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Coculture with *Clostridium difficile* promotes apoptosis of human intestinal microvascular endothelial cells

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Abstract

Objective: The clostridial triose-phosphate isomerase (*tpi*) gene is a housekeeping gene that specifically distinguishes *Clostridium difficile* from other bacteria. This retrospective cohort study was performed to analyze and compare the TPI protein-positive rates in outpatients and hospitalized patients with and without diarrhea (control group).

Methods: Western blotting, methylthiazolyldiphenyl-tetrazolium bromide (MTT) assay, and flow cytometry were used to investigate the pathogenic mechanism of *C. difficile* in the development and progression of diarrhea in patients with inflammatory bowel disease (IBD).

Results: The TPI protein-positive rates were significantly higher in patients with diarrhea but without IBD than in the healthy control group as well as in patients with diarrhea and IBD than in patients with diarrhea but without IBD. Coculture with *C. difficile* inhibited aquaporin-1 protein expression in human intestinal microvascular endothelial cells, which significantly reduced the proliferation of these cells and promoted their apoptosis.

Conclusions: *Clostridium difficile* infection is associated with diarrhea and may be an important risk factor for diarrhea in patients with IBD. Coculture with *C. difficile* may inhibit the proliferation of intestinal mucosal cells and promote their apoptosis, reduce intestinal aquaporin-1 expression, and inhibit intestinal water uptake. *Clostridium difficile* is one cause of *C. difficile*-associated diarrhea.

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Introduction

Infections pose a global threat owing to the emergence of antibiotic-resistant strains. During the past 30 years, the incidence rate *of Clostridium difficile* infection (CDI) has increased worldwide. *Clostridium difficile* is a Gram-positive, spore-forming, anaerobic bacillus. *Clostridium difficile* itself is noninvasive, although some toxigenic strains cause antibiotic-associated diarrhea, colitis, and even fatal pseudomembranous enteritis (collectively termed CDI) through secretion of toxin A, toxin B, and binary toxin.[1] This bacterium considered the major cause of hospital-acquired colitis in patients receiving broad-spectrum antimicrobials or other drugs such as proton pump inhibitors, immunosuppressives, and cancer therapeutics. CDI is also responsible for the exacerbation of inflammatory bowel disease (IBD).[2] IBD is an umbrella term used to describe disorders characterized by chronic inflammation of the digestive tract.[3] Types of IBD include ulcerative colitis and Crohn's disease.[3] Signs and symptoms common to IBD include diarrhea, fever and fatigue, abdominal pain and cramping, blood in the stool, reduced appetite, and unintended weight loss.[4] More than 1 million residents in the United States and 2.5 million in Europe are estimated to have IBD, which is associated with substantial health care costs that can impede patients' career aspirations and reduce their quality of life.[4,5]

Triose-phosphate isomerase (TPI) is an enzyme involved in glycolysis and is a protein specific to *C. difficile*. TPI catalyzes the reversible reaction between dihydroxyacetone phosphate and glyceraldehyde-3-phosphate and thus plays important roles in glycolysis, fatty acid synthesis, gluconeogenesis, and pentose metabolism in *C. difficile*.[6,7] As a housekeeping gene in *C. difficile*,[8] *tpi* can be used to specifically distinguish *C. difficile* from other bacteria.[9] Studies have shown that the highly specific *tpi* gene can be used for rapid screening of *C. difficile* in clinical samples.[10,11]

Human endothelial cells that line the microvasculature play an important "gatekeeper" role in the inflammatory process.[12] They can recruit circulating immune cells into tissues and foci of inflammation.[13] Previous studies have shown that human intestinal microvascular endothelial cells (HIMEC) play a critical regulatory role in inflammation and participate in physiological and pathological events in the gut.[14] However, little data currently exists on how *C. difficile* interacts with HIMECs and influences pathological events in patients with diarrhea.

In this retrospective cohort study, we compared the TPI protein-positive rates in patients with and without diarrhea. We also investigated the effect of *C. difficile* on aquaporin-1 (AQP1) expression in HIMECs and its effects on HIMEC proliferation and apoptosis. Our results might be helpful for understanding the underlying mechanism of the influence of *C. difficile*

on HIMECs and the associated pathological events that occur in patients with diarrhea.

Methods

Patients

From November 2014 to May 2017, stool samples were randomly collected from outpatients and inpatients at Jiangsu People's Hospital, Huai'an First People's Hospital, and Nanfang Hospital affiliated with Southern Medical University in China. All samples were collected within 24 hours after the patients arrived at the hospital and were stored at −80°C prior to use. The inclusion criteria were as follows: the diagnosis of diarrhea met the Diagnostic Criteria for Infectious Diarrhea (WS271-2007, China), and the diagnosis of IBD was confirmed by endoscopy and consistent with the Consensus on Diagnosis and Treatment of Inflammatory Bowel Disease by the Chinese Medical Association (Guangzhou, 2012). The exclusion criteria were as follows: other intestinal infectious diseases (e.g., cholera, dysentery, typhoid, paratyphoid) and a history of chronic diarrhea.

Cell culture

HIMECs were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 15% fetal bovine serum, 100 U/mL of penicillin and streptomycin, 0.584 g/L of glutamine, and 100 mg/L of endothelial cell growth supplement in a 37°C incubator under 5% carbon dioxide (CO_2). The cells were treated with 0.25% trypsin and 0.02% EDTA and passaged twice. Cells that had undergone stable passage were used for the experiments. Morphological changes of the cells were observed under a light microscope, and the subcultured cells were allowed to grow to 70% to 80% confluence 12 to 36 hours before treatment with the bacteria. Primary cells in good growth condition were preserved in cryoprotectant medium and stored in liquid nitrogen for subsequent use. HIMECs were maintained in six-well plates in DMEM containing 15% fetal bovine serum; the plates were then washed with phosphate-buffered saline three times. Next, 500 μL of bacterial solution in the negative control group (10^8 CFU/mL *tpi*-negative *C. difficile*), low-density group (10^8 CFU/mL *tpi*-positive *C. difficile*), and high-density group (2×10^8 CFU/mL *tpi*-positive *C. difficile*) was added into each well and cocultured for 12 hours in a 37°C incubator under 5% CO_2. Warm sterile phosphate-buffered saline was then used to wash each well five times to remove *C. difficile* from the cells, which were later detached via trypsin for flow cytometry and western blotting.

Standard bacterial strain culture and tpi polymerase chain reaction amplification

The standard *C. difficile* strain ribotypes 001, 02, and 078 were preserved in the laboratory of Nanfang Hospital of Southern Medical University. Cycloserine-cefoxitin fructose agar (CCFA) broth and CCFA agar plates were prepared according to the user's manuals of the reagents and stored at 4°C until use (within 1 week). Polymerase chain reaction (PCR) was used to detect the specific *tpi* gene using a 40-μL reaction system containing 1 μL each of the upstream and downstream primers, 20 μL of premixed rTaq DNA polymerase, 16 μL of double-distilled water, and 2.0 μL of the DNA template. The PCR reaction conditions for *tpi* have been previously described in the literature.[15] The PCR product analysis was conducted as follows: 5 μL of the PCR product was electrophoresed in a 1.5% agarose gel (100 V, 100 mA, 35 minutes), and the gel was then stained with ethidium bromide for 20 to 30 minutes. The results were observed with a

gel electrophoresis imager. Positive amplification products were sent to Shanghai Baotong Genomics for sequencing. The bacterial isolates were cultured as follows: the TPI-positive stool specimens were inoculated into CCFA broth containing 0.1% sodium taurocholate and incubated at 37°C for 7 days in an aerobic environment. An equal volume of absolute ethanol was added to the culture, and the mixture was incubated at room temperature for 1 hour followed by centrifugation at 3000 rpm for 10 minutes. The supernatant was discarded. The precipitate was then inoculated into CCFA medium and placed in a self-sealed plastic bag, which was placed in an anaerobic atmosphere generation bag, sealed, and placed in a CO_2 incubator at 37°C for 48 hours. The bacterial isolates were preliminarily characterized according to their colony morphologies and foul odor. Suspected colonies were picked and inoculated into CCFA medium for isolation via anaerobic culture; the colonies were also inoculated onto standard plates and cultured under aerobic conditions. Bacterial isolates that were isolated according to their morphologies and odor characteristics were immediately picked with a sterile cotton swab, eluted with 20% skim milk and sterile distilled water, and then stored at −70°C as a stock until use. The *tpi* was detected using PCR with genomic DNA extracted from the cultured *C. difficile* isolates using a genomic DNA extraction kit.

MTT assay

HIMECs in the exponential growth phase were treated with 2.5 g/L of trypsin and 0.2 g/L of EDTA in a 1:1 ratio and adjusted to a density of 1×10^8/L with RPMI 1640 medium. The cells were seeded into a 96-well plate with 80 μL per well followed by the addition of 80 μL of medium. Next, the cells were cultured at 37°C with 5% CO_2 for 24 hours. The medium was replaced with serum-free medium, and the cells were cultured for an additional 36 hours. Before the end of culture, 40 μL of methylthiazolyldiphenyl-tetrazolium bromide (MTT) was added to each well at a final concentration of 1 g/L followed by culture in the dark. On the day of the MTT assay, all supernatants from the duplicate wells were discarded, and dimethyl sulfoxide was added at 100 μL/well; the plate was then shaken for 10 minutes on a microplate shaker. Wells with medium alone were used as blank controls and set as zero. The optical density was measured at 490 nm.

Flow cytometry

HIMECs in the exponential growth phase (2×10^9/L) were treated with 2.5 g/L of trypsin and 0.2 g/L of EDTA in a 1:1 ratio. The supernatant was aspirated at the end of the culture, followed by digestion with 2.5 g/L of trypsin and 0.2 g/L of EDTA in a 1:1 ratio. The cells were then collected through centrifugation at 1000 rpm for 10 to 15 minutes, fixed with 1.5 mL of cold ethanol overnight, and subjected to flow cytometry.

Western blotting assay

Proteins from the cells were isolated with RIPA lysis buffer (Beyotime, Shanghai, China) containing protease inhibitor. The protein homogenate was centrifuged at 4°C and 12,000 rpm for 30 minutes. Primary antibodies were incubated on the membranes in Tris-buffered saline with Tween 20 at 4°C overnight, followed by incubation with secondary antibodies for 2 hours at 37°C. The florescence was detected with an electrochemiluminescent detection system (UVP Inc., Cambridge, UK) using enhanced chemiluminescence detection reagents. To confirm equal protein loading, the gels were stripped and reprobed with

antibodies to β-actin (Santa Cruz Biotechnology, Dallas, TX, USA).

Ethics

Ethics approval was obtained from the ethics committees of all three hospitals involved in the present study. All patients provided informed consent.

Statistical analysis

Each assay was performed in triplicate and repeated three times independently. All data were analyzed with Graph PRISM 6.0 (GraphPad, Inc., San Diego, CA, USA). Continuous variables were analyzed by Student's t-test or one-way analysis of variance. A P value of <0.05 was considered statistically significant.

Results

Significantly higher C. difficile TPI-positive rates in patients with diarrhea

Of the 509 stool samples obtained from the 509 patients enrolled in the study, 39 samples were positive for TPI, indicating that these individuals carried *C. difficile* in their intestines. The patients with diarrhea exhibited a significantly higher TPI-positive rate than the patients in the control group ($P < 0.01$) (Table 1).

Significantly higher TPI-positive rates in patients with IBD-associated diarrhea

The TPI-positive rate was significantly increased in the patients with IBD-associated diarrhea ($P < 0.01$), suggesting that the TPI protein was likely related to IBD-associated diarrhea (Table 2). However, the mechanism of this association requires further investigation.

Table 1. TPI detection in all patients.

	All patients		
Clinical feature	TPI (+) n = 39	TPI (−) n = 470	P value
Diarrhea			<0.01
−	37	275	
+	2	195	

TPI, triose-phosphate isomerase

Table 2. TPI detection in patients with diarrhea.

	Patients with diarrhea		
Clinical feature	TPI (+) n=39	TPI (−) n=273	P value
IBD			<0.01
+	9	35	
−	30	238	

TPI, triose-phosphate isomerase; IBD, inflammatory bowel disease

Effect of C. difficile on the cell cycle, proliferation, and apoptosis of HIMECs

The effect of *tpi*-positive *C. difficile* on HIMECs was studied through coculture of different concentrations of *tpi*-positive *C. difficile* with HIMECs (blank group: no *C. difficile* added, negative control group: 10^8 CFU/mL of *tpi*-negative *C. difficile*, low-density group: 10^8 CFU/mL of *tpi*-positive *C. difficile*, and high-density group: 2×10^8 *tpi*-positive *C. difficile*). After coculture with *tpi*-positive *C. difficile*, the HIMEC proliferation rate was decreased in each group. A stronger inhibitory effect on cell proliferation was observed in the high-density than low-density group (Figure 1(a)). Changes in the cell cycle and apoptosis indices of the HIMECs were analyzed using flow cytometry after treatment with *tpi*-positive *C. difficile*. The flow cytometry results showed that the cell cycle of the

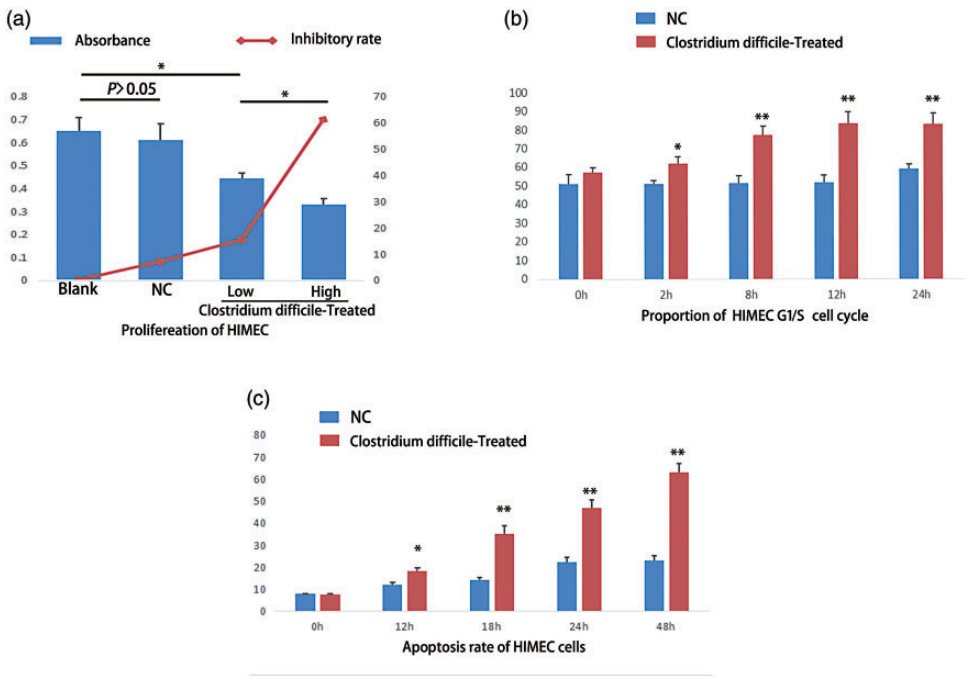

Figure 1. Coculture with *C. difficile* blocks the cell cycle and proliferation and promotes apoptosis of HIMECs. (a) HIMECs showed significant proliferative inhibition after coculture with *C. difficile* when compared with the NC group. (b) A significantly higher percentage of HIMECs were arrested at the G_1/S phase of the cell cycle after coculture with *C. difficile* when compared with the NC group. (c) A significantly higher percentage of HIMECs underwent apoptosis after coculture with *C. difficile* when compared with the NC group. HIMECs, human intestinal microvascular endothelial cells; NC, negative control. *$P < 0.05$ (two-sided); **$P < 0.01$.

HIMECs in the coculture group was arrested at the G_1/S phase (Figure 1(b)). The apoptotic index of the HIMECs was significantly increased after 12 hours of coculture with *tpi*-positive *C. difficile* ($P < 0.05$) (Figure 1(c)).

The changes in Bcl-2 and Bax protein expression were examined by western blotting in HIMECs following coculture. Compared with the control and blank control groups, the relative AQP1 expression level was downregulated in the HIMECs after 18 hours of coculture with high-density *tpi*-positive *C. difficile* (Figure 2). Bcl-2 protein expression was downregulated in the HIMECs after 8 hours of incubation with *tpi*-positive *C. difficile* (Figure 2). In contrast, Bax protein expression in the HIMECs was upregulated after 8 hours of incubation with *tpi*-positive *C. difficile* (Figure 2). The changes in AQP1, Bcl-2, and Bax protein expression in the HIMECs were more obvious in the high-density than low-density group. Taken together, our results show that *C. difficile* is very closely associated with the cell cycle, proliferation, and apoptosis of HIMECs cocultured with *C. difficile*.

Discussion

Although *C. difficile* has been confirmed to be part of the normal gut flora of healthy newborn infants, some researchers have

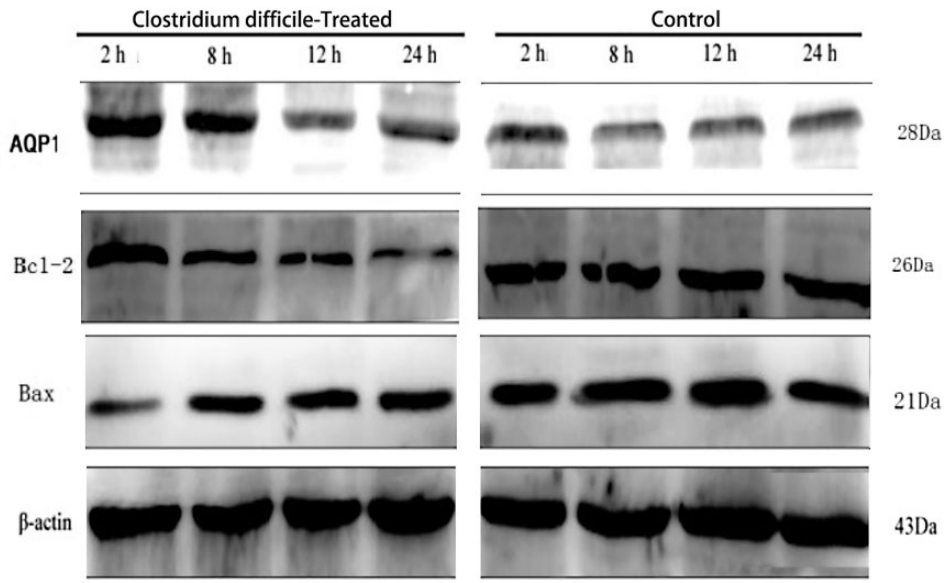

Figure 2. Effect of *C. difficile* on the AQP1 and apoptosis-associated (Bcl-2, Bax) protein expression of HIMECs. Western blot analysis demonstrated a decrease in AQP1 and Bcl-2 and an increase in Bax expression in HIMECs cocultured with *C. difficile*. β-actin served as a loading control. AQP1, aquaporin-1; HIMECs, human intestinal microvascular endothelial cells.

noted that such microorganisms can still produce pathogenic toxins and induce inflammatory reactions by secreting soluble exotoxins in the intestines.[16,17] *Clostridium difficile* can cause a series of clinical symptoms and diseases in humans, including diarrhea, toxic megacolon, and pseudomembranous enteritis. Studies have shown that the toxins TcdA and TcdB produced by *C. difficile* disrupt epithelial cell tight junctions and induce epithelial cell death, resulting in direct damage to the colonic epithelium.[18] In addition, these toxins stimulate the release of proinflammatory cytokines and neutrophil chemokines from colonic epithelial cells, leading to an acute endogenous inflammatory response through neutrophil recruitment.[19] Evidence from studies of *in vivo* poisoning suggests that toxin-induced inflammatory reactions may play an important role in mediating *C. difficile*-associated diarrhea and colitis.[20] However, no study has shown a relationship between *C. difficile* and HIMECs in the development and progression of diarrheal symptoms.

Our study showed significantly higher *C. difficile* TPI-positive rates in outpatients and inpatients with diarrhea than in the normal population. Among the patients with diarrhea, the *C. difficile* TPI-positive rate was higher in those with than without IBD.

In this study, different concentrations of *tpi*-positive *C. difficile* were cocultured with HIMECs. The HIMECs in the high-density coculture group showed proliferative suppression, cell cycle arrest, and apoptotic promotion compared with the low-density and control groups. Aquaporins are a family of proteins that specifically transport water and can significantly increase the permeability of the cell membrane to water; these proteins participate in the regulation

of intestinal water secretion and absorption and the balance of intracellular and extracellular fluid.[21] Many researchers believe that abnormal AQP expression in the intestine leads to disorders of absorption and secretion of water in the colon, and such disorders are involved in the occurrence of diarrhea and constipation.[22] The present study showed that the AQP1 protein was downregulated in HIMECs after coculture with *C. difficile* compared with the control group, suggesting that *C. difficile* influenced the occurrence and progression of diarrhea by suppressing AQP1 protein expression and inhibiting intestinal cell permeability.

In summary, determining the role of *C. difficile* in the occurrence and progression of diarrhea is significant for in-depth research of diarrhea and for efforts in the development of diarrhea treatments.

Declaration of conflicting interest

The authors declare that there is no conflict of interest.

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