also ameliorated the increase in hepatic TNF-α after LPS treatment and attenuated the infiltration of PMN in liver and lung result from LPS. Additionally, we clearly demonstrated that baicalein inhibited iNOS protein expression and NF-κB activation in LPS-stimulated macrophages. This is the first investigation to demonstrate the beneficial effects of baicalein on the circulatory failure in sepsis, in vivo.

The root of *S. baicalensis* contains a number of flavone derivatives, such as baicalin, baicalein and wogonin [36]. It was found that flavonoid was a novel class of natural free radical scavenger, besides α-tocopherol, ascorbic acid and β-carotene, and most of the flavonoids were more potent antioxidants than α-tocopherol [37]. Several lines of evidence indicated that baicalein is the most effective component of *S. baicalensis* in antioxidant and/or free radical scavenging properties [38]. Recently, some work has been done on the protective effect of flavonoids on both cardiomyocytes and neurons [39,40]. These studies showed that the high effectiveness of baicalein on scavenging free radicals could protect neuronal cells from oxidative stress. Baicalein has also been reported to exhibit potent anti-inflammatory effect in vitro, although the detail mechanisms do not clearly [28]. Therefore, we speculate that baicalein, with the anti-inflammatory and antioxidant properties, may exert the capacity to block the deleterious events of endotoxin LPS-induced sepsis.

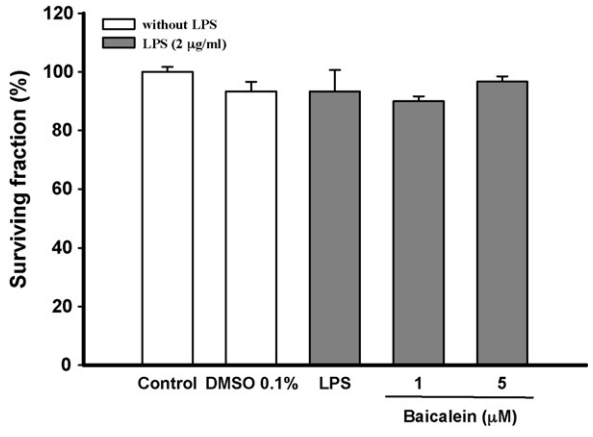

Fig. 8 – Effects of baicalein and LPS on macrophage viability. RAW 264.7 cells were exposed to LPS in the absence or presence of baicalein (1 and 5 μM) for 24 h. Cell viability was determined by the MTT assay as described in Section 2. Data are shown as means ± S.E.s ($n = 6$).

Sepsis is a systemic response to infection, and septic shock is one of the most common causes of death in intensive care units [41]. The most common cause of sepsis is an exposure to the structural component of a Gram-negative bacterial membrane LPS, and key symptoms include hypotension and vasoplegia, which may lead to multiple organ dysfunction and ultimately death [42]. Bacterial LPS in the bloodstream induces overexpression of various inflammatory mediators, such as interleukin 1β, TNF-α, NO and prostaglandin E_2 (PGE_2), and large amounts of inflammatory mediators produced in the body are thought to contribute to the LPS-induced symptoms of septic shock and mortality [43]. We found that, in this experimental model of septic shock, the increase in plasma levels of NO metabolites and TNF-α by LPS was downregulated by baicalein treatment, suggesting that the increased survival of mice by baicalein treatment in this animal model of sepsis

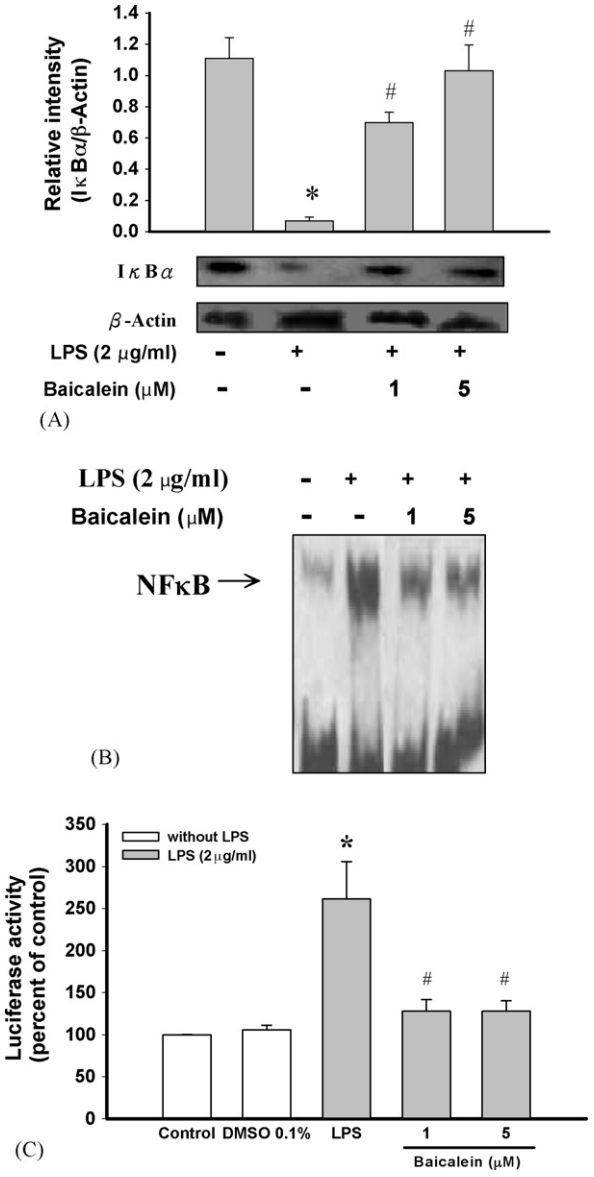

Fig. 10 – Effects of baicalein (1 and 5 μM) on IκBα (A) and NF-κB activity (B) and NF-κB-dependent luciferase reporter activity (C) in RAW 264.7 cells activated by LPS. Cells were transfected with the reporter gene and incubated for 24 h with or without baicalein in the presence of LPS, after which the luciferase activity was determined, as described in Section 2. Data are shown as means ± S.E.s ($n = 6$). $^{*}P < 0.05$: LPS vs. control, $^{\#}P < 0.05$: LPS/baicalein vs. LPS.

might have been mediated by inhibiting the production of NO and TNF-α.

Among inflammatory mediators, the production of NO by iNOS in various systemic vessels after LPS treatment contributes to the hyporeactivity to vasoconstrictors [44–46]. Results as shown in Fig. 2A and B, baicalein improved NE-induced vasocontraction and ACh-induced vasorelaxation, indicating that it can preserve vascular contractile and endothelial-dependent relaxing functions in sepsis. Furthermore, baicalein reduced the L-arginine-induced relaxation after LPS treatment (Fig. 2C), implying that it could suppress the activity of iNOS. This point was further supported by the results in which baicalein markedly attenuated plasma nitrate/nitrite concentration (Fig. 4B) and iNOS protein expression in aortas tissue induced by LPS (Fig. 5).

The large amounts of NO produced in response to bacterial LPS or cytokines play an important role in endotoxemia and inflammatory conditions [47]. Drugs that inhibit NO production by inhibiting iNOS gene expression and enzyme activity have beneficial therapeutic effects on the treatment of sepsis [48]. The inducibility of iNOS by LPS depends primarily on the transcription factor NF-κB [49]. In vitro models, such as macrophage cells or other cell lines are useful materials with a steady high-level production of NO. Previous study had shown that baicalein (25 μM) can inhibit LPS-induced NO generation and iNOS protein expression in macrophage. However, the detail mechanisms do not clearly [28]. To investigate the possible mechanism of action of baicalein effect, we used RAW 264.7 cells. As shown in Fig. 10, cotreatment of RAW 264.7 cells with baicalein interfered with NF-κB DNA binding activity and NF-κB-dependent reporter gene expression, which is thought to be associated with the inhibitory effects of baicalein on iNOS gene expression. As mentioned earlier, ubiquitous degradation of IκBs is key regulatory step in NF-κB activation [50]. Here, we clearly demonstrated that LPS-induced degradation of IκBα was inhibited by baicalein in RAW 264.7 cells. Thus, all observations indicate that baicalein can block the activation of NF-κB, at least in part, by inhibiting the removal of IκBα from the NF-κB/IκBα complex. The detailed molecular mechanism of baicalein on the upstream elements of the IκBα signal transduction pathway needs to be further investigated.

There is well evidence that septic or endotoxic shock is also associated with the generation of ROS [51]. NO may combine with $O_2^{\bullet -}$ to form the more-potent reactive oxygen metabolite, the $ONOO^-$, which decomposes to form $^{\bullet}OH$ [20]. Both $ONOO^-$ and $^{\bullet}OH$ are responsible for cellular lipid peroxidation, protein oxidation and mitochondrial impairment function, which cause further damage to tissues and can induce cell death [19]. Results in this study demonstrated that post-treatment with baicalein significantly suppressed the superoxide anion production in aorta induced by LPS (Fig. 4A). Moreover, LPS induced multiple organ injuries/dysfunctions, which were further evidenced by histologic findings of PMN infiltration in the lung and liver (Fig. 6). Treatment with baicalein not only ameliorated the deterioration of hymodynamic changes (hypotension and bradycardia) but also attenuated liver and lung abnormalities caused by LPS. Thus, the beneficial effects of baicalein on sepsis may be associated with its antioxidant properties.

In response to endotoxaemia, the organism provokes release of pro-inflammatory cytokines (TNF-α) from activated monocytes, macrophages, neutrophils and other immune cells into surrounding tissues, thereby causing tissue damage and organ failure [52]. In response to this pro-inflammatory reaction, the body also produces an anti-inflammatory response [52]. IL-10 participates in this compensatory anti-inflammatory response. In the present study, baicalein ameliorated the increase in hepatic TNF-α after LPS injection, possibly mediated by its anti-inflammatory and anti-oxidant properties. Baicalein also attenuated hepatic IL-10; whether

the inhibitory effects of baicalein are a result of a reduction in TNF-α remains to be determined.

In conclusion, this is the first in vivo experiment to demonstrate the beneficial effects of baicalein on circulatory failure induced by LPS. This protective effect by baicalein in animals with sepsis/septic shock seems to be derived from free radical scavenging activity and antioxidant capacity (e.g., scavenging $O_2^{\bullet -}$ and $NO^{\bullet}$). In addition, the baicalein effect may be mediated partly by inhibition of TNF-α production and inflammatory cell infiltration. The multi-factorial beneficial effects of baicalein may contribute to the higher survival rate. Furthermore, the inhibitory effects of baicalein on iNOS expression may be mediated by suppressing the activation of NF-κB. Thus, baicalein may prove to be a valuable protective agent against septic shock.

Acknowledgements

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