Changes of the immunological barrier of intestinal mucosa in rats with sepsis

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BACKGROUND: Sepsis has become the greatest threat to in-patients, with a mortality of over 25%. The dysfunction of gut barrier, especially the immunological barrier, plays an important role in the development of sepsis. This dysfunction occurs after surgery, but the magnitude of change does not differentiate patients with sepsis from those without sepsis. Increased intestinal permeability before surgery is of no value in predicting sepsis. The present study aimed to observe the changes of intestinal mucosal immunologic barrier in rat models of sepsis induced by cecal ligation and puncture.

METHODS: Sixty Sprague-Dawley rats were randomly divided into a sepsis group (n=45) and a control group (n=15). The rats in the sepsis group were subjected to cecal ligation and puncture (CLP), whereas the rats in the control group underwent a sham operation. The ileac mucosa and segments were harvested 3, 6 and 12 hours after CLP, and blood samples were collected. Pathological changes, protein levels of defensin-5 (RD-5) and trefoil factor-3 (TFF3) mRNA, and lymphocytes apoptosis in the intestinal mucosa were determined. In an additional experiment, the gut-origin bacterial DNA in blood was detected.

RESULTS: The intestinal mucosa showed marked injury with loss of ileal villi, desquamation of epithelium, detachment of lamina propria, hemorrhage and ulceration in the sepsis group. The expression of TFF3 mRNA and level of RD-5 protein were decreased and the apoptosis of mucosal lymphocyte increased (P<0.05) in the sepsis group compared with the control group. Significant differences were observed in RD-5 and TFF3 mRNA 3 hours after CLP and they were progressively increased 6 and 12 hours after CLP in the sepsis group compared with the control group (P<0.05, RD-5 F=11.76, TFF3 F=16.86 and apoptosis F=122.52). In addition, the gut-origin bacterial DNA detected in plasma was positive in the sepsis group.

CONCLUSION: The immunological function of the intestinal mucosa was impaired in septic rats and further deteriorated in the course of sepsis.

KEY WORDS: Sepsis; Mucosal immunology; Defensin-5; Trefoil factor family 3; Cecal ligation and puncture

INTRODUCTION

Sepsis, a systematic inflammatory response syndrome, is considered as an immunologic unbalance.[1] It has become the greatest threat to in-patients with a mortality of over 25%.[2] It has proved that the dysfunction of gut barrier, especially the immunological barrier, plays an important role in the development of sepsis.[3] Kanwar et al[4] found that gut barrier dysfunction occurs after surgery, but the magnitude of change does not differentiate patients with sepsis from those without sepsis. Increased intestinal permeability before operation has been found to be of no value in predicting sepsis. In the present study, we observed the changes of the intestinal mucosal immunologic barrier in rat models of sepsis induced by cecal ligation and puncture.
METHODS

Animals
Sixty healthy and clean Sprague-Dawley rats, weighing 140-220 g, were supplied by the Animal Experiment Center of Sun Yat-Sen University, and the number of check was 2006A064. The rats were randomly divided into a control group (n=15) and a sepsis group (n=45). And the rats of the sepsis group were further divided into 3 sub-groups 3, 6 and 12 hours after operation (n=15 each group).

Sepsis models
Sepsis models were induced through cecal ligation and puncture (CLP). The rats were anesthetized by an abdominal injection of 20% urethane (1.2 mg/kg). A ventral midline incision was made, and the cecum was exposed and most of it was ligated at the one-third distance away from the ileocecal junction. At the end of the appendix, 2 holes were punctured with an 18-gauge needle, a 1.0 cm strip was placed at each hole for drainage, and at last the abdomen was closed. The criteria for successful sepsis models included that the mortality of female and male rats was 50% and 80% respectively at the tenth day post-operation in the preliminary experiment, which met with the interim standard.

Sample collection
At 3, 6 and 12 hours after the sepsis models were established, the rats were killed and their ileum was removed for pathological observation. A piece of 5 cm long ileum was resected and washed with cold CMF-HBSS, and then incubated in CMF-HBSS containing 30 mmol/L EDTA for 20 to 25 minutes. The specimen of the ileum was strongly vibrated with Vortex-Genie 2, USA to remove the epithelium. Finally the product was washed twice with CMF-HBSS and was centrifugated at 2000 r/min (Supercentrifuge, 5415D, Eppendorf, Germany). The sediments were collected for RT-PCR detection. Meanwhile, 1.5 mmol of vein blood was drawn from each rat.

Pathological examination
The removed ileum was fixed in 10% neutral formalin for 24 hours. Paraffin-embedded sections (5 μm thick) were subjected to staining with hematoxylin and eosin. Intestinal mucosal morphological injury was observed under a light microscope (Olympus, CX30, Japan). Pathological examination was performed by an experienced pathologist who was unaware of the details.

MRNA expression of RD-5 and TFF3
Intestinal mucosa was separated from the gut and total RNA was extracted by Trizol (15596-026, Invitrogen, California, USA). AMV reverse transcriptase and oligo (dt) 15 primers were used for reverse transcription. Two μg of total RNA was collected as a template and preserved at -20 °C. DNA was amplified in the presence of 2.5 units of TaqDNA polymerase (Life Technologies) according to the manufacturer's protocol with some modifications. The DNA polymerase was TaKaRa Ex Taq. The primers used were amplified (PTC-200, MJ Research, USA) as follows: glyceraldehyde-phosphate dehydrogenase (GAPDH): forward, 5'-AGACAGGCCGCAT.

CTCTGTGF3, reverse 5'-CTTGGCCGTGGTAGAGTCAF3'; RD-5: forward, 5'-ATCGGTGGCCTCAGAACTC-3′, reverse 5'-TCGCAGCCATTGAAATT-3′. Thirty-three cycles were used (94 °C for 3 minutes, 94 °C for 30 seconds, 55 °C for 30 seconds, 72 °C for 30 seconds, and 72 °C for 10 minutes). The primers used were amplified as follows: GAPDH: forward, 5'-GCTGGGTCCTCACCTGAAGG-3′, reverse, 5'-GGATGAACCTT GCCAACAGGCC-3'; TFF3: forward 5'-AGGCCAGAATTTGGTGCC-3', reverse 5'-TCAAATGTGACATCTCGTC-3'. Forty cycles were used (48 °C for 45 minutes, 94 °C for 2 minutes, 94 °C for 30 seconds, 58 °C for 60 seconds, 68 °C for 2 minutes and 68 °C for 7 minutes). Ten μg amplified product was subjected to agarose gel electrophoresis and densitometric scanning by a gel image analyser (UVP, GDS-8000pc, California, USA). The ratio RD-5 and GAPDH, TFF, and GAPDH represented the expression of RD-5 and TFF3 mRNA.

Apoptosis of ileal mucosal lymphocytes
Apoptosis-related DNA fragmentation was determined by the TUNEL assay with the apopTag peroxidase in situ apoptosis detection kit (Roche, Switzerland). Samples were processed according to the manufacturer's instructions. Only TUNEL-positive cells showing signs of brown were counted. An arbitrary cutoff of TUNEL-positive cells/100 crypts was determined as the minimum number to report.

DNA of intestinal bacteria detected by PCR
Escherichia coli, which accounted for 80% of gut-origin strains, were tested. The DNA was extracted from blood samples by the TM Blood DNA kit (B15111 D3392-01 E.Z.N.A., Omega Bio-Tek, USA). PCR experiments were performed using a set of primers: BG-1 and BG-4, which were derived from the β-galactosidases gene of E.coli and found in most E.coli. The primes used in PCR were as follows: BG-1: 5'-CTTTGCGCTGTTT...
CCGGCACCAGAA-3’, BG-4: 5’-AACCACCGCACGATAGAGATTCGGG-3’. PCR was performed in a 25-μl reaction mixture containing 0.25 μl eTaq-ase, 2.5 μl buffer, 2 μl dNTP, 0.5 μl BG-1, 0.5 μl BG-4, and 0.5 μl template. The conditions for PCR were as follows: 30 cycles at 94 °C for 5 minutes, 94 °C for 1 minute, 60 °C for 30 seconds, 72 °C for 1 minute, 72 °C for 10 minutes, and final extension for 10 minutes at 4 °C. The products were resolved by electrophoresis with 1.5 % agarose gel.

Statistical analysis
All the results were expressed as mean±SEM. Multiple comparisons were performed with analysis of variance by SPSS 13.0 package. A P value of 0.05 was considered statistically significant.

RESULTS
Histopathological changes of ileal mucosa
Intestinal mucosal structure was distinct, and villi were complete in the control group; while serious ileal mucosal injury was characterized by loss of glandular structure, intestinal epithelial villi, edema of the lamina propria, mesenteric chyladenectasis, capillary bleeding, and ulcers in the sepsis group (Figure 1).

Expression of RD-5 mRNA and TFF3 mRNA
The expressions of RD-5 mRNA and TFF3 mRNA were decreased in the sepsis group compared with the control group (P<0.05), and they were decreased with the time (Figures 2, 3; Table 1).

Apoptosis of lymphocytes of ileal mucosa
The severity of epithelial apoptosis was significantly increased at each time point after CLP in the sepsis group compared with the control group (P<0.05). (Figure 4; Table 1).

Results of intestinal bacterial DNA
Bacillus coli DNA was positive in the sepsis group but negative in the control group (Figure 5).
Figure 2. The expression of RD-5 mRNA of ileal mucosa (randomly five rats from each group). A: control group; B: sepsis group at 3 hours; C: sepsis group at 6 hours; D: sepsis group at 12 hours.

Figure 3. The expression of TFF3 mRNA of ileal mucosa (randomly five rats from each group). A: control group; B: sepsis group at 3 hours; C: sepsis group at 6 hours; D: sepsis group at 12 hours.

Figure 4. The apoptosis of lymphocytes in ileal mucosa (TUNLE staining, original magnification×400). A: control group; B: sepsis group at 3 hours; C: sepsis group at 6 hours; D: sepsis group at 12 hours.
DISCUSSION

Uncontrolled SIRS is an important pathologic basis of the development of sepsis, whereas dysfunction of the intestinal barrier contributes to the development of SIRS. [7] Kauwar et al [4] reported that intestinal barrier dysfunction may be associated with the decreased immune function of the intestinal mucosa. RD-5 is a native immune substance secreted by Paneth's cells in the small intestine. In vitro test has proved that antimicrobial spectrum of RD-5 is wide, and plays an important role in the protection of intestinal barrier function. [8,9] TFF3 is produced by goblet cell, and could protect and restore intestinal epithelial cells by interacting or linking with myxogluco protein, blocking C3 deposition, and changing objective gene. [10,11] Approximately 40% to 50% of lymphocytes are in the intestinal epithelium in peripheral circulation [12] and they participate in intestinal immunologic surveillance and the first-line defense. In the past few years, the above-mentioned indexes have been used to evaluate the immune function of the intestine in many countries, but studies mostly concentrated on single factor analysis. [13,14] This study was to observe the changes of the immunologic barrier of intestinal mucosa in septic rats.

Yao et al [15] reported that intestinal mucosa was seriously impaired in septic rats, and the destroyed integrity of the mucosa caused the bacterial translocation of the intestine and led to endotoxemia. Although intestinal mucosal injury is not correlated with increased intestinal permeability and the damage of intestinal epithelial integrity doesn't necessarily cause bacterial translocation, if intestinal mucosal injury is combined with decreased intestinal mucosal immune function, it is possible that bacteria in the intestine may transfer to the other organs. In the present study, we observed that when the expression of RD-5 and TFF3 mRNA decreased significantly and epithelial apoptosis increased significantly in rat models of sepsis, bacillus coil DNA was amplified in the blood. This indicated that decreased intestinal mucosal immune function promoted the bacterial translocation.

In the sepsis group of our study, the injury of intestinal immune function progressively increased with the time after CLP, possibly because the acute stress of intestinal injury caused by sepsis led to ischemia and hypoxia, ischemia-reperfusion injury, nutrition disorders, excessive release of many inflammatory cytokines such as TNF-α, IL-1, IL-6, PAF. [16,17] With the development of sepsis, intestinal epithelial apoptosis increased and intestinal defense function and self-restoring

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<tr>
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<th>RD-5 Mean±SD</th>
<th>TFF3 Mean±SD</th>
<th>Lymphocyte apoptosis Mean±SD</th>
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<tr>
<td>Control</td>
<td>0.65±0.03</td>
<td>1.34±0.12</td>
<td>6.10±0.80</td>
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<td>Sepsis at 3 hours</td>
<td>0.59±0.06</td>
<td>1.06±0.10</td>
<td>12.52±0.62</td>
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<tr>
<td>Sepsis at 6 hours</td>
<td>0.42±0.05</td>
<td>0.84±0.07</td>
<td>18.38±0.59</td>
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<tr>
<td>Sepsis at 12 hours</td>
<td>0.23±0.06</td>
<td>0.50±0.07</td>
<td>28.11±0.72</td>
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<tr>
<td>F</td>
<td>11.76</td>
<td>16.86</td>
<td>122.52</td>
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<td>P</td>
<td>0.00</td>
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Table 1. Comparison of the expressions of RD-5 and TFF3 mRNA and lymphocyte apoptosis (mean ± SD, n=15)
function decreased, thus forming a vicious cycle "from intestinal barrier dysfunction to sepsis". Therefore, early intervention and increase of intestinal immune function in patients with sepsis may have significant effect on the prognosis.

We also observed the expression of RD-5 and TFF3 at the gene level, and in the future we plan to study at the protein level. In addition, researchers pointed out that the intestinal immune system may interact with the body immune system via complement, acute-phase protein, cytokines, and recirculation of lymphocytes. The ischemia-reperfusion injury in the intestine caused by sepsis may also affect the immune organs far away from the intestine, but the mechanism awaits further investigation.

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**Contributors:** Jiang LY proposed the study and wrote the first draft. All authors contributed to the design and interpretation of the study and to further drafts.

**REFERENCES**