Chlorogenic acid alleviates sepsis-induced endothelial barrier dysfunction and the underlying mechanism

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Research

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Abstract

Background

Chlorogenic acid (CGA) has been shown to improve inflammatory cytokines in patients with ulcerative colitis. Vascular endothelial barrier dysfunction is closely related to sepsis. We hypothesize that CGA plays an important role in treating sepsis by protecting the mesenteric endothelial barrier.

Methods

Severe sepsis was established by cecal ligation and puncture (CLP) in SD rats. Immediately after the induction of sepsis, CGA (20 mg/kg) was administered intravenously, and the effects of CGA on mesenteric vascular leakage, hemodynamics, liver and kidney blood flow, and the ultrastructure of the mesenteric endothelial cells were determined. The transendothelial electrical resistance and the Transwell permeability assays were used for observing endothelial barrier function in vascular endothelial cells (VECs). ZO-1 and VE-cadherin expression were observed by immunohistochemistry and western blotting.

Results

FITC-BSA leakage from mesenteric micro-vessels was significantly increased after septic shock, which was significantly improved by CGA. Liver and kidney blood flow were increased by 41.2% and 38.4%, respectively, after CGA administration compared with the septic shock group. Hemodynamic status was significantly decreased after septic shock, and significantly improved by CGA. The 72-h survival rate of septic shock rats in the CGA group (50%) was significantly higher than the septic shock group (6.25%). CGA improved the tight junction opening after septic shock and also significantly up-regulated the expression of ZO-1 and VE-cadherin. CGA also protected endothelial hyperpermeability against lipopolysaccharide-stimulated VEC injury by increasing the expression of ZO-1 and VE-cadherin *in vitro*.

Conclusion

CGA was beneficial for endothelial barrier function in rats with septic shock, which is the major contribution to CGA with respecting to improving hemodynamic status and organ perfusion. The underlying mechanism involved CGA up-regulation of ZO-1 and VE-cadherin.

Background

Sepsis is a life-threatening organ dysfunction caused by a dysregulated host response to infections that can affect multiple organs in the body. Sepsis is a major cause of worldwide mortality,¹ which is most often attributed to multi-organ dysfunction syndrome (MODS). Severe vascular endothelial barrier failure is associated with sepsis. Hyperpermeability of vascular endothelial cells (VECs) is identified as the key

factor in the progression to MODS in septic patients.² Thus, recovery of endothelial barrier integrity is essential for maintaining vascular homeostasis and tissue fluid balance for treatment of severe sepsis and related multiple organ dysfunction.

Sepsis-induced dysfunction of VECs could include barrier dysfunction, inflammation and coagulation disorders. These abnormalities may lead to severe hypotension, tissue edema, leukocyte adhesion, and hemostasis imbalance and vasomotor tone alteration.^{3,4} Various options are available for treating VEC injury, such as inhibition of inflammatory signaling pathways, tissue factor natural inhibitors, and cellular therapy, but the clinical efficacy is not fully established.^{5,6} In our study, we reported that CGA could improve sepsis-induced vascular endothelial permeability.

Chlorogenic acid (CGA) is a natural polyphenol with antioxidant, antiviral, antimicrobial, free radical scavenger, and anti-inflammatory activity.⁷ It has been demonstrated that CGA has the ability to suppress oxidative stress. For example, Kim et al⁸ found that CGA inhibits H₂O₂-induced apoptotic primary cortical neurons death via up-regulation of the anti-apoptotic protein, Bcl-2. Previous studies have demonstrated that CGA has a therapeutic effect on ulcerative colitis, cardiovascular disease, and diabetes;⁹ however, it is unclear whether or not CGA can protect vascular endothelial barrier in patients with septic shock. Recent studies have shown that CGA may ameliorate the high mobility group box 1 (HMGB1) and enhance host defense in sepsis.¹⁰ Moreover, some studies have shown that endothelial barrier disruption participates in the occurrence of vascular hyperpermeability following septic shock,¹¹ and the peripheral membrane proteins (ZO-1 and VE-cadherin) play an important role in regulation of the endothelial barrier function.^{12,13} Previous studies have also shown that CGA treatment has a preventive role in blood retinal barrier (BRB) breakdown in patients with diabetic retinopathy by improving the expression of ZO-1.¹⁴ Accordingly, we hypothesized that CGA can improve vascular leakage by regulating ZO-1 and VE-cadherin proteins.

Therefore, the CGA effects on mesenteric vascular leakage, hemodynamics, liver and kidneys perfusion, the ultrastructure of the mesenteric endothelial cells and its relationship with ZO-1 and VE-cadherin proteins were investigated in a rat model of cecal ligation and puncture (CLP)-induced septic shock.¹⁵

Materials And Methods

Animal Management

This study was approved by the Research Council and Animal Care and Use Committee of Research Institute of Surgery (Army Medical University), and was in accordance with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication, 8th edition, 2011).

Preparation of septic shock model in rats

Healthy adult Sprague–Dawley (SD) rats, 12–14 weeks old for both sexes and weighing 180–220 g, were anesthetized with sodium pentobarbital (45 mg/kg ip; Sigma, St. Louis, MO, USA). One hundred and twenty rats were randomly divided into three groups (40 per group), and designated as the sham, sepsis, and CGA groups. The sepsis rat model was induced by cecal ligation and puncture (CLP), as described previously.¹⁶ In brief, an abdominal midline incision was made and the cecum was exposed and ligated. The ligated cecal stump was twice-punctured at a 1-cm distance with a triangular needle (the needle size was approximately 1.5 mm in diameter). Feces were allowed to flow into the abdominal cavity. After closure of the abdomen, the rats were returned to the cages and allowed food and water *ad-libitum*. The rats in the sham group underwent sham operations following the same procedure as the sepsis group but without CLP and treatment. The rats in the CGA group received a cauda vein injection of CGA (20 mg/kg; Sigma) immediately after CLP establishment. CGA was dissolved in normal saline (NS) to prepare a solution with a concentration of 10 mg/mL. After 24 h, and in separate experiments, the dynamics of bovine serum albumin labeled with fluoresceine-isothiocyanate (FITC-BSA) leakage from the mesenteric micro-vessels were determined by intravital microscopy. The liver and kidney blood flow, hemodynamic parameters, and blood gases were observed. The mesenteric micro-vessels and superior mesenteric vein (SMV) were collected for histologic evaluation and protein level determination.

Cell culture and treatment

VECs were obtained from the mesenteric vein of SD rats by enzymatic digestion as described by our research team.¹⁷ VECs were cultured in endothelial cell medium (ECM, Cat. No.1001; ScienCell, USA). Before each experiment, VECs (3–5 passages) were serum-starved for 24 h.

VECs were seeded on inserts of a 6-cell culture plate (0.4- μ m pore size, #3450; Corning, USA). VECs under lipopolysaccharide (LPS) stimulation (100 ng/ml, Escherichia coli serotype O111:B4; Sigma) were co-cultured with CGA (20 μ mol/L; Sigma)^[8]. CGA was dissolved in a sterile phosphate buffer solution to prepare a solution with a concentration of 2 mol/L. Then, the trans-endothelial electrical resistance (TEER) of VECs was assessed using a Voltohmmetre (World Precision Instruments, Inc., USA) every 30 min until 24 h. The penetration rate was measured after completing the TEER analysis. FITC-BSA was added to the inserts, and 200 μ L of the supernatant was collected every 10 min followed by the addition of 200 μ L of fresh basal medium. The penetration rate was calculated by the total supernatant fluorescence optical density (OD)/FITC-BSA stock solution fluorescence OD. In separate experiments, the ZO-1 and VE-cadherin in VECs was determined by immunofluorescence and Western blotting at 24 h.

Vascular leakage in mesenteric micro-vessels

FITC-BSA was used to evaluate albumin leakage across the mesenteric venular wall by virtue of inverted intravital microscopy (C11440; Hamamatsu, Japan). In brief, the rat abdomen was opened after anesthesia via a middle incision (2–3 cm length). The ileocecal portion of the mesentery (a 10–15 cm portion of the caudal mesentery) was exteriorized and mounted on a transparent plastic stage. The mesentery was kept warm and moist by continuous superfusion with saline solution at 37 °C. The animals were intravenously injected with 50 mg/kg of FITC-BSA. After 10 min of baseline observation,

50 mg/kg of FITC-BSA was injected intravenously and allowed to circulate for 5 min, then the fluorescence intensity of FITC-BSA was recorded and of the FITC-BSA⁺ area per vessel was quantified.

Liver and kidney blood flow

Liver and kidney blood flow were measured by a Laser-Doppler Perfusion Imager (PeriCam PSI ZR, Sweden). Briefly, the liver and kidneys were exposed and a computer-controlled optical scanner was positioned above the surface of the liver and kidneys at a distance of 14 cm. A color-coded image to denote the specific relative perfusion level was displayed on a video monitor, and all images were evaluated with PIMsoft software (PeriCam PSI ZR, Sweden).

Hemodynamic parameters and blood gases

The rats were anesthetized with 3% sodium pentobarbital, and the left femoral artery and left ventricle were catheterized with a polyethylene(PE) catheter for measurement of the mean arterial pressure (MAP) and hemodynamic parameters, including the left ventricular systolic pressure (LVSP) and maximum rate of increase and decrease of the left intraventricular pressure [$\pm dp/dt \max$] (SP844, Power Lab; AD Instruments, Castle Hill, NSW, Australia). After instrumentation, the rats were allowed to equilibrate for 10 min, 0.3–0.5 mL of blood was collected for measurement of blood gas using a blood gas analyzer (ABL90 Flex; Radiometer, Denmark). To avoid additional blood loss in the rats, an equal volume of blood was reinfused after each blood sample was obtained.

Immunohistochemistry Rat Mesentery Tissue

The rat mesenteric micro-vessels in each experimental condition were harvested for examination by immunohistochemistry. Briefly, mesenteric tissues were aseptically harvested from the gut using micro-scissors and forceps after superficially dripping 4% phosphate-buffered paraformaldehyde for 25 min, then fixing with 4% paraformaldehyde at 4 °C overnight. Subsequently, samples were washed extensively with PBS and incubated for 30 min at 37 °C with 0.1% Triton X-100 in PBS. After rinsing three times with 0.1M PBS, samples were incubated with blocking buffer containing 5% BSA. Then, the samples were rinsed in PBS and labeled with antibodies against VE-cadherin (555289; BD Biosciences, USA), ZO-1(33-9100; Invitrogen, USA) and CD31 (ab24590; Abcam, USA).

Ultrastructure of the tight junction in rat mesenteric micro-vessels

The rat mesenteric micro-vessels in each experimental condition were harvested for examination by electron microscopy. Briefly, after superficially dripping 3% glutaraldehyde in 0.1 M PBS for 20 min, the mesentery was cut into blocks smaller than 1 mm³ and further fixed by immersing in the 3% glutaraldehyde for 1 h at room temperature, then overnight at 4 °C. After being rinsed, the tissues were post fixed with 1% osmium tetroxide in 0.1 M PBS for 2 h at 4 °C, dehydrated, then embedded in Epon 812(Olympus, Tokyo, Japan). Ultrathin sections were stained with uranyl acetate and lead citrate and observed with a transmission electron microscope (JEM 1400; JEOL Instruments, Japan).

Western blot analysis of ZO-1 and VE-cadherin expression

Western blot analysis of ZO-1 and VE-cadherin in SMV was performed using specific antibodies against ZO-1 (1:1000), VE-cadherin (1:1000), and β -actin (1:7000; Sigma). Bands were detected with fluorescent secondary antibodies and quantified with an Odyssey CLx Infrared Imaging System (LI-COR; Odyssey, Lincoln, NE, USA).

Statistics

Data are shown as the mean $\pm$ standard error of the mean (SEM). Differences among several groups were tested using ANOVA. All data were assessed using the SPSS 11.0 and GraphPad Prism software (San Diego, CA, USA). A $P < 0.05$ was considered statistically significant. "N" in the figure legends refers to the number of subjects (samples or rats).

Results

Role of CGA in protecting the endothelial barrier function in vivo and in vitro

To determine the effects of CGA on vascular leakage following sepsis, the dynamics of FITC-BSA leakage from the mesenteric micro-vessels was measured. Albumin leakage from the mesenteric micro-vessels increased in the septic shock group; the rate of increase was 243.3%. CGA treatment significantly ameliorated the albumin leakage; the rate of decrease was 37.9% compared with the LPS group (Fig. 1A and B).

To determine the effect of CGA on endothelial barrier function *in vitro*, we next investigated the role of CGA in regulating the TEER and the endothelial monolayer permeability to FITC-BSA. The data showed that the TEER of VECs was significantly decreased and the permeability of FITC-BSA was increased 24 h after LPS stimulation. CGA significantly increased the TEER and reduced the permeability of FITC-BSA by 34.3% and 44.8%, respectively, compared to the LPS group (Fig. 1C and D).

Mechanism underlying CGA protection of the endothelial barrier function in vivo and in vitro

To determine the potential involvement of tight and adherens junctions with ZO-1 and VE-cadherin in the endothelial barrier after septic shock, we examined the patterns of protein expression by immunohistochemistry and western blotting. Examination by confocal microscopy revealed a decrease in the expression of ZO-1 and VE-cadherin in mesenteric micro-vessels after septic shock, which was relieved by treatment with CGA (Fig. 2A). This result was supported by the western blot findings (Fig. 2B). Further study also showed that CGA effectively prevented the LPS-induced decrease in ZO-1 and VE-

cadherin expression *in vitro*. These results indicated that CGA improved the vascular endothelial barrier function by increasing the expression of ZO-1 and VE-cadherin.

Effects of CGA on tight junctions in endothelial cells of the mesentery micro-vessels

Because fluid leakage for macromolecules such as albumin is carried out via widening of the intercellular cleft, we assessed the effect of CGA on tight junctions in endothelial cells of mesenteric micro-vessels using electron microscopy. The results showed that endothelial cells formed smooth, continuous, and high-density tight junctions in the sham-group. After sepsis was established, the integrity of tight junctions was disrupted and usually coincided with erythrocyte diapedesis. The sepsis-induced alterations in the endothelium ultrastructure were significantly reduced by treatment with CGA injection (Fig. 3).

Effects of CGA on the liver and kidney perfusion in rats with septic shock

To determine whether or not CGA ameliorated the micro-circulation by improving vascular leakage, liver and kidney perfusion were measured. The results showed that both liver and kidney perfusion were significantly decreased after septic shock. Specifically, liver and kidney perfusion decreased by 73.8% and 78.3%, respectively, compared with the sham-operated group. Liver and kidney blood flow increased by 41.2% and 38.4%, respectively, compared with the septic shock group (Fig. 4A and B). These results suggest that CGA significantly protects vital organ perfusion by decreasing vascular leakage.

Effects of CGA on hemodynamic parameters in rats with septic shock

To evaluate whether CGA is beneficial for hemodynamics, changes in MAP, LVSP, and $\pm$ dP/dtmax were monitored. The hemodynamic parameters significantly decreased after septic shock; the MAP decreased to 63 mmHg (Fig. 5A) and LVSP decreased to 84 mmHg in the septic shock group (Fig. 5B). CGA administration significantly improved hemodynamics. The MAP in the CGA group reached 105 mmHg (Fig. 5A). The LVSP and $\pm$ dP/dtmax also increased significantly (Fig. 5B-D).

The blood lactic acid (Lac) level is an important prognostic index, which reflects tissue oxygen delivery, metabolic state, and organ dysfunction. The results showed that the Lac level after septic shock increased significantly. CGA antagonized the septic shock-induced increase in the Lac level and the level of Lac decreased from 3.57 mmol/L in the septic shock group to 2.54 mmol/L in the CGA group (Fig. 5E).

Effects of CGA on survival of rats with septic shock

Septic shock results in high mortality in rats. Indeed, only one of 16 rats in the septic shock group survived to 72 h, and the average survival time was 15 ± 16 h. CGA at doses of 5 and 10 mg/kg did not effectively improve the survival time and rate. The 72-h survival rates were 12.5% (2/16) and 18.75%

(3/16), respectively. CGA, at a dose of 20 mg/kg, significantly prolonged the survival time and increased the survival rate. The 72-h survival rate was 50% (8/16), and the survival time was 59 ± 51 h. (Fig. 6A and B).

Discussion

Vascular endothelial barrier dysfunction is the major cause of sepsis-induced organ dysfunction. Previous studies have shown that CGA protects against vital organ injury, such as acute liver and acute kidney injury, by inhibiting inflammasome activation.^{18,19} The present study showed that CGA significantly improved vascular leakage in septic shock rats, then enhanced tissue perfusion in vital organs including the heart, and thus improved hemodynamic status and survival time. Improvement in the endothelial barrier by CGA was closely related to increased expression of ZO-1 and VE-cadherin.

Sepsis is defined as a “dysregulated host immune response to infection leading to organ failure”. Recently, endothelial system dysfunction has been suspected to be detrimental to the recovery of septic patient and contribute to the high morbidity and mortality.^{2,20} Previous studies have demonstrated that the inter-alpha-inhibitor ameliorates endothelial inflammation in septic human patients,²¹ indicating that improvement in the endothelial barrier function may be an important measure for protecting organ function during sepsis. In this study we used CLP to induce sepsis, and found that CGA significantly improved vascular leakage and vital organ function, thus, prolonging survival by protecting endothelial barrier function in septic shock, which may provide a new measure for the clinical treatment of septic patients.

It is well-known that the tight junction (TJ) protein, ZO-1, and the adherens junction (AJ) protein, VE-cadherin, are the best index representing endothelial barrier function. TJ and AJ proteins are essential for maintaining the integrity of the endothelial barrier by restricting paracellular permeability and regulating vessel maturation and stability. In sepsis, tissue hypoperfusion exacerbates vascular endothelial injury by various mechanisms, including reducing ZO-1 and VE-cadherin protein expression, widening the intercellular cleft, and promoting plasma protein and fluid leakage. This finding is consistent with our results that FITC-BSA leakage in the mesenteric vessels increased and the expression of ZO-1 and VE-cadherin was down-regulated in CLP rats. Furthermore, we used LPS to stimulate VECs *in vitro* and measured the TEER and permeability of FITC-BSA, and the results *in vitro* were in agreement with the *in vivo* findings. Taken together, these results suggest that the vascular endothelial barrier disruption was related to the decreased expression of TJ and AJ proteins.

Previous studies have indicated that one possible molecular mechanism involved in endothelial dysfunction is excessive oxidative stress. A number of studies have reported that traditional Chinese medicinal herbs have scavenging effects on hydroxyl radical and superoxide anion radical. CGA is one of the main active ingredients of many Chinese herbal medicines, such as honeysuckle. A recent study showed that CGA attenuated Pb-induced renal injury in mice by inhibiting oxidative stress.²² In addition, studies have shown that CGA has a preventive role in diabetic retinopathy by preserving tight junction

protein levels;¹⁴ however, the effect of CGA on the expression of ZO-1 and VE-cadherin following sepsis is unclear. Our study suggests that the beneficial effect of CGA on CLP-induced endothelial barrier injury might be attributed to the increased ZO-1 and VE-cadherin proteins; however, how CGA regulates the expression of ZO-1 and VE-cadherin in the endothelium in septic shock is unknown. Previous studies have shown that mitogen activated protein kinase (MAPK) is involved in sepsis-associated lung injury and the MAPK signaling pathway plays an important role in regulating the expression of ZO-1 and VE-cadherin.²³ Petecchia et al²⁴ reported that extracellular signal-regulated kinase (ERK) and MAPK signaling pathway affects the expression of TJ proteins in intestinal epithelial cells, and also showed that blocking the ERK1/2 and MAPK pathways improved inflammation-induced down-regulation of ZO-1 and occludin expression in epithelial cells. Chu et al²⁵ demonstrated that 5-methoxytryptophan protected VE-cadherin and prevented endothelial hyperpermeability by blocking p38 MAPK activation. Recently, Gao et al²⁶ showed that CGA alleviates inflammation-induced ulcerative colitis in mice by inhibiting the MAPK/ERK/ c-Jun N-terminal kinase (JNK) signaling pathway. Zheng et al²⁷ also found that the protection of CGA against monocrotaline-induced liver disease is attributed to inhibiting the PI3K and MAPKs signaling pathways; however, the precise mechanism underlying septic shock requires further investigation.

Our results showed that CGA is beneficial in septic shock at a dose of 20 mg/kg. Previous studies have shown that CGA injected intravenously at a dose of 20 mg/kg immediately after the induction of sepsis improves animal survival and inhibits immune cell apoptosis.¹⁰ Our pilot experiments showed that CGA (5 and 10 mg/kg) slightly prolonged survival time and increased survival rate. CGA (20 mg/kg) significantly improved the survival rate of septic shock rats. Therefore, 20 mg/kg of CGA was used in the present experiments.

Several approaches have been used to produce sepsis or septic shock in the laboratory, including cecal ligation and puncture, intravascular infusion of endotoxin or live bacteria, bacterial peritonitis, and pulmonary infection. Different models have their own advantages and limits.²⁸ At present the most commonly used models of sepsis are LPS-induced endotoxemia and CLP-induced sepsis. The LPS-induced septic model is simply achieved with good stability and controllability. This model can rapidly generate abundance of inflammatory factors, but cannot imitate the pathologic and physiologic changes in septic patients. The CLP model exhibits the pathogenic features observed in clinical situations but has lower stability and poor control.^{15,29,30} In the present study we used the CLP model, which better mimics the pathologic and physiologic processes in patients with sepsis. Moreover, the severity of sepsis was controlled in our laboratory. In addition, we found that the greater omentum encased the punctured cecum and affected the success rate of the model. The greater omentum was ligated and excised before establishing the model, thus, significantly enhancing the success rate of the model. In our experiments if the MAP was ≥ 70 mmHg 12 h after surgery, the septic shock model was considered a success. Specifically, the success rate of septic shock was approximately 89%.

Although our experiments demonstrated that CGA protected vascular leakage in septic shock, this study had the following limitations that require further investigation: ①Further investigation is necessary to elucidate the exact mechanisms underlying the effect of CGA on the expression of ZO-1 and VE-cadherin. ②We should use a specific MAPK inhibitor to verify whether or not CGA protects CLP-induced endothelial barrier disruption via the MAPK signaling pathway. ③ Because we only verified the protective role of CGA in septic shock rats, it is unknown whether or not CGA has a consistent protective function in large animals and humans, and this merits further investigation.

Conclusions

CGA is beneficial in rats with septic shock. CGA can protect tissue perfusion and vital organ function by improving vascular endothelial barrier function. The mechanism is closely related to increased expression of ZO-1 and VE-cadherin. These findings provide a new treatment strategy for severe sepsis, especially when accompanied by endothelial dysfunction.

Abbreviations

CGA: Chlorogenic acid; CLP: cecal ligation and puncture; VECs: vascular endothelial cells; HMGB1: high mobility group box 1; BRB: blood retinal barrier; SEM: mean $\pm$ standard error of the mean; SMV: superior mesenteric vein; LPS: lipopolysaccharide; TEER: trans-endothelial electrical resistance; MAP: mean arterial pressure; LVSP: left ventricular systolic pressure; $\pm dp/dt$ max: maximum rate of increase and decrease of the left intraventricular pressure; Lac: lactic acid; TJ: tight junction; AJ: adherens junction; MAPK: mitogen activated protein kinase; ERK: extracellular signal-regulated kinase; JNK: c-Jun N-terminal kinase.

Declarations

Acknowledgements

None.

Authors' contributions

ZSZ performed the experiments, collected data, YW, YZ XYP and MYX prepared the manuscript and participated in the animal study and data analysis. TL and LML conceived the study, participated in design and coordination, and edited the manuscript. All authors read and approved the final version of the manuscript.

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collection/analyses of data, decision to publish, or manuscript preparation.

Availability of data and materials

All materials are commercially available, and data are presented in this article are available per the open access policy.

Ethical Approval and Consent to participate

This study was approved by the Research Council and Animal Care and Use Committee of Research Institute of surgery, Army Medical University. Our methods conformed to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication, 8th edition, 2011).

Consent for publication

Not applicable.

Competing interests

No conflict of interest exists in the submission of this manuscript.

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Figures

Figure 1

Effect of CGA on albumin leakage in mesenteric micro-vessels in vivo and co-culture of VECs with CGA following stimulation with LPS in vitro. A: Representative images of albumin leakage. B: Quantitative evaluation of albumin leakage presented as the percent change, n=8. C: CGA was added into VECs and the TEER of each group was measured, n=3. D: CGA was added into VECs and FITC-BSA penetration of each group was measured, n=8. Sham: sham-operated; Sep: septic shock; CGA: chlorogenic acid treatment. Data are shown as the mean±SEM. ***P<0.001 vs. Sham or Nor, ##P<0.01 vs. Sep or LPS (1-way ANOVA). Scale bar: 50µm(A).

Figure 2

Expression of ZO-1 and VE-cadherin in vivo and in vitro. A: IHC for ZO-1 and VE-cadherin in mesenteric micro-vessels. B: Expression of ZO-1 and VE-cadherin in SMV, n=3. C: VECs treated with CGA were analyzed by immunofluorescence for ZO-1 and VE-cadherin. D: VECs treated with CGA were analyzed by Western blot, n=3. Sham: sham-operated; Sep: septic shock; CGA: chlorogenic acid treatment. Data are

expressed as the means $\pm$ SEM. *** $P < 0.001$ vs. Sham or Nor, ## $P < 0.01$ vs. Sep or LPS (1-way ANOVA)
Scale bar: 50 μ m (A and C).

Figure 3

Representative TEM images of tight junctions (TJs) of rat mesenteric micro-vessels. Note the tight junctions (arrowheads) and erythrocyte diapedesis (*). EC- endothelial cell, RBC- red blood cells, TJ - tight junction, L-lumen. Sham: sham-operated; Sep: septic shock; CGA: chlorogenic acid treatment.

Figure 4

Effect of CGA on liver and kidney perfusion. A: Representative images of liver and kidney blood flow by laser-Doppler perfusion imager (Dark blue represents the lowest perfusion value and red represents the highest perfusion value displayed). B: Quantitative evaluation of liver and kidney blood flow, n=8. Sham: sham-operated; Sep: septic shock; CGA: chlorogenic acid treatment. Data are shown as the mean±SEM. ***P<0.001 vs. Sham, ##P<0.01 vs. Sep (1-way ANOVA).

Figure 5

Effects of CGA on hemodynamic status and blood lactic acid following septic shock in rats. A: mean arterial pressure (MAP), n=8; B: maximum left ventricular systolic pressure (LVSP), n=8; C and D: maximum increase and decrease rate of left intraventricular pressure ($\pm dp/dt_{max}$), n=8; E: lactic acid (Lac), n=8. Sham: sham-operated; Sep: septic shock; CGA: chlorogenic acid treatment. Data are expressed as the mean $\pm$ SEM. ***P<0.001 vs. Sham, ##P<0.01 vs. Sep (1-way ANOVA).

Figure 6

Effects of CGA on survival of septic shock rats. A: Survival rate, B: Survival time. $n=16$ for survival time and rate; Sham: sham-operated; Sep: septic shock; CGA: chlorogenic acid treatment. *** $P<0.001$ as compared with the sham-operated group, ## $P<0.01$ as compared with the Sep group.