American Journal of Emergency Medicine 33 (2015) 1665–1670

Contents lists available at ScienceDirect

American Journal of Emergency Medicine

journal homepage: www.elsevier.com/locate/ajem

Original Contribution

Shenfu injection alleviates intestine epithelial damage in septic rats☆,☆☆

Xi Xing, MS, Ronglin Jiang, MS *, Lingcong Wang, MD, Shu Lei, MD, Yihui Zhi, MD, Yanchun Wu, MS, Meifei Zhu, MS, Liquan Huang, MS, Guolian Xia, MS, Zheqi Chen, MS

ICU, The First Affiliated Hospital of Zhejiang Chinese Medical University, Hangzhou, 310006 Zhejiang Province, China

ARTICLE INFO

Article history:
Received 11 June 2015
Received in revised form 29 July 2015
Accepted 2 August 2015

ABSTRACT

Background: Shenfu injection (SFI) promotes tissue microcirculation and oxygen metabolism. We aimed to assess its effects on intestinal epithelial damage in septic rats.
Methods: Fifty Sprague-Dawley rats were randomly divided into sham operation (Sham), sepsis (cecal ligation and puncture [CLP]), and SFI (low-dose, middle-dose, high-dose) groups (n = 10). For Sham animals, the abdominal cavity was opened and closed. For other groups, severe sepsis was induced by CLP. After surgery, saline (Sham and CLP rats) and SFI (treatment groups) were administered intraperitoneally. Samples were collected 12 hours after injection. Serum tumor necrosis factor α, diamineoxidase, and D-lactate levels and ileal mucosal damage and ultrastructural change, as well as protein and messenger RNA expression of tight junction markers, including Claudin-3 and zonula occludens protein-1 in ileal mucosa's epithelial cells, were assessed. All animal experiments were carried out under aseptic conditions.
Results: Compared with Sham animals, serum tumor necrosis factor α, DAO, and D-lactic acid levels in CLP animals were significantly higher; the ileal mucosal damage was more severe; and the expression levels of tight junction markers were significantly decreased. These indexes were significantly improved in SFI groups, in a concentration-dependent manner, compared with CLP rats. Sham animals displayed orderly arranged ileal mucosal villi, continuous tight junctions between epithelial cells, intact organelles, and microvilli. Compared with CLP animals (with obvious damage in these structures), an overt improvement was observed in SFI groups, especially in the high-dose SFI group, with tight junctions clearly visible between epithelial cells.
Conclusions: Shenfu injection significantly alleviates intestinal epithelial damage in septic rats, in a dose-dependent manner.

1. Introduction

Severe sepsis is treated by early fluid resuscitation and appropriate antibiotics, although a high mortality rate of 30% to 50% is still observed [1]. Before 1986, the gastrointestinal (GI) tract was believed to play a passive role in the pathophysiology of sepsis. In 1986, Carrico et al [2] first proposed the GI tract to be the driving force behind multiple-organ dysfunction syndrome in sepsis. In 1987, the concepts of bacterial translocation from the gut and gut-origin sepsis were proposed by Border et al [3]. To date, many studies have demonstrated the GI tract's significance in the initiation and progression of systemic inflammatory response syndrome, sepsis, and multiple-organ dysfunction syndrome. Researchers have also demonstrated the importance of the intestinal mucosa's mechanical barrier in preventing bacterial translocation from the gut, with the intercellular tight junctions playing a critical role [4]. Tight junctions consist of the tight junction proteins occludin, claudins, junctional adhesion molecules, and tricellulin. These proteins form a tight junction complex with junctional complex proteins, such as zonula occludens protein-1 (ZO-1), via the cell cytoskeleton system [5]. Tight junction was demonstrated to be highly correlated with intestinal permeability [6].

However, the protective effect of Shenfu injection (SFI) on tight junctions of intestinal mucosa's epithelial cells is not well understood, and the underlying mechanisms need to be elucidated. In this study, we hypothesized that SFI could significantly alleviate intestinal epithelial damage in septic rats. Therefore, a rat model of severe sepsis was established by cecal ligation and puncture (CLP). The tight junction changes in intestinal mucosa's epithelial cells and the protective effects of different SFI doses were examined. In addition, the mechanisms by which SFI protects the intestinal mucosa's mechanical barrier were examined. Our data reveal SFI's preventive effects on the pathophysiological processes of sepsis, indicating that SFI can further improve the success rate of resuscitation in patients with severe sepsis.

2. Materials and methods

2.1. Animal grouping

The study protocol was approved by the hospital' Animal Research Ethics Committee. A total of 50 clean-grade healthy male Sprague-Dawley rats weighing 200 ± 20 g were provided by the experimental

☆ Source of support: This work is supported by Prevention and Control of Major Diseases, Zhejiang Chinese Medicine Research Program (No. 2012ZGG001).
☆☆ Conflict of interest: The authors declare that they have no conflict of interest.
* Corresponding author. Tel.: +86 13958095268; fax: +86 21 64085875.
E-mail address: jiangrl0420@sina.com (R. Jiang).

http://dx.doi.org/10.1016/j.ajem.2015.08.001
0735-6757/

animal center of Zhejiang Chinese Medical University, China. Animals were housed for 2 weeks under normal conditions in the experimental animal center of Zhejiang Chinese Medical University at 20°C ± 1°C and 50% to 60% humidity, under 12:12-hour light/dark cycle, with a ventilation rate of 8 to 15 times/h. Using a random number table, rats were subdivided into 5 groups (n = 10): sham operation (Sham), sepsis (CLP), and SFI (low-dose, LSF; middle-dose, MSF; high-dose, HSF) groups.

2.2. Animal model establishment and treatment

A rat model of severe sepsis was induced by CLP [7]. Briefly, all rats were deprived of food, but with free access to water for 12 hours prior to surgery. Anesthesia was carried out with an intramuscular injection of 5% ketamine (0.2 mL/100 g body weight). After fixation on the operating panel, rats were submitted to abdominal hair removal and skin disinfection, and a ventral midline incision (1.5 cm) was made. For Sham rats, the abdominal cavity was opened and closed immediately, but was not ligated or perforated. For rats of the remaining 4 groups, the abdominal cavity was opened to find the cecum, whose mesentery was carefully dissected; then, the cecum root was ligated, avoiding damage to the ileum and mesenteric vessels. Punctures were made through the cecum by perforating at 3 locations using a 21-gauge needle. The cecum was gently compressed until fecal pellets were extruded. The bowel was then returned to the abdomen and the incision closed. At the end of the operation, all rats were resuscitated immediately with normal saline (5 mL/100 g body weight) subcutaneously. Ten minutes after operation, Sham and CLP animals received 20 mL/kg of normal saline; the rats of the LSF, MSF, and HSF groups received 5 mL/kg SFI plus 15 mL/kg of normal saline, 10 mL/kg SFI plus 10 mL/kg normal saline, and 20 mL/kg SFI, respectively, by tail vein injection. Afterward, the rats were placed in cages at a constant temperature (22°C) and allowed free access to food and water. The animals were constantly observed until they recovered from anesthesia. Dead animal number, animal vigor, hair and stool characteristics of survival rats, and number of open abdominal cavities were evaluated every 2 hours.

2.3. Animal model verification and specimen collection

Twelve hours after operation, survival rats were anesthetized with 5% ketamine. To verify whether the animal model was successfully established, rats in Sham and CLP groups were examined. Heart rate (HR) was measured as follows: needle electrodes were inserted into both upper extremities and left lower extremity and connected to the RM-6280 Physiologic Recording System (Junyue electrical equipment factory, Dongguan, China). Rectal temperature (T) was monitored using a thermometer. Heart blood samples were collected as follows: hair removal and skin disinfection were performed in the area under the xiphoid; then, a needle attached to a 5-mL syringe was inserted upward into the heart at 30°, and 2 mL blood was drawn from heart into a blood culture flask. The abdominal cavity was opened and swabbed with a sterile swab stick to collect 1 mL peritoneal exudates. The peritoneal swabs and blood culture specimens were immediately sent to the Clinical Pathogenic Microbiological Laboratory of the First Affiliated Hospital of Zhejiang Chinese Medicine University for bacterial culture [8]. For all rats, blood samples (2 mL) were collected from the abdominal aorta; a 2-cm section of terminal ileum was extracted for further analysis; a portion of blood specimen was immediately sent to the Clinical Laboratory of the above mentioned hospital for white blood cell (WBC) amounts, serum aminotransferase (ALT), aspartate transferase (AST), blood urea nitrogen (BUN), and creatinine levels, and creatine kinase isoenzyme MB (CK-MB) activity.

2.4. Detection of serum tumor necrosis factor α, diamineoxidase, and D-lactic acid levels

Serum tumor necrosis factor α (TNF-α), diamineoxidase (DAO), and D-lactic acid concentrations were measured using enzyme-linked immunosorbent assay kits following the manufacturer's instructions (Shanghai Xitang Biotechnology, LTD, Shanghai, China).

2.5. Ileal mucosal damage index

Ileum tissue sections (3-5 μm) were stained with hematoxylin and eosin (HE) and observed under a XZT-302 microscope (Shanghai Yuguang Detection Equipment Co, Ltd, Shanghai, China) at ×100 magnification. Histologic evaluation was performed according to the Chiu scoring method [9].

2.6. Ultrastructure of ileal mucosal epithelium

Ileum tissue (1 mm^3) fixed by glutaraldehyde was obtained and fixed again with 1% osmic acid at 4°C for 1 hour. Then, samples were dehydrated twice with ethanol gradient (50%, 70%, 80%, 90%, and 100%) and acetone. After dehydration, the tissue was embedded in acetone-Epon812 and penetrated. The resulting tissue block was placed into a capsule with diallyl phthalate, which was allowed to polymerize for 72 hours (24 hours each at 35°C, 45°C, and 60°C). After polymerization, the tissue block was trimmed, and ultramicrotomy was performed. The thin sections were double stained with lead citrate and uranyl acetate, and the ultrastructure of ileal mucosa's epithelium was observed on a JEM-12003X transmission electron microscope (JEM, Akishima, Japan).

2.7. Western blot analysis of tight junction protein expression

Twenty milligrams of ileal tissue was minced, resuspended in 200 μL RIPA buffer, and homogenized. Protein concentration was determined by the bicinchoninic acid method. Thirty micrograms of total protein extract was subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis and transferred onto a membrane. After blocking with 5% nonfat milk for 2 hours followed by 3 washes for 30 minutes in Tween-tris buffered saline buffer (10 mmol/L Tris-HCl [pH 7.4], 150 mmol/L NaCl, 0.05% Tween-20), the membrane was incubated with rabbit anti–Claudin-3 (1:2000; Abcam, Cambridge, UK) and anti-ZO-1 (1:2000; Abcam) polyclonal antibodies, overnight at 4°C. The membrane was washed 3 times and incubated with horseradish peroxidase–labeled goat antirabbit secondary antibody for 2 hours. Signals were detected on a Bio-Rad (Hercules, CA) gel imaging system, and protein bands were semiquantified with the Quantity One software (Bio-Rad Laboratories, Hercules, CA). The relative tight junction protein level was derived as an optical density value of the tight junction protein/optical density value of β-actin from the same sample.

2.8. Determination of messenger RNA expression of tight junction genes

The messenger RNA (mRNA) expression of tight junction genes in ileal mucosa's epithelial cells was assessed by reverse transcriptase polymerase chain reaction (PCR). Total RNA was extracted using TRIzol reagent (Life Technologies, Waltham, MA). Then, first-strand cDNA was synthesized with the Super RT cDNA Kit (Kangwei Shiji Biotechnology, LTD, Beijing, China) following the manufacturer's instructions. Real-time PCR was performed on an ABI 7900 Real-Time PCR System (Applied Biosystems, Foster City, CA), with initial denaturation at 95°C for 10 minutes, followed by 35 to 40 cycles of 95°C for 15 seconds and 60°C for 1 minute. The primers used are as follows: 5′-CATCGCAGCTACTTGCCAGT-3′ (forward) and 5′-TTTTTTTTTTTTTTTTGCAAAAACGA-3′ (reverse) for Claudin-3; 5′-CCATCTTTGGACCGATTGCTG-3′ (forward) and 5′-TAATGCCCGAGCTCCGATG-3′ (reverse) for ZO-