Original Article

Enterogenous infection of *Candida albicans* in immunocompromised rats under severe acute pancreatitis

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BACKGROUND: Opportunistic infection of *Candida albicans* (*C. albicans*) has become a serious problem in immunocompromised patients. The study aimed to explore the mechanism of enterogenous infection of *C. albicans* in immunocompromised rats under severe acute pancreatitis (SAP).

METHODS: Sprague Dawley (SD) rats ($n=100$) were randomly assigned into 5 groups as the following: blank group, cyclophosphamide+ceftriaxone+SAP group, cyclophosphamide+ceftriaxone group, cyclophosphamide+SAP group, and cyclophosphamide group. The rats were sacrificed at 5 and 10 days, and their jejunum, colon, mesenteric lymph nodes, pancreas, intestinal content, and blood were quickly collected to detect *C. albicans*. A region of the 25S rRNA gene was chosen and amplified by polymerase chain reaction (PCR) to differentiate *C. albicans* genotypes. The amplified products were further sequenced and compared to judge their homology.

RESULTS: Compared with the Cyclophosphamide group, the combination of immunosuppressants and broad-spectrum antibiotics significantly increased the colonization of *C. albicans* in intestine in 5 and 10 days. Pure SAP stress did not increase the opportunistic infection of *C. albicans*. The PCR products of *C. albicans* isolates all belonged to the genotype A family, and sequence alignment showed that the amplified fragments were homologous.

CONCLUSION: The damage of immune system and broad-spectrum antimicrobial agents are important risk factors for opportunistic fungal infection. Intestinal tract is an important source for genotype-A *C. albicans* to translocate and invade into bloodstream.

KEY WORDS: *Candida albicans*; Immunosuppression; Severe acute pancreatitis; Genotype

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INTRODUCTION

The increasing incidence of acquired immunodeficiency syndrome (AIDS) and the recent development of new and more aggressive treatment procedures for patients with malignancies and organ transplants have resulted in an increase in the number of immunocompromised patients with fungal infections.^[1,2] Among the *Candida* species, *Candida albicans* (*C. albicans*) is still considered as the most important fungal pathogen.^[1] Compared with patients discharged without a diagnosis of invasive fungal infection (IFI), those diagnosed with IFI are associated with a

significantly longer hospital stay and higher mean cost.^[3] In the United States, approximately \$1.89 billion in annual hospital costs may be attributable to IFIs.^[3]

It is generally believed that the main reservoir for *C. albicans* in humans is the gastrointestinal tract (GI) and that systemic infections predominantly originate from this reservoir.^[4,5] Under normal conditions when the intestinal mucosal barrier is intact, and the host's innate immune system is functioning, *C. albicans* behaves like commensal members of the gastrointestinal microflora. There is a homeostasis between *C. albicans* and the host.^[6] However,

when the balance is disrupted, the yeast can break through the intestinal mucosal barrier and cause invasive candidiasis and candidemia.^[6] Under critical illness such as severe acute pancreatitis (SAP), the structure and function of intestinal mucosa would be damaged, leading to gut barrier dysfunction.^[7,8]

Although recent microbiological studies have been focused on the pathogenic role of intestinal bacterial sepsis, the presence and activity of fungi have not been extensively explored, and what would happen in immunocompromised patients under SAP has puzzled researchers for years. In this paper, we tried to explain the phenomenon of enterogenous infection of *C. albicans* in immunocompromised rats under SAP.

METHODS

Animals

The experimental protocol and procedures used in this study were in strict accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and approved by the Experimental Animal Ethics Committee of Fudan University. A total of 100 SD rats (body weight, 170–220 g) were collectively housed in cages in the experimental room for at least 3 days before the start of experiments.

Experimental design

The rats were randomly assigned into 5 treatment groups with 20 in each group. The rats of each group were further divided into 2 subgroups with 10 in each subgroup according to different experiment time (5 or 10 days). The four groups were as follows:

1) Blank (B): Rats in this group didn't receive any treatment during the experiment.

2) Cyclophosphamide+ceftriaxone+SAP (CCS): Rats in this group all received intraperitoneal (IP) injection of cyclophosphamide (200 mg/kg) on the 1st day of experiment. Meanwhile, they were injected with ceftriaxone (100 mg/kg) through the tail vein once a day during the whole experiment. On the 4th day, rats in the 5-day subgroup received IP injection of L-arginine (2.0 g/kg) twice to establish SAP model, which was established on the 9th day in the 10-day subgroup.

3) Cyclophosphamide+ceftriaxone (CC): Rats in this group all received IP injection of cyclophosphamide (200 mg/kg) on the 1st day of the experiment. Meanwhile, they were injected with ceftriaxone (100 mg/kg) through the tail vein once a day during the whole experiment.

4) Cyclophosphamide+SAP (CS): Rats in this group all

received IP injections of cyclophosphamide (200 mg/kg) on the 1st day of the experiment and intravenous saline once a day during the whole experiment. On the 4th day, rats in the 5-day subgroup received IP injection of L-arginine (2.0 g/kg) twice to establish SAP model, which was established on the 9th day in the 10-day subgroup.

5) Cyclophosphamide (C): Rats in this group all received IP injections of cyclophosphamide (200 mg/kg) on the 1st day of experiment and intravenous saline once a day during the whole experiment.

We observed the behavioral changes of rats in each treatment group after the experiment. Each change would be recorded, such as appetite, weight, excrement, hair coat and emotion.

Histologic changes of intestinal mucosa and pancreas

After the animals were sacrificed by cervical dislocation, the specimens of pancreas and gut were fixed in formalin solution and embedded in paraffin. Sections were routinely chipped and stained with hematoxylin and eosin (HE) for morphologic analysis by light microscopy.

Detecting the presence of *C. albicans* in multiple organs

As soon as the experimental rats were sacrificed, the blood was collected by cardiac puncture. Then, the jejunum, colon, mesenteric lymph nodes, pancreas and intestinal content were quickly collected. The intestinal tissues were cleaned with PBS to minimize surface contamination from organisms present in the lumen. The homogenized tissues and blood were plated into Sabouraud dextrose agar (SDA). The proliferation of *C. albicans* was observed after 48-hour incubation at 37 °C.

Molecular analysis of *C. albicans* isolates

The isolates were identified as *C. albicans* on the basis of their cultural and morphological characteristics on SDA and colony color on CHROM agar *Candida*.

DNAs were extracted using the Fungal DNA Kit (OMEGA) according to the manufacturer's instructions.

The primer pairs whose sequences span the site of the transposable intron in the 25S rDNA were as described by McCullough.^[9] The primer sequences were as follows: CA-INT-L, 5'-ATAAGGGAAGTCGGCAAAA TAGATCCGTAA-3' and CA-INT-R, 5'-CCTTGGCTGTG GTTTCGCTAGATAGTAGAT-3'. The reactions contained 1 L of each primer (100 pM), 2 L DNA template, 5.0 L 10×Ex Taq buffer, 4 L dNTP mixture, 0.5 L ExTaq DNA polymerase (TaKaRa, Japan), and the total volume was adjusted to 50 L with H₂O. The PCR conditions were as follows: denaturation at 94 °C for 5 minutes, 30 cycles of

94 °C for 30 seconds, 60 °C for 30 seconds, and 72 °C for 1 minute, and a final extension at 72 °C for 10 minutes. All reaction products were characterized by electrophoresis on 1.5% agarose gels in 1×TAE (Tris-acetate-EDTA) at 140 V for 30 minutes and stained with ethidium bromide for visualization using an ultraviolet transilluminator.

The target fragments in the gels were extracted with a Gel Extraction Kit (OMEGA, USA), cloned into pMD18-T vector (TaKaRa, Japan), and then sequenced using a 3730 XL DNA analyzer (Applied Biosystems, USA) at Life Technologies (Shanghai, China). The sequence analysis and multiple alignments were performed using DNASTar Software.

Statistical methods

All data were compared with Fisher's exact test. Differences were considered to be statistically significant if the *P* value was less than 0.01. All analysis was performed with Stata 10 software.

RESULTS

The behavioral changes of rats in each treatment group

The rats in group C showed no remarkable changes after the experiment. However, the animals in other groups changed sharply as follows: 1) they ate and drank less, resulting in weight loss; 2) their excrement increased and became loose; 3) their hair coat shed and tarnished; and 4) they became agitated and irritable.

Presence of *C. albicans* in multiple tissues

The culture results have been summarized in Table 1. Regardless the experiment time (5 or 10 days), the combination of immunosuppressants and broad-spectrum antibiotics significantly increased *C. albicans* colonization in intestine than single use of cyclophosphamide ($P<0.01$) (Table 1). However, no significant difference was observed between the groups C and CS ($P>0.01$). There were no statistical differences in positive rate of *C. albicans* from intestinal content between the 10-day subgroup and the

5-day one in each treatment group ($P>0.01$) (Table 1).

In the 5-day subgroup, rats from the group CC seemed to be more apt to suffer *Candidemia* than those in group C ($P=0.011$). With the extension of experiment days, this statistic difference disappeared, and there was no significant difference between the groups CC and C. It seemed that SAP stress or the combination of SAP and ceftriaxone did not increase the risk of *Candidemia* compared with group C. As for the culture result of jejunum and colon, the positive rates in groups CCS, CS, and CC seemingly increased in comparison with group C, though there was no statistic difference between these groups with Fisher's exact test (Table 1).

Histologic changes of intestinal mucosa and pancreas

There were no significant pathological changes in pancreatic tissues from groups C and CC. However, edema, acinar and fat necrosis, hemorrhage, as well as monocyte and neutrophil infiltration were observed in the pancreas of all rats from groups CS and CCS (Figure 1). The intestinal mucosa of rats without SAP showed normal villous architecture and glands, with no hemorrhage (Figure 2). Significant pathological changes in the intestinal tissues were observed in SAP models, presenting with mucosal and submucosal swelling, congestion, and inflammatory cell infiltration, shedding of some villi, and mucosal erosion (Figure 2).

Genotyping of *C. albicans* isolates

Among the 600 samples, *C. albicans* could be isolated from 104 samples (Table 1). We made the gene analysis of all *C. albicans* strains isolated from multiple tissues. The PCR products of representative genotypes of *C. albicans* were exemplified in Figure 3. The PCR products appeared as a single band at 450 bp, defining the genotype A.

Analysis and comparison of genomic sequences

The sequences spanning the site of the transposable group I intron of the 25S rRNA gene were chosen for alignment. By amplifying and comparing the sequence of

Table 1. Culture of *C. albicans* from multiple tissues

Tissues	Group B		Group C		Group CC		Group CS		Group CCS	
	5 days	10 days	5 days	10 days	5 days	10 days	5 days	10 days	5 days	10 days
Bloodstream	0 [*]	0	0	0	6 ^a	4	2	0	4	4
Mesenteric lymph nodes	0	0	0	0	0	0	0	0	0	1
Pancreas	0	0	0	0	0	0	0	0	1	0
Intestinal content	0	0	1	0	7 ^a	6 ^a	4	4	4	6 ^a
Jejunum	0	0	1	0	5	3	4	2	3	3
Colon	0	0	1	1	6	5	5	3	4	4
Total samples 600	Total positive samples 104									

Compared with the cyclophosphamide group, ^{*} $P<0.01$; ^a: The numbers of positive samples from which *C. albicans* could be isolated.

Figure 1. Pancreatic histology in the blank group and SAP groups (HE staining; original magnification $\times 100$). **A:** normal pancreatic histology of rats in the blank group; **B:** destruction of acinar structures, with visible scattered bleeding spots, saponification spots, and inflammatory infiltrates after SAP induction.

Figure 2. Intestinal histology in the blank group and SAP groups (HE staining; original magnification $\times 100$). **A:** normal intestinal histology of rats in the blank group; **B:** mucosal and submucosal swelling, congestion, inflammatory cells infiltration, shedding of some villi, mucosal erosion in SAP group.

Figure 3. Genotype of *C. albicans* isolates from multiple tissues. All lanes were genotype A (450 bp).

25S rRNA gene of *C. albicans* isolated from each cultured tissue (blood, mesenteric lymph nodes, pancreas, intestinal content, jejunum, and colon), we found that these sequences were almost identical (Table 2), demonstrating that the intron was partially present throughout their genomes.

DISCUSSION

C. albicans is a ubiquitous commensal organism and has been considered as a major pathogen for immunocompetent as well as immunocompromised patients. Excessive use of antifungal agents has been implicated in the emergence of antifungal resistance in *C. albicans* and constitutes a serious clinical problem in hospitals by affecting the natural balance

of the intestinal microflora in patients.^[10]

In this study, obvious *C. albicans* colonization and proliferation were observed in intestine and circulation of rats whose immune system was damaged by cyclophosphamide and intravenously continuously injected with ceftriaxone for 5 days. Previous studies have discovered that broad-spectrum antimicrobial agents customarily used for the treatment of bacterial infection in patients and animals substantially alter the bacterial flora of the gut, and thus facilitate the uninhibited growth of yeasts.^[11] Among these agents, ceftriaxone is excreted in the bile, resulting in high intestinal concentrations, and can significantly increase the alimentary tract colonization by yeasts in experimental animals and human.^[12] Antineoplastic agents causing neutropenia and mucositis, such as cyclophosphamide, damage the natural host defenses and predispose the patients to disseminated fungal infection.^[12] Our study found that the rate of optimistic infection of *C. albicans* did not decrease with the extension of ceftriaxone exposure time, implying that over use of broad-spectrum antimicrobial agents was an important risk factor of optimistic infection of *C. albicans*, and this harm could not be reduced with the prolonging of time.

Studies in both animal models and humans have documented that gut permeability is increased shortly after the onset of pancreatitis, and clinically the magnitude of

Table 2. Sequence alignment of the PCR products

Groups	Blood	Intestinal contents	Jejunum	Colon	Lymph nodes	Pancreas
Group CC (5 days)						
Blood	—	99.6	99.6	100.0	—	—
Intestinal contents	99.6	—	99.4	75.8	—	—
Jejunum	99.6	99.4	—	99.4	—	—
Colon	100.0	75.8	99.4	—	—	—
Group CC (10 days)						
Blood	—	97.8	99.6	98.3	—	—
Intestinal contents	97.8	—	99.6	98.3	—	—
Jejunum	99.6	99.6	—	98.3	—	—
Colon	98.3	98.3	98.3	—	—	—
Group CS (5 days)						
Blood	—	98.1	100.0	100.0	—	—
Intestinal contents	98.1	—	100.0	99.8	—	—
Jejunum	100.0	100.0	—	100.0	—	—
Colon	100.0	99.8	100.0	—	—	—
Group CS (10 days)						
Blood	—	—	—	—	—	—
Intestinal contents	—	—	99.4	89.8	—	—
Jejunum	—	99.4	—	92.1	—	—
Colon	—	89.8	92.1	—	—	—
Group CCS (5 days)						
Blood	—	—	100.00	99.8	—	92.1
Intestinal contents	—	—	100.00	99.8	—	90.7
Jejunum	100.00	100.00	—	99.8	—	96.1
Colon	99.8	99.8	99.8	—	—	99.4
Pancreas	92.1	90.7	96.1	99.4	—	—
Group CCS (10 days)						
Blood	—	99.4	99.6	99.4	98.2	—
Intestinal contents	99.4	—	99.8	100.0	99.4	—
Jejunum	99.6	99.8	—	99.8	95.6	—
Colon	99.4	100.00	99.8	—	95.8	—
Lymph nodes	98.2	99.4	95.6	95.8	—	—
Group C (5 days)						
Blood	—	—	—	—	—	—
Intestinal contents	—	—	99.0	99.8	—	—
Jejunum	—	99.0	—	98.7	—	—
Colon	—	99.8	98.7	—	—	—
Group C (10 days)						
Blood	—	—	100.0	99.8	—	92.1
Intestinal contents	—	—	—	—	—	—
Jejunum	—	—	—	—	—	—
Colon	—	—	—	—	—	—

gut barrier failure has been shown to correlate with the development of sepsis, multiple organ failure syndrome, and an increased risk of death.^[13] In SAP, waterfall-style release of inflammatory factors such as TNF- α led to ischemia-reperfusion injury of gut mucosa which resulted in serious oxidative stress and activation of caspase-3 pathway and severe apoptosis of gut mucosa.^[8] However, in our study, although SAP could have a destructive effect on the intestinal mucosa barrier as shown in the pathological images (Figure 2), the rate of opportunistic colonization and blood stream dissemination of fungi in groups CCS and CS did not increase compared with group CC ($P>0.01$). Unlike bacteria, which were thought to be the main cause of superinfection leading to pancreatic necrosis that peaks during the first 4 days following the onset of symptoms, *C. albicans* could not break through intestinal mucosa barrier and invade into distant organs as free as bacteria after SAP, suggesting that inherent intestinal mucosa still keeps its barrier function against the yeast in some ways.^[14,15] Hence, when clinicians are faced with infection after SAP, pathogenic bacteria, not

fungi, should be taken into first account.

Molecular typing of an infectious agent is important for epidemiological studies and for the development of appropriate infection control strategies. Different methods have been developed to differentiate isolates of *C. albicans* and to determine the relationships between the genotypes and the diseases.^[16,17] McCullough developed a PCR-based method using a primer pair designed to span the region covering the site of the transposable group I intron of the 25S rRNA gene (rDNA).^[9] This method has been shown to be able to classify *C. albicans* strains into 5 genotypes based on the length of the amplified PCR product as genotype A (450 bp), B (840 bp), C (450 and 840 bp), D (1 080 bp), and E (1 400 bp).^[9] The classification of genotypes using this form of PCR relies on the presence of group I introns of varying sizes in the 25S rRNA. The method detecting the presence and the size of the intron in the 25S rDNA is particularly easily adapted for use in reference laboratories for the rapid identification of large numbers of isolates.^[16] In this study, a PCR primer pair designed to span the 25S rRNA gene was employed to genotype *C. albicans* isolated from various anatomical sites of infected rats. The results showed that the PCR products appeared as a single band at 450 bp, which meets the criteria of genotype A and in accordance with the researches of Adachi.^[18–20] Previous studies have shown that *C. albicans* of genotype A are more prone to invade the blood stream and more virulent than the other genotypes.^[19] Meanwhile, *C. albicans* of genotypic subgroup A were the dominant strains in the biofilm and more resistant to the antifungal agent flucytosine.^[9,21] The prevalence of genotypes B and C was higher in non-invasive *C. albicans* strains. In our research, the PCR products of *C. albicans* isolated from multiple tissues were a single band of 450 bp, suggesting that *C. albicans* of genotype A were more likely to proliferate and invade into important organs in the immunocompromised rat model. Meanwhile, it also indicated that genotype A may have a greater tendency to be transmitted between rats in the same environment.^[22] The present study confirmed the invasiveness of *C. albicans* of genotype A and indicated that *C. albicans* originated from intestine tract would be more resistant to the antifungal agent flucytosine. When antifungal agents are used for the treatment of *C. albicans* infection in such patients, the antifungal effect should be evaluated, and the dose should be optimized.

Researchers commented that the presence or absence of transposable group I intron in 25S rDNA gene would be important in the detection of invasiveness of *C. albicans*.^[19] The aim of this study was to determine the correlation between *C. albicans* isolated from multiple

tissues of infected rats. By amplifying and comparing the sequence of PCR products of *C. albicans* isolated from each cultured tissue (blood, mesenteric lymph nodes, pancreas, intestinal content, jejunum, and colon), we found that the amplified gene segments were almost same regardless the anatomical sites of isolation, suggesting that these *C. albicans* might be originated from the same strain or translocated from the same infected source. Therefore, GI remains to be an important source for disseminated infections of *C. albicans* in immunocompromised rats.

In conclusion, GI is an important source for *C. albicans* of genotype A to break through the intestinal mucosa barrier and translocate to other organs. The damage of immune system and broad-spectrum antimicrobial agents are important risk factors for opportunistic fungal infection. Clinically significant *C. albicans* infection could not be induced by pure stress factors like SAP. To understand the mechanisms of *C. albicans*-bacterial interactions in the circulation and exploit it for therapeutic strategies, further studies using *in vitro* or animal model systems as well as large-scale, multicentre clinical data are needed.

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Ethical approval: All applicable international, national, and/or institutional guidelines for the care and use of animals were followed. All procedures performed in studies involving animals were in accordance with the ethical standards of the institution or practice at which the studies were conducted.

Conflicts of interest: All authors have seen and approved the content of "Instructions to Authors" and the submission version of the paper. All authors declare that they have no conflict of interest.

Contributors: Zhao XW conceived the study. Lei Y analyzed all data and wrote the final version of the manuscript. Dan X, Cui YH and Yang CH assisted in animal experiment. Zhou YJ participated in study design and coordination. Tang JG helped to draft the manuscript. Zhao XW and Yan L contributed equally to this paper.

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