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Protective effect of citrulline on the intestinal mucosal barrier of mice during sepsis

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Abstract: Excessive inflammation caused by sepsis can disrupt gut mucosal barrier and aggravate sepsis. The purpose of the study was to confirm whether citrulline can protect the intestinal mucosal barrier during sepsis. Citrulline was used to pretreat the sepsis mouse model and then endotoxin levels, intestinal mucosal permeability, intestinal mucosal morphology and tight junction protein expression were detected to analyze the effect of citrulline on the gut barrier during sepsis. Statistics revealed that, the amount of endotoxin and intestinal mucosal penetration and the morphological score of the intestinal mucosa of septic mice with citrulline treatment were significantly decreased ($P<0.05$), while the claudin-1 and occludin protein expression levels were obviously increased in septic mice with citrulline treatment ($P<0.05$). This study defined the protective effect of citrulline on the intestinal mucosal barrier of septic mice. Future studies should examine whether it has the same effect on patients with sepsis.

Keywords: Sepsis, citrulline, intestinal mucosa.

INTRODUCTION

Sepsis refers to the systemic inflammatory response during which the host exhibits a inappropriate reaction to infection, which in turn leads to serious organ failure (Shanker-Hari *et al*., 2016). There are ~18 million new cases of sepsis every year worldwide, which are increasing at a rate of 1.5-8.0% annually and the mortality rate of severe sepsis is ~50% (Prescott and Angus, 2018). There are two influencing factors of sepsis: Invasion and defense, namely, the invasion of bacteria and endotoxins and the defense function of the body. There are more than 250 different species of intestinal bacteria, a total number of 10^{13} cells which is greater than that of whole human cells (Allam-Ndoul *et al*., 2020). However, intestinal bacteria and their endotoxins generally were no harm to physical health, as the intestinal bacteria maintain an ecological balance and the body has an intestinal mucosal barrier defense. This barrier mainly includes mucosal epithelial cells and tight junctions (Wardill and Bowen, 2013; Yu and Li, 2014). Occludin and claudins proteins are important components of intestinal tight junction (Chelakkot *et al*., 2018). During the progression of sepsis, various inflammatory factors and endotoxins can influence the expression of tight junction proteins by damaging epithelial cells, giving rise to tight junction destrucion and intestinal mucosal barrier damage (Perrone *et al*., 2012). Intestinal mucosal barrier damage increases permeability, as well as the translocation of bacteria and endotoxins, further aggravating sepsis and even causing multiple organ failure (Haussner *et al*., 2019; Yoseph *et al*., 2016). Therefore, protecting intestinal barrier is very valuable during sepsis.

Citrulline is produced in the urea cycle or as a by-product of the catalysis of arginine to nitric oxide (NO) (Wijnands *et al*., 2012). In addition, citrulline can be converted into arginine in the body and its negative feedback loop regulates NO production. During sepsis, complex of endotoxin and plasma protein binds to the CD14 receptor of monocytes/macrophages and induces TNF-α synthesis (Brocco *et al*., 2012). Secondary inflammatory factors are then released, which amplify inflammatory signal and form a cascade effect (Armada *et al*., 2015). The upregulated TNF-α and IL-6 have a damaging effect on the intestinal mucosa (Suzuki *et al*., 2011; Ye *et al*., 2006). Citrulline exhibit inhibition of proinflammatory factors in a sepsis rat model, thereby regulating the body's balance of proinflammatory and anti-inflammatory factors (Asgeirsson *et al*., 2011; Cai *et al*., 2016). Therefore, we hypothesized that citrulline can protect intestinal mucosal barrier during sepsis. This study revealed the influence of citrulline pretreatment on the intestinal mucosal barrier by constructing mouse model of sepsis. Ethical approval (No. 202171) for this study was obtained from the Affiliated Hospital of Putian University.

MATERIALS AND METHODS

Establishment of a sepsis animal model

The sum of 50 C57BL/6 male mice were obtained from Guangxi Medical University. Prior to surgery, mice were kept in a specific pathogen-free room at 25˚C and 50% humidity for 7 days. The animals were assigned as Sham, sepsis and citrulline groups. The surgical procedure to induce the experimental sepsis mouse model was executed as described before (Rittirsch *et al*., 2009). Briefly, following anesthesia with 40mg/kg sodium pentobarbital solution injected into the abdominal cavity. The hair in the abdominal region was shaved and an incision about 2cm was made in mid-abdomen. The

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abdominal cavity was cut layer by layer and the cecum was located in the right abdominal cavity, removed and placed on a sterile gauze. The middle of cecum was ligated with No. 1 thread. Then the cecum distal to the ligation site was punctured with a 21G needle to squeeze out 100mg feces into the abdominal cavity (fig. 1A). The abdominal cavity was then sutured. Following resuscitation from anesthesia, the mice were placed back into the cage for breeding. The surgery performed for mice in the sham group was similar to the above operation, with the exception that cecal ligation and puncture (CLP) was not performed. In the citrulline group, septic mice were constructed after L-citrulline (Guangzhou RiboBio Co., Ltd.) was administered by gavage for 7 consecutive days prior to the surgery at a dose of 200mg/kg daily.

Collection of specimens

At 24, 48 and 72h post-operation, 1 ml blood was drawed from the heart under anesthesia with 40 mg/kg sodium pentobarbital by intraperitoneal injection and 0.5cm FITC-dextran 40S (FD40S; MilliporeSigma) was by intra-gastric administration way at a concentration of 22mg/ml 5h before blood collection. Then, two 1-cm long sections of the ileum were cut out, soaked in 4% paraformaldehyde. Following specimen collection, the mice were euthanized with CO_2 at a flow rate of 30% cage volume displaced/min.

The blood naturally coagulated at 4^{o}C and was centrifuged at 1,000 x g for 10 min. The supernatant was then obtained and frozen at -80^{o}C until required for further experiments.

Citrulline concentration detection

The citrulline concentration in mice was detected using a citrulline ELISA kit (Xiamen Lun Changshuo Biological Technology Co., Ltd.). The optical density (OD) was surveyed by a microplate reader with 450nm wavelength (BioTek Instruments, Inc.). The citrulline concentration in serum of mice was then calculated according to the standard concentration curve.

Endotoxin detection

Serum endotoxin concentration was detected using a horseshoe crab kit (Xiamen Bioendo Technology Co., Ltd.). Endotoxin standard solutions were prepared at different concentration gradients. The endotoxin standard solution and serum samples were then added to the 96-well plate to detect the OD value by a microplate reader with 405nm wavelength using (BioTek Instruments, Inc.). The experimental procedure was repeated three times. Based on the standard curve of different standard endotoxin concentration, the endotoxin level of each sample was calculated.

Intestinal permeability testing

Different concentration gradients of FD40S standard solutions were prepared. The standard product and each serum sample were then added to the cuvette and analyzed by a fluorescence spectrophotometer (Shimadzu Corporation). The excitation light and emission light wavelength of the instrument were set to 480 and 520nm, respectively. The experimental surgery was repeated three times. The FD40S concentration was calculated based on the standard curve of different standard FD40S concentrations.

Histopathological scoring of the ileum

The ileal tissue was stained by H&E method (Cardiff *et al*., 2014). The stained slides were examined under a microscope (Olympus BX53; Olympus Corporation) at a magnification of x200. The ileal mucosa was histopathologically scored as follows (Bouboulis *et al*., 2018): 0, Normal intestinal mucosal epithelium without damage; 1, widened space under the mucosal epithelium and presence of hyperemic capillaries; 2, further expanded and lifted space under the mucosal epithelium, accompanied by peeling of the epithelium and lamina propria; 3, some of the mucosal epithelium had fallen off; 4, the mucosal epithelium of the intestinal mucosa had completely fallen off and was accompanied by capillary hemorrhage; and 5, the lamina propria of the intestinal mucosa had disintegrated and was accompanied by associated bleeding and ulcers.

Immunohistochemical score of tight junction proteins in the ileum

The ileum sections were immersed with 4% paraformaldehyde for 24h, then embedded in paraffin and cut into 5um thick sections. Using the dyeing method described previously (Jiang *et al*., 2021), the ileal tissue was subjected to immunohistochemistry to determine claudin-1 and occludin expression. The dilution ratios of claudin-1 (Cat. No. Ab15098; Abcam) and occluding (cat. no. 168986; Abcam) antibodies were both 1:100 and the slides were hatched with the primary antibody solution in a 4^{o}C refrigerator wet box overnight. Then the slides were hatched with goat-anti-rabbit secondary antibody solution at 25^{o}C for 30 min. Tight junction protein expression was examined under the microscope and yellow or brown particles were observed. Tight junction protein expression value of each slice was calculated by image J software (Version 1.8.0; National Institutes of Health). Then the ratio of the staining intensity was used to compare the difference in protein expression among the sham, CLP and citrulline groups.

STATISTICAL ANALYSIS

SPSS 17.0 software (SPSS, Inc.) was used to analyze the experimental data. Unpaired T-test was applied to identify statistical comparisons between two independent sample means. Mann-Whitney U test was used for non-normally distributed data. A one-way ANOVA, followed by a least significant difference post-hoc test, was applied to contrast the sample means among multiple groups.

RESULTS

Evaluation of the sepsis mouse model

Except for the 2 mice that died during the construction of the sepsis model and 3 mice that died after the model was established, the remaining mice were included in the experiment. Following successful modelling, the septic mice exhibited erected hair, a trembling body and shaky walking (fig. 1B). When the abdominal cavity was opened, the color of the ligated cecum turned black and purulent exudate appeared around the cecum (fig. 1C).

Citrulline concentration is increased in the serum of septic mice with citrulline treatment

In the septic mice with citrulline treatment, the citrulline concentration was significantly higher than that in sepsis mice 24, 48 and 72 h post-surgery (131.37±22.61 vs. 22.99±2.58, 105.88±18.09 vs. 16.46±2.73 and 70.95±21.13 vs. 14±3.45 mg/l, respectively; all $P<0.05$; fig. 2). The citrulline concentration in all groups gradually decreased over time.

Intestinal permeability of septic mice with citrulline treatment is significantly decreased

Compared with sham group, sepsis mice exhibited higher FD40S concentration at 24, 48 and 72h post-surgery (8.227±1.944 vs. 4.25±1.24, 19.79±3.41 vs. 5.41±1.31 and 22.96±2.31 vs. 6.04±1.55μg/ml, respectively; all $P<0.05$; fig. 3A). Furthermore, in contrast to sepsis group, septic mice with citrulline treatment exhibited lower FD40S concentration at 48 and 72h (14.054±3.326 vs. 19.793±3.411 and 19.384±2.806 vs. 22.964±2.312μg/ml, respectively; both $P<0.05$; fig. 3A).

Endotoxin concentration of septic mice with citrulline treatment is significantly decreased

Compared with sham group, sepsis mice exhibited higher endotoxin concentration at 24, 48 and 72h post-surgery (0.47±0.1 vs. 0.22±0.08, 0.74±0.15 vs. 0.25±0.11and 0.92±0.13 vs. 0.24±0.06 EU/ml, respectively; all $P<0.05$; fig. 3B). Furthermore, in contrast to sepsis group, septic mice with citrulline treatment exhibited lower endotoxin concentration after 48 and 72h (0.48±0.15 vs. 0.74±0.15and 0.65±0.14 vs. 0.92±0.13, respectively; both $P<0.05$; fig. 3B).

Ileal tissue damage of septic mice with citrulline treatment is obviously alleviated

Compared with sham group, sepsis mice exhibited higher histopathological score of the ileum 72h after surgery (3.8±0.84 vs. 0.6±0.89; $P<0.05$; fig. 4). However, in contrast to sepsis group, septic mice with citrulline treatment exhibited lower histopathological score of the ileum 72h after surgery (2.2±1.1 vs. 3.8±0.84; $P<0.05$; fig. 4).

Citrulline increases expression of tight junction proteins in septic mice

3 days after surgery, sepsis mice exhibited lower expression of claudin-1 and occludin in the ileal tissue in contrast to the sham group (0.64±0.17 vs. 1.03±0.08 and 0.66±0.17 vs. 1.02±0.14, respectively; $P<0.05$; fig. 5). However, compared with sepsis group, septic mice with citrulline treatment exhibited higher expression of claudin-1 (0.85±0.14 vs. 0.64±0.17; $P<0.05$; fig. 5) and higher expression of occludin (0.89±0.13 vs. 0.66±0.17; $P<0.05$; fig. 5).

DISCUSSION

The intestine is generally considered to be a driving factor for a variety of serious diseases, including sepsis (Yoseph *et al*., 2016). Intestinal mucosal barrier is a significant factor in the resistance of the intestine against the attack of bacteria and endotoxins. The composition of this barrier mainly includes epithelial cells and tight junction proteins (Bjarnason *et al*., 1995; Li *et al*., 2009). Sepsis usually leads to epithelial cell apoptosis (Perrone *et al*., 2012) and tight junction destruction (Yoseph *et al*., 2016; Li *et al*., 2009), resulting in increased permeability (Haussner *et al*., 2019; Suzuki, 2013). The damage of intestinal mucosal barrier can lead to bacteria translocation and endotoxins into the circulatory system, exacerbating sepsis (De-Souza and Greene, 2005) and even leading to multiple organ failure (Piton and Capellier, 2016; Schulz *et al*., 2015). Therefore, protecting the intestinal mucosal barrier is particularly important for patients with sepsis.

Citrulline has been reported to exert therapeutic effects in kidney (Romero *et al*., 2013), liver (Cai *et al*., 2016; Rajcic *et al*., 2021) and cardiovascular disease (Ochiai *et al*., 2012). This study assessed the role of citrulline on the intestinal tract during sepsis. First, during sepsis, the destruction of the intestinal epithelium of mice following citrulline pretreatment was found to be decreased and the secretion of tight junction proteins increased. These two factors are the morphological elements of intestinal barrier. Second, the reduction of endotoxin concentration and intestinal permeability also suggested effectiveness of citrulline in protecting the intestinal barrier.

There are several kinds of tight junction proteins, including claudin, occludin, zonula occludens, junctional adhesion molecule, afadin and anti-tight junction-associated protein (Gunzel and Yu, 2013). Among them, claudin-1 (Saeedi *et al*., 2015) and occludin (Balda *et al*., 2000) are commonly expressed in the intestine.

It has been reported in the previous literature that the blood citrulline level can be used as a marker of intestinal diseases (Uyanga *et al*., 2021) and that milk rich in citrulline can promote the secretion of tight junction

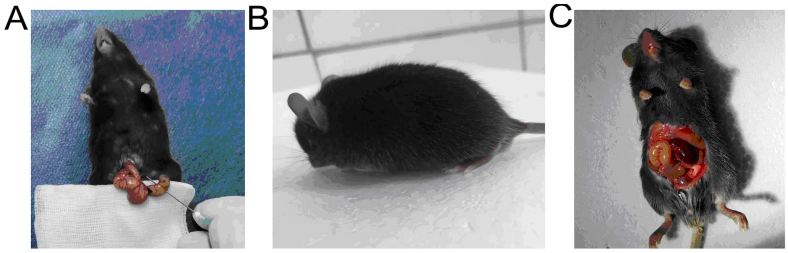

Fig. 1: Construction of sepsis mouse model. (A) Cecal ligation and puncture surgery in mice. (B) External and (C) intra-abdominal manifestations of the sepsis model.

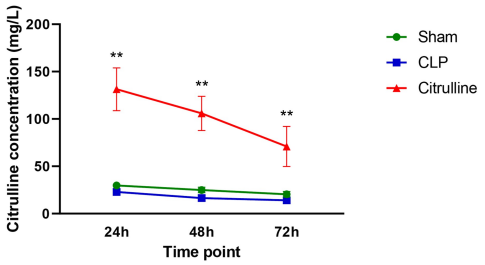

Fig. 2: Significant increase of citrulline concentration in citrulline group compared with CLP group 24, 48 and 72h post-modeling. **P<0.01. CLP, cecal ligation and puncture.

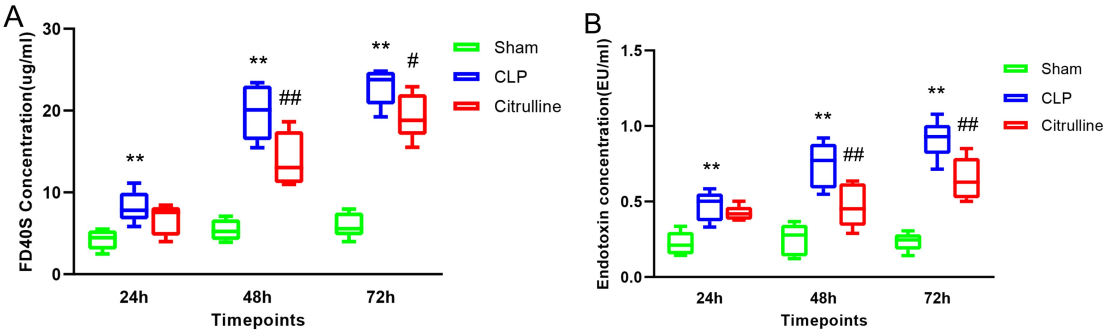

Fig. 3: FD40S and endotoxin concentrations in three groups of mice. (A) FD40S and (B) endotoxin concentration in each group. **P<0.01, CLP vs. sham group; #P<0.05 and ##P<0.01, citrulline vs. CLP group. FD40S, FITC-dextran 40S; CLP, cecal ligation and puncture.

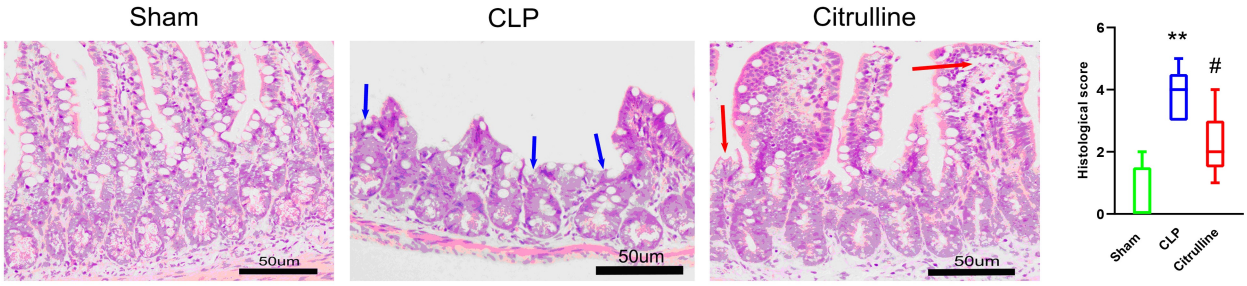

Fig. 4: Histopathological scores of the mouse ileum from three groups of mice. Small blue arrows show epithelial shedding with ulcers and bleeding; Big red arrows show widening of subepithelial space and partial epithelial shedding. **P<0.01, CLP vs. sham group; #P<0.05, citrulline vs. CLP group. Scale bar, 50-µm. CLP, cecal ligation and puncture.

protein by IPEC-J2 cells (Ho *et al.*,2020). There is currently no report on citrulline in the therapy of sepsis. The present study focused on the changes in the intestinal mucosal barrier during sepsis and confirmed the therapeutic function of citrulline in sepsis. The results provided a novel insight into potential treatment strategies for sepsis.

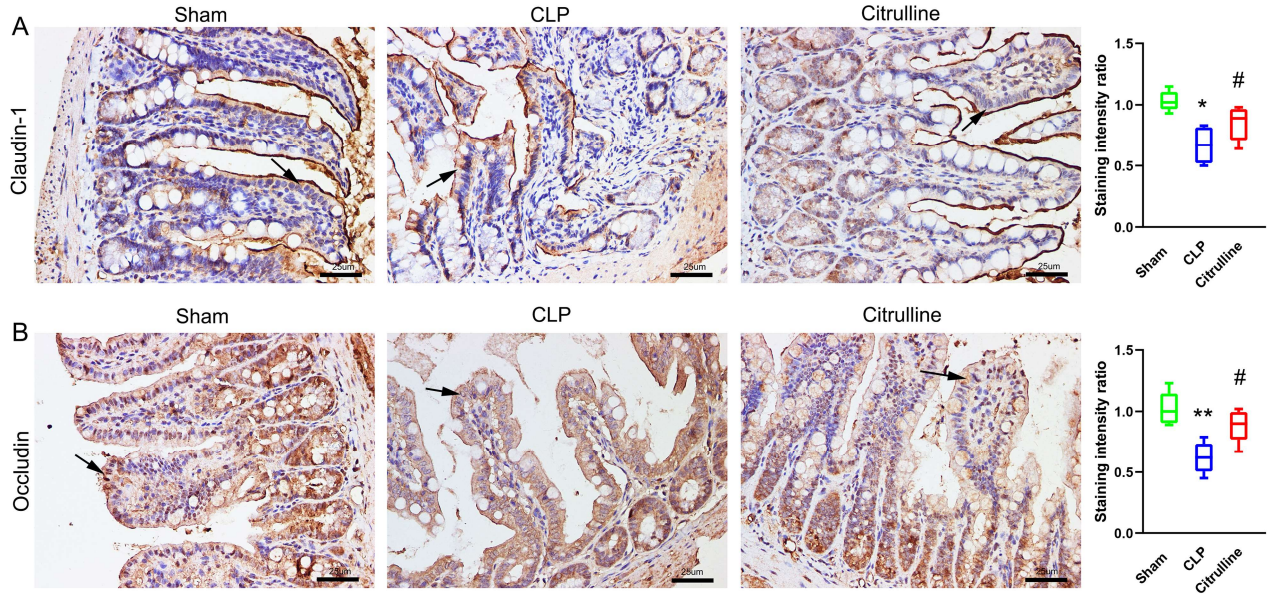

Fig. 5: Tight junction protein expression in mouse ileum. (A) Claudin-1 and (B) occludin protein expression in mouse ileum. Arrows show the claudin-1 and occluding protein expressions. *P<0.05, CLP vs. sham group; **P<0.01 CLP vs. sham group; $^{\#}$P<0.05, citrulline vs. CLP group. Scale bar, 25-µm. CLP, cecal ligation and puncture.

However, this study was not without its limitations. First, the number of experimental animals used was relatively small. Secondly, the experiments were conducted on animals and the results have not been verified in humans. Other experiments involving human bodies need to be further carried out.

CONCLUSION

Citrulline was shown to exert a protective action over the intestinal mucosal barrier of mice during sepsis and may prove useful in the treatment of sepsis. Determining whether citrulline exerts the same protective effect on patients with sepsis is required in future studies.

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