Original Research Article

In vitro and *in vivo* antiseptic activities of caffeoylquinic acid

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Abstract

Purpose: To evaluate the antiseptic effect of caffeoylquinic acid (CA) in in vivo and in vitro models.
Methods: In vivo sepsis was produced in rats via cecal ligation and puncture (CLP) method. Four groups of rats were used: control group, untreated CLP group, and two CA groups treated with caffeoylquinic acid (50 and 100 mg/kg, p.o.) for 30 days before the induction of sepsis. Following the induction of sepsis, histological assessment of lung tissue was carried out using hematoxylin and eosin, and isolectin B4 staining. In addition, in vitro tests were performed on RAW264.7 cells in which inflammation and oxidative stress were induced by lipopolysaccharide (LPS).
Results: Treatment with CA significantly (p < 0.05) enhanced the survival of lung cells, relative to the CLP group. Lung histopathology revealed that pretreatment with CA did not attenuate the increased infiltration of macrophages in the alveoli. Results from in vitro studies showed that CA attenuated LPS-induced nitric oxide (NO) levels, but had no significant effect on the level of LPS-induced pro-inflammatory cytokines in RAW264.7 cells (p < 0.05).
Conclusion: These results reveal that CA attenuates NO and TNF-α levels in LPS-stimulated macrophages, thereby decreasing inflammation-associated sepsis. Thus, CA may have beneficial effects on lung injury as a result of its antioxidant and anti-inflammatory activities.

Keywords: Caffeoylquinic acid, Sepsis, Oxidative stress, Cytokines, Cecalligation, Puncture

INTRODUCTION

Sepsis is an inflammation that occurs due to microbial infection. It is one of the major causes of death [1]. A report has revealed that in USA, septic shock causes the death of more than 75,000 patients every year [2]. Multiple organ dysfunctions occur in several lung disorders such as adult respiratory distress syndrome (ARDS) and acute lung injury (ALI) [3]. The management of ARDS/ALI and treatment of sepsis pose difficult challenges to health care [4]. Studies have shown that LPS promotes sepsis condition in infection caused by gram negative bacteria by stimulating inflammation in macrophages. The inflammation in macrophages is enhanced by production of nitric oxide (NO), tumor necrosis factor α (TNF-α), interleukin 1β (IL-1 β) and IL-6 [5]. It has been reported that the LPS-induced

sepsis in macrophages is attenuated by inducing the expressions of heme-oxygenase-1 (HO- 1) [6]. Thus, HO-1 could be a therapeutic target for the regulation of inflammation.

Caffeoylquinic acid (CA) has been isolated from *Aster tataricus* (Asteraceae), and traditionally used for medical purposes [7]. The parent moieties in CA are caffeic and quinic acid obtained from natural sources [8,9]. Caffeoylquinic acid possesses several pharmacological effects such as anticancer, anti-inflammatory and antioxidant activities, and its anti-inflammatory activity is due to downregulation of the synthesis of inflammatory cytokines [10,12].

The present study investigated the *in vitro* and *in vivo* effects of CA on sepsis.

EXPERIMENTAL

Animals

Male albino Wistar rats (120 - 150 g) were procured from Shangai Animal House, China, and housed under standard conditions. The rats were acclimatized to laboratory conditions for 7 days and allowed *ad libitum* access to normal standard chow and tap water. The protocol for the study was approved by Institutional Animal Ethical Committee of The First Affiliate Hospital of Xi'an Medical University, China (approval no. IAEC/HXMU/2017/11). The study was carried out in line with the guidelines of Association for the Assessment and Accreditation of Laboratory Animal Care International (AAALAC) for experimentation and animal use [13].

Induction of sepsis

The Wistar rats were assigned to four groups (8 rats/group): control group, untreated CLP group, and two CA groups that received CA at separate doses of 50 and 100 mg/kg p.o. for 30 days before the induction of sepsis. The animals were anesthetized and sepsis was induced using the CLP method. Incision was done on the midline of abdomen for laparotomy and sutured thereafter to ligate the exposed cecum. Then, an 18-gauge needle was used to puncture the cecum twice. The cecum was ligated with suture and placed properly in the abdominal region. However, in control group, laparotomy was performed and closed without the ligation of cecum. All of the animals received volume resuscitation with both intravenous and intraperitoneal saline (4 mL/100 g of body weight). Thereafter, they were placed in cages and allowed free access to normal standard chow and water.

Histopathology studies

Histological changes were assessed after 18 h of CLP (as a late stage of sepsis). All the animals were sacrificed by cervical dislocation 18 h after the induction of sepsis. Lung tissues were excised and fixed by embedding in 2 % formaldehyde. The tissue samples were washed with PBS and 30 % sucrose was used for cryoprotection. Tissue sections were sliced in a microtome, fixed onto glass slides, and preserved at 20 $^{\circ}$C after fixing onto glass slides. For each animal, each slide contained six sections collected at 100 mm intervals throughout the entire tissue specimen. The tissue slides were stained with hematoxylin and eosin, and isolectin B4 stain for ease of observation of histological changes. Macrophages of alveoli were determined by lectin binding according to standard methods [14]. Tissue sections were washed for 20 min in PBS buffer containing Triton X-100, $MnCl_2$, $MgCl_2$ and $CaCl_2$. Thereafter, peroxidase-conjugated *Griffonia simplicifolia* isolectin B4 (0.025 mg/mL)-containing buffer was used to bath the tissue sections overnight at a temperature of 4 $^{\circ}$C. The tissue sections were then rinsed with PBS, and incubated with 3,3-diaminobenzidine (DAB) substrate for 5 min for the visualization of sites containing macrophage-bound peroxidase-lectin conjugates. They were thereafter mounted in EntellanNeu after counterstaining with thionine.

In vitro studies

Cell culture

Macrophages (murine RAW264.7) were cultured in Dulbecco's modified Eagle's medium supplemented with 10 % fetal bovine serum, streptomycin, penicillin and $NaHCO_3$ (1.5 g/L). Twenty four-well plates were used to seed the cells which were maintained in a 95 % O_2 and 5 % CO_2 atmosphere at 37 $^{\circ}$C. The cells were treated with LPS after 30 min with different concentrations of CA.

Cell viability assay

Cells (2×10^5/mL) were kept in 24-well plates and incubated at 37 $^{\circ}$C for 24 h. The cell culture was incubated for 6 h with different concentrations of CA (0 - 100 mg/mL), and then incubated with 3-4,5-dimethylthiazole-2-yl-2,5-diphenyl tetrazolium bromide (0.5 mg/mL) for 4 h at 37 $^{\circ}$C. The resultant formazan crystals were dissolved in

DMSO and the absorbance of the solution was read at 570 nm.

Assessment of NO

Griess reagent was used to measure the quantity of nitrite in the cell culture for the estimation of NO. Cells were treated for 30 min with CA prior to LPS treatment, and thereafter with LPS (1 mg / mL) for 6 and 24 h. Griess reagent was added to equal amount of cell culture and the resultant solution was incubated at room temperature for 10 min. The absorbance of the solution was read at 540 nm in an ELISA test reader.

Evaluation of cytokines

Cells were treated for half an hour with CA prior to LPS treatment, and then treated with LPS (1 mg / mL) for 6 and 24 h. The levels of cytokines were assayed using ELISA kits as per the instructions on the kit manual.

RESULTS

Effect of CA on the histopathology of lung tissue

Effect of CA on the histopathology of lung tissues in CLP-induced sepsis is shown in Figure 1a. There was significant decrease in the survival of cells in CLP group, when compared to control group. However pre-treatment with CA significantly enhanced cell survival, relative to the CLP group. In addition, it was observed that the CLP group showed infiltration of interstitial zone, and increased inflammation that caused enhancement of thickness of the alveolar septa (Figure 1b). Pretreatment with CA did not attenuate the increased infiltration of macrophages in the alveoli.

Effect of CA on cell viability

The effect of CA on the viability of cells was assessed by MTT assay as shown in Figure 2. It was observed that treatment of RAW264.7 cells with CA (10 - 100 µg/mL) for 6 and 24 h produced no cytotoxic effects.

Effect of CA on the production of NO

The effect of CA on the production of NO was assessed by estimating the concentration of nitrite in the LPS-treated RAW264.7 cells. It was observed that LPS enhanced the level of nitrite in the cells in the absence of CA. However, treatment with CA significantly decreased the level of nitrite in the LPS-treated cells, when compared to those without CA treatment.
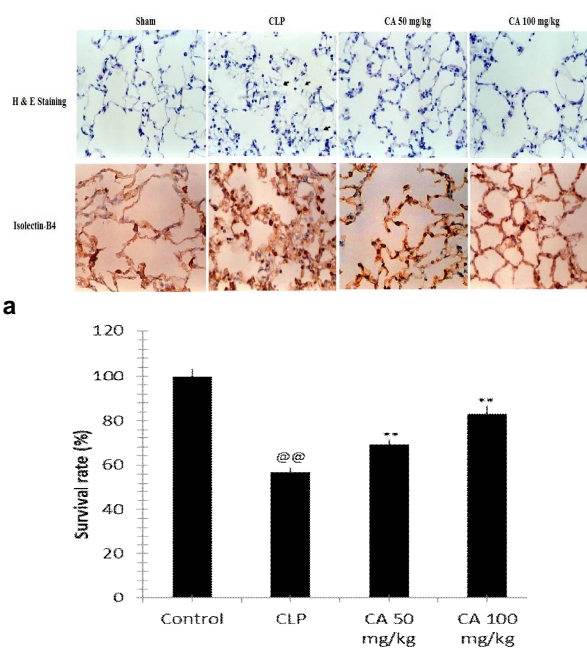

Figure 1: Effect of CA on the histopathology of lung tissues in CLP-induced sepsis. a: histopathology of lung tissues by H & E staining; b: percentage survival. Values are mean ± SD (n = 8); $^{@@}$ $p < 0.01$, compared to control group; ** $p < 0.01$, compared to CLP group
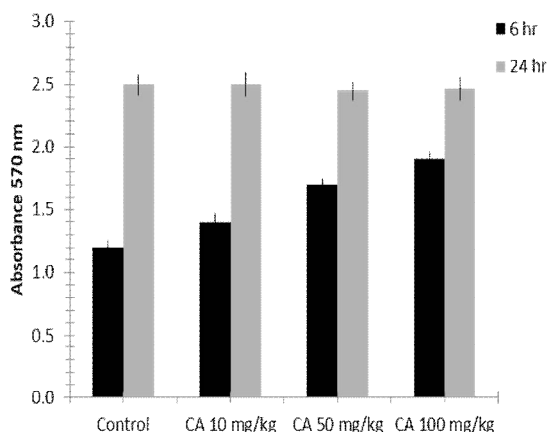

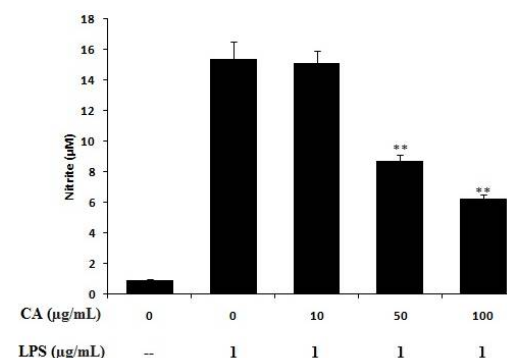**Figure 2:** Effect of CA on the viability of RAW264.7 cells as assessed by MTT assay

Figure 3: Effect of CA on the production of NO in LPS-treated RAW264.7 cells. Values are mean ± SD (n = 6); ** $p < 0.01$, compared to LPS-only group

Effect of CA on the level of cytokines

The effect of CA on the level of cytokines in LPS-treated RAW264.7 cells is shown in Figure 4. Level of cytokines i.e. IL-1β and TNF-α were assessed in LPS-stimulated RAW264.7 cells, with and without CA treatment. There were significant decreases in the levels of pro-inflammatory cytokines after 6 and 24 h of LPS administration in the two groups.
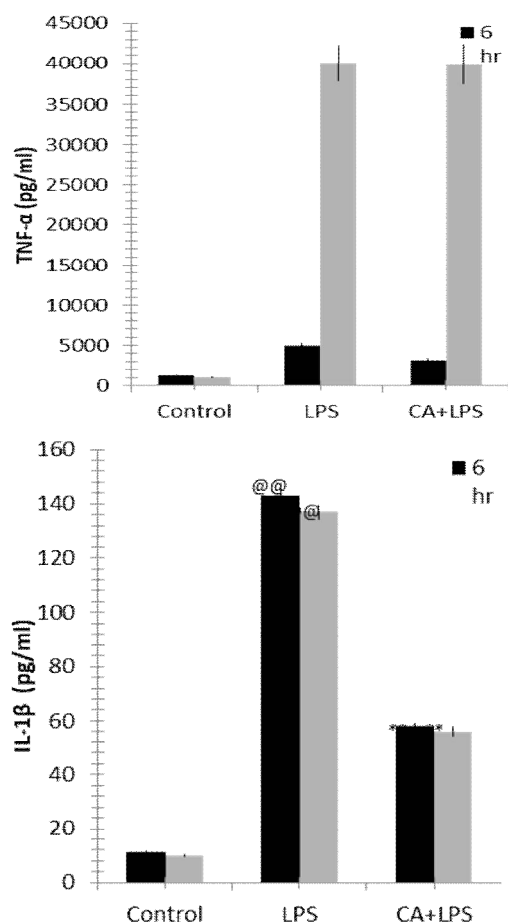

Figure 4: Effect of CA on the levels of cytokines in LPS-treated RAW264.7 cells. Data are expressed as mean ± SD (n = 6)

DISCUSSION

This study evaluated the beneficial effect of CA against sepsis in *in vivo* and *in vitro* models. CLP method was used for the production of sepsis in rats since its pathogenesis resembles that of human sepsis [13]. The study results are also in agreement with those obtained in human sepsis, since the survival rate in CLP-treated group was significantly decreased, when compared to the control group [14]. However, pretreatment with CA significantly enhanced the survival rate of the cells, relative to the CLP group.

The results of the present study suggest that inflammation of alveolar septa was enhanced due to infiltration of interstitial cells in the lung tissue of CLP-induced septic rats. Histopathology of CA-treated group suggested that treatment with CA did not attenuate the infiltration of macrophages in the alveoli. The alveolar macrophages provide defense against antigens in the respiratory system by enhancing the synthesis of pro-inflammatory cytokines and engulfing the pathogens [16]. Thus, the result of this study reveals that CA produces beneficial effect against sepsis related to its immune responses.

In vitro model studies were also performed for the assessment of mechanism involved in the CA-mediated protection against inflammation in sepsis. It was observed that treatment with CA had no cytotoxic effects at several concentrations (from 10 - 100 µg/mL). Studies have revealed that altered levels of TNF-α attenuate lung injury by reducing the expressions of iNOS [17]. Clinically, it has been proven that septic shock gets worse in the patient if TNF receptor is blocked [18]. The result of this study reveals that treatment of RAW264.7 cells with CA in presence of LPS did not alter the level of TNF-α and thereby produces its anti-inflammatory property in septic shock condition.

CONCLUSION

The findings of this study indicate that treatment with CA attenuates lung injury by reducing oxidative stress and inflammatory mediators. Thus, CA possesses the potential for clinical application in the management of sepsis.

DECLARATIONS

Conflict of interest

No conflict of interest is associated with this study.

Authors' contribution

We declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by the authors. Zhaojie Li

designed the protocol, supervise the work and write the manuscript. Xin Shang, Mingzhi Long, Kai Li and Yanfeng Liu helps in the statistical analysis and histopathology study.

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