Original Article

Effect of *Candida albicans* on Intestinal Ischemia-reperfusion Injury in Rats

Lei Yan[1], Chun-Rong Wu[2], Chen Wang[3], Chun-Hui Yang[2], Guang-Zhi Tong[4], Jian-Guo Tang[2]

[1]Department of Emergency Medicine, Zhongshan Hospital, Fudan University, Shanghai 200032, China
[2]Department of Trauma, Emergency and Critical Care Medicine, Shanghai Fifth People's Hospital, Fudan University, Shanghai 200240, China
[3]Department of Respiratory, Taizhou Municipal Hospital, Taizhou, Zhejiang 318000, China
[4]Division of Swine Infectious Diseases, Shanghai Veterinary Research Institute, Chinese Academy of Agriculture Sciences, Shanghai 200240, China

Abstract

Background: Inflammation is supposed to play a key role in the pathophysiological processes of intestinal ischemia-reperfusion injury (IIRI), and *Candida albicans* in human gut commonly elevates inflammatory cytokines in intestinal mucosa. This study aimed to explore the effect of *C. albicans* on IIRI.

Methods: Fifty female Wistar rats were divided into five groups according to the status of *C. albicans* infection and IIRI operation: group blank and sham; group blank and IIRI; group cefoperazone plus IIRI; group *C. albicans* plus cefoperazone and IIRI (CCI); and group *C. albicans* plus cefoperazone and sham. The levels of inflammatory factors tumor necrosis factor (TNF)-α, interleukin (IL)-6, IL-1β, and diamine oxidase (DAO) measured by enzyme-linked immunosorbent assay were used to evaluate the inflammation reactivity as well as the integrity of small intestine. Histological scores were used to assess the mucosal damage, and the *C. albicans* blood translocation was detected to judge the permeability of intestinal mucosal barrier.

Results: The levels of inflammatory factors TNF-α, IL-6, and IL-1β in serum and intestine were higher in rats undergone both *C. albicans* infection and IIRI operation compared with rats in other groups. The levels of DAO (serum: 44.13 ± 4.30 pg/ml, intestine: 346.21 ± 37.03 pg/g) and Chiu scores (3.41 ± 1.09) which reflected intestinal mucosal disruption were highest in group CCI after the operation. The number of *C. albicans* translocated into blood was most in group CCI ([33.80 ± 6.60] ×10^2 colony forming unit (CFU)/ml).

Conclusion: Intestinal *C. albicans* infection worsened the IIRI-induced disruption of intestinal mucosal barrier and facilitated the subsequent *C. albicans* translocation and dissemination.

Key words: *Candida albicans*; Infection; Inflammation; Intestinal Mucosa Barrier; Ischemia-reperfusion Injury

Introduction

Intestinal ischemia-reperfusion injury (IIRI) in human is a frequent event entailing high morbidity and mortality, representing a pivotal complication in many clinical cases and surgical procedures such as small bowel transplantation, cardiopulmonary bypass, strangulated hernias, abdominal aortic aneurysm surgery, and neonatal necrotizing enterocolitis.[1,2] Ischemia-reperfusion (IR) is detrimental to the intestine not only for disrupted nutrient absorption by the mucosa but also for damaged barrier integrity, leading to possible translocation of pathogenic microorganisms across the gut wall and consequently systemic sepsis.[2,3] As an essential part of the intestinal mucosa, epithelial enterocytes and tight junction proteins are vulnerable to IIRI, causing inflammatory activation of the innate immune response.[4] Inflammatory factors, such as tumor necrosis factor (TNF)-α, interleukin (IL)-6, and IL-1β, are produced in the intestine during IIRI and contribute to a local and systemic inflammatory response, leading to damages to local and remote organs, such as gastrointestinal (GI) tract and lungs, causing multiple organ failure and death, resulting in up to 80% mortality.[5]

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Address for correspondence: Dr. Jian-Guo Tang, Department of Trauma, Emergency and Critical Care Medicine, Shanghai Fifth People's Hospital, Fudan University, Shanghai 200240, China
E-Mail: tangjianguo@5thhospital.com

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Candida albicans is an opportunistic fungal pathogen commonly found in human GI tract.[6] Under physiological conditions, there is a dynamic balance between microorganisms in the intestinal lumen and the multifactorial host defense mechanisms. However, when the balance is lost, yeast can break through the intestinal mucosal barrier and cause invasive candidiasis and candidemia.[7] In many clinical cases, such as excessive use of antibiotics, immunosuppression, and organ transplantation, the load of *C. albicans* in GI tract would rise rapidly. The fungal infection was accompanied by the significant elevation of inflammatory cytokines.[8] Early during *C. albicans* infection, inflammatory cytokines such as TNF-α, IL-6, and IL-1β participate in the control of fungus.[9] However, these inflammatory cytokines produced in IIRI also participated in organ disruption.[5] Recent microbiological studies have been focused on the pathogenic roles of intestinal bacteria in IIRI; however, the presence and activity of fungi have not been extensively studied. Marotta *et al*. had shown that protein-calorie malnutrition aggravates the gut translocation of *C. albicans* triggered by mesenteric IR injury in a mice model.[10] However, whether *C. albicans* in GI tract would aggravate IIRI and systemic inflammatory response syndrome (SIRS) has puzzled the researchers for years. Therefore, the present study aimed to explore the effect of inflammation caused by intestinal *C. albicans* infection on the pathophysiology of IIRI.

Methods

Experimental animals and grouping

Animals

The experimental protocol and procedures used in this study were approved by the Experimental Animal Ethics Committee of Fudan University, and in strict accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. Fifty specific pathogen-free female Wistar rats (body weight, 170–220 g) were collectively housed in cages in the experimental room for at least 3 days before the start of the experiments (male rats are prone to fight with each other, which would result in trauma and infection and affect the results of research). The rats were maintained at 22°C under a 12:12 h light–dark cycle. They were provided with clean laboratory diet and tap water ad libitum and housed in a clean-air and pathogen-free room with restricted access.

Experimental design

The rats were randomly assigned to five treatment groups according to computer-generated random numbers as the following:

I. Blank and sham (BS) (n = 10): Rats were given purified water during the entire experiment. They were exposed to laparotomy without clamping the superior mesenteric artery (SMA) when undergoing surgery on the 16th day
II. Blank and IIRI (BI) (n = 10): Rats were given purified water during the entire experiment. On the 16th day, they were exposed to laparotomy with occlusion of the SMA for 60 min, followed by 120 min of reperfusion period
III. Cefoperazone plus IIRI (CI) (n = 10): Rats were given 2% cefoperazone in drinking water from the 1st to 16th day.[11] On the 16th day, they were exposed to laparotomy with occlusion of the SMA for 60 min, followed by 120 min of reperfusion period
IV. *C. albicans* plus cefoperazone and IIRI (CCI) (n = 10): Rats were given 2% cefoperazone in drinking water from the 1st to 16th day. They were inoculated with 1 ml 0.9% NaCl containing 10^8 CFU live *C. albicans* cells by oral gavage on the 5th day. On the 16th day, they were exposed to laparotomy with occlusion of the SMA for 60 min, followed by 120 min of reperfusion period
V. *C. albicans* plus cefoperazone and sham (CCS) (n = 10): Rats were given 2% cefoperazone in drinking water from the 1st to 16th day. They were inoculated with 1 ml 0.9% NaCl containing 10^8 CFU live *C. albicans* cells by oral gavage on the 5th day and were exposed to laparotomy without clamping the SMA when undergoing surgery on the 16th day.

Microorganism

C. albicans isolates (American Type Culture Collection [ATCC] 10231) were obtained from the ATCC. All of the microorganisms were cultured aerobically at 37°C for 48 h. At the end of the incubation, microorganisms were harvested by centrifugation, washed three times in 0.9% NaCl, and adjusted to a concentration of 10^8 colony forming unit (CFU)/ml in 0.9% NaCl.

Rat model of intestinal ischemia-reperfusion injury

During all the procedures, the animals had spontaneous breathing and the body temperature was maintained at 37°C using a water-circulating heating pad. A midline laparotomy was performed under deep anesthesia with sodium pentobarbital. The SMA was identified and isolated; then, a small nontraumatic vascular clamp was applied for 1 h.[12,13] Ischemia was recognized by lack of pulse or by pale color of the intestine. After the ischemic phase, the clamp was removed and the intestine was allowed to reperfusion for 2 h. The return of the pulses, the reestablishment of the pink color, and enhanced intestinal peristalsis were taken as the signs of intestinal reperfusion. In BS and CCS groups, SMA was isolated but not clamped. After 2 h, the blood and small intestine were sampled for analysis.

Histologic changes of intestinal mucosa and injury score

After sacrifice of the animals, the removed small intestinal tissue samples were immediately fixed in 10% neutral-buffered formalin, embedded in paraffin, and sectioned. The sections were processed by hematoxylin-eosin (HE) staining and examined under light microscopy. Histological scores were evaluated by two independent investigators who were blind to the grouping; two sections were observed per rat. The degree of histopathological changes was graded semiquantitatively using the histological injury scale described by Chiu *et al*.[14] as follows: 0 - normal mucosal villi; 1 - development of

a subepithelial space, usually at the apex of the villi with capillary congestion; 2 - extension of the subepithelial space with moderate lifting of the epithelial layer from the lamina propria; 3 - massive epithelial lifting down the sides of the villi and ulceration at the villous tips; 4 - denuded villi with dilated capillaries and increased cellularity of the lamina propria; and 5 - degradation and disintegration of the lamina propria, hemorrhage, and ulceration.[14]

Measurement of inflammatory mediators and diamine oxidase

After 120 min of removing of the vascular clamp, blood was collected into heparinized tubes and plasma was isolated via centrifugation (3000 ×*g* for 10 min). As soon as the experimental rats were sacrificed, their intestinal tissues were quickly removed and cleaned with phosphate-buffered saline (PBS). The tissues were homogenized with PBS and centrifuged at 3000 ×*g* for 10 min. The tissue supernatants and plasma were stored at −20°C until further assays, respectively. The levels of TNF-α, IL-6, IL-1β, and diamine oxidase (DAO) were determined by enzyme-linked immunosorbent assay (ELISA) according to the manufacturer's instructions (R&D, USA), and the concentrations of cytokines were determined spectrophotometrically by the absorbance at 450 nm. A standard curve was constructed using the cytokine standards available in the ELISA kit. The cytokine concentrations for unknown samples were calculated according to the standard curve.

The presence of *Candida albicans* in gastrointestinal tract and blood

The homogenized tissues and blood were plated on Sabouraud dextrose agar (Bianzhen Biological Technology, Nanjing, China). Colonies of *C. albicans* were observed after 48-h incubation at 37°C. To obtain Colony-Forming Units, we calculated the number of colonies on the medium.

Statistical methods

Data were entered and analyzed using Statistical Package for Social Sciences version 21.0 (IBM, USA, New York). All data were presented as means ± standard error (SE). If the data of cytokines met the standards of both normal distribution and homogeneity of variance, they were analyzed by the one-way analysis of variance with the least significant difference test used as *post hoc* analysis. Otherwise, they were analyzed using Kruskal–Wallis test followed by *post hoc* Wilcoxon rank-sum tests and Bonferroni correction. The percentages of culture-positive samples among the total tissues and blood were compared between groups with Fisher's exact test. Differences were considered to be statistically significant if the $P < 0.05$.

Results

Mucosal inflammation and injury score

The intestine of rats in group BS displayed smooth villus and healthy glands with few inflammatory cells infiltrating in the mucosal epithelial layer [Figure 1a]. Compared with sham groups, damaged intestinal wall, loss of villous structure, and severe necrosis were observed in group BI [Figure 1b]. The rats of group CCI showed denuded villi, ulceration, hemorrhage, and inflammatory cells infiltrating near the basement membrane of the intestine [Figure 1d]. Hence, the mucosal injury scores were highest in group CCI. Animals in group CI exhibited milder mucosal damage than those in group CCI whereas the lifting of epithelial layer from lamina propria and the ulceration were still observed [Figure 1c].

Consistent with the histologic changes of intestinal mucosa, the intestinal injury scores were significantly higher in IIRI groups compared with sham groups, and infection of *C. albicans* increased the histologic scores [Figure 1f].

In *Candida*-infected animals, the presence of fungal hyphae indicated the colonization of fungi in the intestinal mucosa [Figure 1, arrows]. Infectious *C. albicans* hyphae were present in the lumen in groups CCS, CCI, and CI [Figure 1d and 1e]. Taking advantage of the disruption of intestinal mucosa after IIRI, some fungi, in the form of hyphae or pseudohyphae, even invaded into small intestinal wall, laying the foundation for the subsequent candidemia and disseminated candidiasis.

Changes of inflammatory mediators and diamine oxidase

Inflammatory cytokines such as TNF-α, IL-6, and IL-1β may

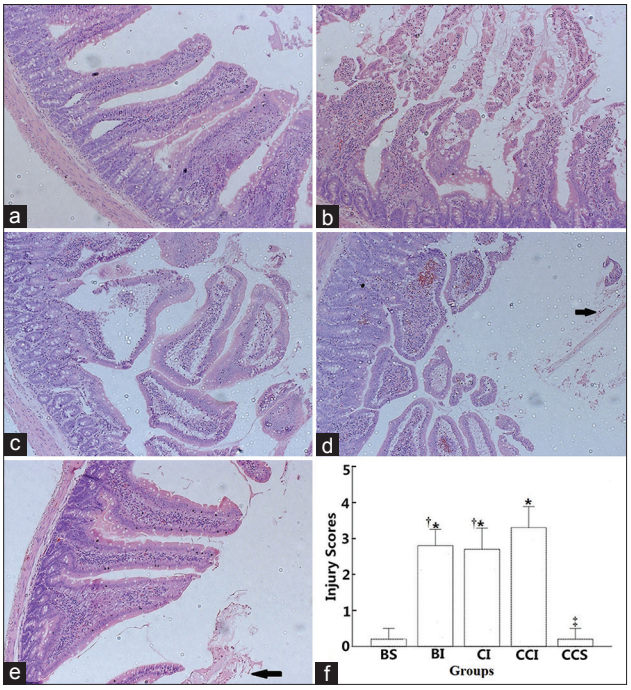

Figure 1: Histologic changes of intestinal mucosa and injury score. a-e: HE staining (Original magnification × 100). BS: Blank and sham (a); BI: Blank plus intestinal ischemia-reperfusion (b); CI: Cefoperazone plus intestinal ischemia-reperfusion (c); CCI: *Candida albicans* plus cefoperazone and intestinal ischemia reperfusion injury (d); CCS: *Candida albicans* plus cefoperazone and sham (e); Intestinal *C. albicans* hypha is emphasized by arrow. **P* < 0.05 compared with group BS; †*P* < 0.05 compared with group CCS; ‡*P* < 0.05 compared with group CCI (f).

be important mediators in IIRI and may also contribute to the defense mechanisms of the host in response to *C. albicans* infection.[5,9] As shown in Figure 2, the levels of TNF-α, IL-6, and IL-1β in serum and intestinal tissue significantly increased after IIRI compared with those in sham groups. Excessive *C. albicans* infection in the gut increased the serum and intestinal IL-6 and TNF-α compared to the blank groups (IL-6: $\mu = 5.11, 3.21$; TNF-α: $\mu = 4.30, 3.23$, $P < 0.01$) [Figure 2a, 2b, 2e, and 2f]. We found that the levels of IL-1β in serum from the *C. albicans* infection group (CCS: 10.28 ± 1.07 pg/ml) were similar to those in the blank group (BS: 9.88 ± 1.16 pg/ml) [Figure 2c]. With the double strikes of IIRI and *C. albicans* infection, the levels of TNF-α, IL-6, and IL-1β in both serum and intestine increased significantly in group CCI than that of other groups. However, no statistical difference in TNF-α and IL-6 was found between groups CI and BI (IL-6: $\mu = 1.11, 1.27$; TNF-α: $\mu = 0.73, 1.23$, $P > 0.01$).

DAO is an enzyme synthesized primarily in GI mucosal cells, and the level of DAO has been used as an indicator of the integrity and functional mass of the intestinal mucosa.[15-17] Compared with groups BS (10.43 ± 3.56 pg/ml, 94.82 ± 4.12 pg/g) and CCS (12.03 ± 2.14 pg/ml, 96.52 ± 5.24 pg/g), the serum and intestinal DAO activity significantly increased in groups BI (38.47 ± 1.49 pg/ml, 297.93 ± 18.70 pg/g), CI (36.47 ± 5.70 pg/ml, 282.51 ± 35.50 pg/g), and CCI (44.13 ± 4.30 pg/ml, 346.21 ± 37.03 pg/g). However, no statistical difference was found between CI and BI groups ($\mu = 0.75, 1.06$, $P > 0.05$). Meanwhile, *C. albicans* infection seemed to have no significant influence on the levels of DAO in group CCS compared with group BS ($\mu = 1.51, 0.69$, $P > 0.05$) [Figure 3].

Presence of *Candida albicans* in gastrointestinal tract and blood

After 48-h incubation at 37°C, pure *C. albicans* was isolated

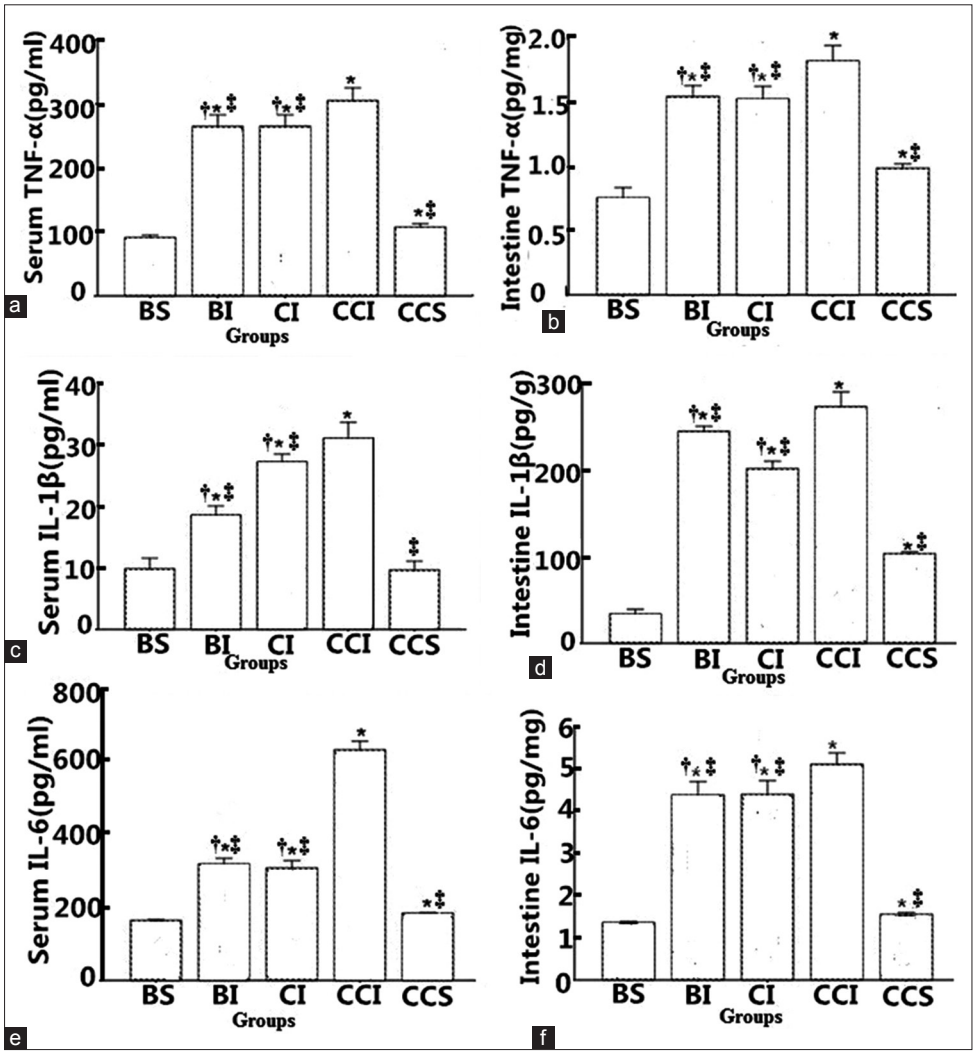

Figure 2: *Candida albicans* increased the local and systemic inflammatory response after IIRI. Serum and intestine levels of TNF-α (a,b), IL-6 (e,f), and IL-1β (c,d) were determined by ELISA after operation, $*P < 0.01$ compared with group BS; $^{\dagger}P < 0.05$ compared with group CCS; $^{\ddagger}P < 0.05$ compared with group CCI. CCI: *Candida albicans* plus cefoperazone and intestinal ischemia-reperfusion injury; BS: Blank and sham; CCS: *Candida albicans* plus cefoperazone and sham; IIRI: Intestinal ischemia-reperfusion injury; TNF-α: Tumor necrosis factor-α; IL: Interleukin; ELISA: Enzyme-linked immunosorbent assay.

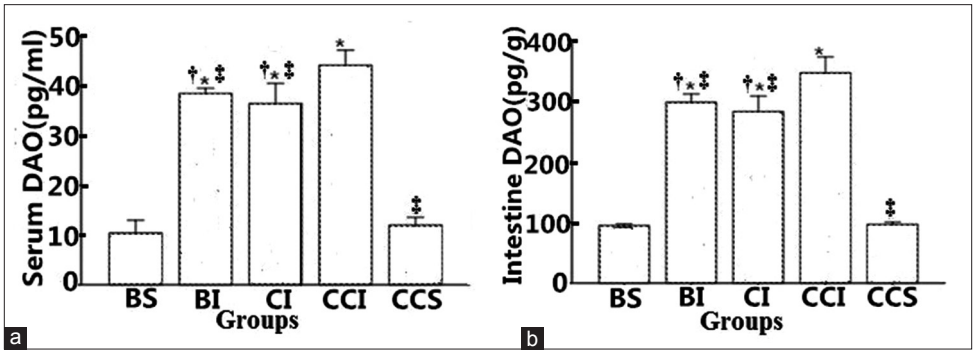

Figure 3: *Candida albicans* elevated DAO in serum (a) and intestine (b). *$P < 0.01$ compared with group BS; †$P < 0.05$ compared with group CCS; ‡$P < 0.05$ compared with group CCI. CCI: *Candida albicans* plus cefoperazone and intestinal ischemia-reperfusion injury; BS: Blank and sham; CCS: *Candida albicans* plus cefoperazone and sham; DAO: Diamine oxidase.

from the intestines of all rats in groups CCS, CCI, and CI. The rates of positive-culture from blood of groups CCS, CCI, and CI were 10%, 40%, and 20%, respectively. As for groups BS and BI, *C. albicans* was not isolated from either intestine or blood [Figure 4]. The number of *C. albicans* colonies in blood and intestine is shown in Table 1.

Discussion

In this research, intestinal *C. albicans* infection increased the inflammatory factors, such as TNF-α, IL-6, and IL-1β, worsened the IRI-induced disruption of intestinal mucosal barrier, had a bad influence on the damaged intestinal mucosa, and facilitated the subsequent *C. albicans* dissemination. This devastating effect could be found in pathological changes of intestine, e.g., inflammatory cells accumulated in the areas infected by *C. albicans*.[18] As a result, *C. albicans* broke through intestinal mucosa barrier and translocated into blood.

IIRI in human is a common event in a variety of clinical conditions, such as small bowel transplantation, trauma, burn injuries, septic shock, and heart and aortic surgery.[2,3] The pathophysiological processes of IIRI *in vivo* are complex, and inflammation may play an important role. Our results showed that the levels of TNF-α, IL-6, and IL-1β increased sharply after IIRI in rats. Previous researches have shown that in the early stages of IIRI, the production of oxygen-free radicals causes polymorphonuclear neutrophils (PMN) activation and inflammatory cytokine production. The activation of PMN and inflammatory cytokines further lead to the release of oxygen-free radicals, thereby contributing to intestinal injury.[19,20] Inflammation involves a sequential activation of signaling pathways which results in the production of pro- and anti-inflammatory mediators during IIRI.[21] Among the proinflammatory mediators, TNF-α and TNF-αR1 systems play central roles in the physiological regulation of intestinal barrier function and TNF-α can induce intestinal epithelial barrier dysfunction.[21] TNF-α also acts as an initiator to induce the expression of other cytokines such as IL-6.[21] IL-6 and IL-1β are a triad of cytokines involved in inflammation and innate response activation triggered by tissue damage.[22] Meanwhile, IL-1β

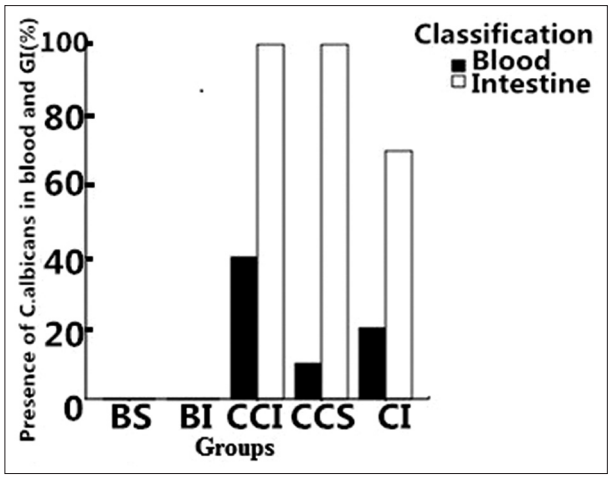

Figure 4: Presence of *Candida albicans* in gut and blood. The disruption of intestinal mucosa after IIRI facilitated *Candida albicans* blood translocation. The incidence of *Candida albicans* blood invasion was closely related to fungal load in the gut. IIRI: Intestinal ischemia-reperfusion injury.

Table 1: *Candida albicans* load in blood and intestine ($n = 10$, each)

Group	Blood (×10³ CFU/ml)	Intestine (×10⁶ CFU/g)
CCI	33.80 ± 6.60	22.0 ± 7.3
CCS	0.14 ± 0.07	14.4 ± 6.9
CI	2.26 ± 1.09	0.007 ± 0.005
BI	0	0
BS	0	0

CCI: *Candida albicans* plus cefoperazone and intestinal ischemia-reperfusion injury; CCS: *Candida albicans* plus cefoperazone and sham; CI: Cefoperazone plus intestinal ischemia-reperfusion injury; BI: Blank and intestinal ischemia-reperfusion injury; BS: Blank and sham.

is considered as a marker of acute inflammation, resulting in the activation of macrophages during early stages of tissue injury.[23] These cytokines all play important roles in the pathophysiology of IIRI, which could explain the sharp increase of cytokines after IIRI compared with sham groups.

In this study, cefoperazone was added into drinking water to inhibit intestinal commensal bacteria, which might create proper conditions for *C. albicans* proliferation.[11,24] Meanwhile, our research showed that

IIRI strongly promoted *C. albicans* infection [Figure 4 and Table 1]. Conversely, *C. albicans* augments the inflammation after IIRI too [Figure 2]. Galectin-3 is an intestinal lectin that binds to specific *C. albicans* glycans and involved in inflammation.[25] Compared with rats in Group BS, *C. albicans* infection in group CCS triggered TNF-α, IL-6, and IL-1β expression and production. It is suggested that these cytokines contribute to the defense mechanisms of the host in response to microbial colonization and invasion.[25-28] TNF-α is one of the major secretory products of macrophages with an important role in host defense against disseminated candidiasis.[29,30] IL-1β is an important proinflammatory cytokine that is required for the induction of immune responses involving IL-17-producing helper T-cells that are integral to antifungal defense by mobilizing neutrophils.[28] IL-6 is a regulator of the cellular response to *C. albicans* infection, which may have a proinflammatory effect by inhibiting the suppressor activity of $CD4^+$ $CD25^+$ regulatory T-cells or by affecting the differentiation of T lymphocytes to IL-17-producing T-cells.[31-33] In view of the complex role of cytokines in the host defenses against infection and in the tissue-destructive effects of inflammation on the hosts, we sought to explore their effect on aggravating IRI of intestine with *C. albicans* infection. In our research, the levels of TNF-α, IL-6, and IL-1β reached the highest values in animals under both *C. albicans* infection and IIRI operation. Compared with rats in the CCS group, rats in CCI group also got IIRI operation; therefore, the intestinal inflammation and pathological results of group CCI showed the additional effect of IIRI.

Our studies also suggested that *C. albicans* enteral infection may aggravate local or SIRS after IIRI, which would have a bad influence on the recovery of damaged intestinal mucosa and increase mortality of patients after operation. This devastating effect could be found in pathological changes of intestine, e.g., inflammatory cells accumulated in the areas infected by *C. albicans*.[18] Histological evaluation of the disruption of intestinal mucosal barrier [Figure 1] showed that the histological damage index reached the highest values in animals suffered from both *C. albicans* infection and IIRI [Figure 1].

DAO regulates cell proliferation through degradation of polyamine, an indispensable substance for mitosis and meiosis. DAO also rapidly detoxifies dietary histamine, preventing the allergy-like symptoms of histamine excess.[16,17] The activity of DAO is associated with the maturation and integrity of small intestinal mucosa.[16,17] In our study, with the increase of inflammation mediators, the levels of DAO reached the peak in group CCI after IIRI. As similar to histological damage index, the levels of DAO increased in IIRI groups compared with the sham groups.

Results of experimental studies indicated that significant *C. albicans* infection of the rat intestine would aggravate the disruption of intestinal mucosal barrier after IIRI, and this effect could attribute to the aggravation of inflammatory reaction. Meanwhile, systemic inflammation would also increase intestinal permeability.[34] In addition, IIRI damages the integrity of intestinal barrier. The disruption of intestinal barrier would be a sally port for the pathogenic microorganism to invade into blood and translocate to the distant organs. Therefore, in this research, blood *C. albicans* cultures were positive in groups CCI and CI. The preoperative anesthetics might also decrease the intestinal blood flow, resulting in aggravated disruption and delayed recovery of intestinal mucosa.[35] Therefore, an increase in intestinal permeability due to anesthetics might explain the systemic invasion of *C. albicans* in group CCS. With the double blow of IRI operation, the load of *C. albicans* in the blood in group CCI would be more than those in other groups at the same time point [Table 1].

Several limitations were encountered in our study. First of all, in our research, *C. albicans* intestinal infection trigger inflammation was on the contrary to previous reports by Mason *et al*.[36] A major reason is that Mason *et al*. only examined HE-stained histological samples of intestine without detecting inflammatory mediator. Hence, they believe that colonization with *C. albicans* during antibiotic treatment does not trigger overt inflammation in the murine cecum. Besides, *C. albicans* load in the intestine in our research is much more than that in research by Mason *et al*. Second, the short term *C. albicans* colonization in the rat model does not correctly reflect the situation of clinical patients. Indeed, the amount of *C. albicans* in intestine cannot be substantially sustained over time in patients as already described elsewhere.[37] Further prospective study to elucidate the role of *C. albicans* might be needed in the clinical field. Finally, the precise microbial and host factors which favor *C. albicans* as a cause of GI and systemic candidiasis were not studied.

In conclusion, the results of our experimental studies demonstrated that a significant fungal infection of the intestinal mucosa could aggravate the disruption of intestinal mucosal barrier after IIRI and facilitate the subsequent translocation and dissemination of *C. albicans*. Our study indicated that ecological imbalance in the intestinal flora may worsen IIRI and facilitate the development of SIRS. However, more detailed researches are needed to further elucidate the molecular mechanism underlying the disruption of intestinal mucosal barrier and the enterogenous invasion of *C. albicans* during IIRI.

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Conflicts of interest

There are no conflicts of interest.

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