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Original Article

Effect of Clostridium butyricum and its components in different concentrations on epithelial-mesenchymal transition of ulcerative colitis

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Abstract: Objective: To investigate the effect and potential mechanism of Clostridium butyricum (CB) in different concentrations on epithelial-mesenchymal transition (EMT) of ulcerative colitis. Methods: Fifty C57BL/6 mice were randomly divided as a control group, a DSS group, a low-dose, a middle-dose and a high-dose CB treated group. DAI, colon length, and tissue damage for each group was assessed. Expression of E-cadherin and Vimentin in the colon was detected by real-time qPCR and IHC. *In vitro*, the control, TGF-β1, low-dose, high-dose supernatant and dead CB groups were monitored for loss of cell polarity and expression of phenotype by real time qPCR and Western blotting. Results: Compared to the control group, DAI and colon length in the DSS group was significantly deteriorated (*P<0.05*) with most crypt loss and infiltrating inflammatory cells as significantly down-regulation of E-cadherin and up-regulation of Vimentin by IHC staining (*P<0.05*). Not like the high-dose CB group, DAI, colon length and tissue damage in the low-dose and middle-dose CB group was critically ameliorated on the final experimental day (*P<0.05*) with up-regulation E-cadherin and down-regulation vimentin. *In vitro*, supernatant and the dead CB group, especial the high-dose dead CB, could prevent loss of cell polarity. Dead and high-dose supernatant of CB could significantly down-regulate vimentin mRNA (*P<0.05*), when supernatant of both CB groups could up-regulate expression of E-cadherin mRNA by regulating TGF-β1 mRNA. At the protein level, high-dose supernatant of CB could increase expression of E-cadherin and decrease that of vimentin. Conclusion: Clostridium butyricum could dose-dependently suppress experimental colitis because of its components inhibiting EMT undergoing down-regulation TGF-β1.

Keywords: Clostridium butyricum, ulcerative colitis, EMT, dose-dependent

Introduction

Ulcerative colitis (UC) is an inflammatory bowel disease (IBD), characterized by abdominal pain, diarrhea, and bloody stool and severely threatens human health. However, the pathogenic mechanism is not clear [1]. Recent studies show that UC is associated with loss of intestinal epithelial cells (IECs) and fibrosis [2-4]. Dextran sodium sulfate (DSS) has been widely used to induce experimental colitis in order to study the mechanism of UC and evaluate the therapeutic effect. This process is similar to the clinical UC syndromes and pathogenic mechanisms of UC [5-8]. Currently, epithelial-mesenchymal transition (EMT) is considered as the major factor in the development of IBD because of a similar process in loss of IECs and improving the fibrosis. This process includes loss of epithelial phenotype, loss of cell polarity of epithelial cells, and transition to mesenchymal-like cells [9, 10]. Therefore, inhibiting EMT may improve the outcome of UC. Recent studies have suggested that probiotics are important medicines for treating UC because of regulating the colonic immune and keeping the colonic epithelium barrier intact [11]. Clostridium butyricum (CB), as the most important microflora in intestine, plays the important role in clinical UC and experimental colitis model. The mechanism of CB is associated with keeping the balance of microbiotic flora in the colon and energy

Table 1. Classification of histological damage scale in colon tissues

Inflammation	Extent	Crypt damage	Percentage involvement	Scores
None	None	None	None	0
Mild	Mucosa	Basal 1/3 damaged	1-25	1
Severe	Mucosa and submucosa	Basal 2/3 damaged	26-50	2
	Transmural	Only surface epithelium intact	51-75	3
		Entire crypt and epithelium lost	76-100	4

Table 2. Primer sequences of target genes in tissues

Genes	Primer sequences
TGF-β1	Forward 5'-AGCTGCGCTTGCAGAGATTA-3'
	Reverse 5'-CAGCCACTCAGGCGTATCAG-3'
Vim	Forward 5'-GCACTAGCCGCAGCCTCTAT-3'
	Reverse 5'-GCGAGAAGTCCACCGAGTCT-3'
E-cad	Forward 5'-GATCCTGAGCTGCCTCACAA-3'
	Reverse 5'-CAGCCTGAACCACCAGAGTG-3'

Table 3. Primer sequences of target genes in cells

Genes	Primer sequences
TGF-β1	Forward 5'-AATTCCTGGCGTTACCTTGG-3'
	Reverse 5'-TCTCCTTGGTTCAGCCACTG-3'
Vim	Forward 5'-GCACTGCCGCAGCCTCTAT-3'
	Reverse 5'-AGCGAGAAGTCCACGGAGTC-3'
E-cad	Forward 5'-GCTGCCACCAGATGACGATA-3'
	Reverse 5'-ACTTCCGGTCTGGCATCAAG-3'

production to restore the intact of epithelium which depends on the production of butyrate [12-14]. Here, we use DSS-induced colitis mice model to study the dose-dependent therapeutic effect of live CB on experimental colitis and EMT. Moreover, we also study the effect of potential elements of CB in inhibiting the cell-signaling mechanism of EMT.

Materials and methods

Main reagents

Fifty male six-week-old C57BL/6 mice, 18-20 g, were provided by Experimental Animal Center of Shandong University and all experiments were approved by the Animal Care and Use Committee of the Shandong University of Medical Science. Dextran sodium sulfate (36-50 kDa, Shanghai MP biomedical), human recombinant TGF-β1 (Peprotech), RNAprep Pure Cell/Bacteria Kit and RNAprep Pure Tissue Kit (TIANGEN BIOTECH Co.LTD), All-in-One™ First-Strand cDNA Synthesis Kit and All-in-One™ qPCR Mix (GeneCopoeia.Inc), anti-E-cadherin (24E10, CST) and anti-Vimentin (D21H3, CST) were obtained. Live Clostridium butyricum power (Ataining, East Sea Pharmaceutical Co.Ltd) was maintained in MRS culture medium under anaerobic condition at 37°C for 12 hours. The small intestinal cell line IEC-6 (ATCC, CRL-1592) was cultured in DMEM with 10% fetal bovine serum (FBS) and 100 U/ml penicillin G and 100 μg/ml Streptomycin (Solarbio) in 37°C cell culture box supplemented with 5% CO_2 and 95% air.

Methods

Animal model: Fifty male C57BL/6 mice were randomly divided in five groups (10 mice in each group), including the normal control group, DSS-induced colitis model (DSS group), low-dose Clostridium butyricum treated group (low-dose CB group), middle-dose Clostridium butyricum treated group (middle-dose CB group) and the high-dose Clostridium butyricum treated group (high-dose CB group). Except mice in the normal control group drinking with double distilled water for fourteen days, all other mice began drinking 3.5% DSS solution from Day 8 to Day 14 to induce animal UC model. Before drinking DSS solution, mice of low-dose, middle-dose and high-dose CB group separately received gastric intubation of 10^7 CFU/ml/mice, 10^8 CFU/ml/mice and 10^9 CFU/ml/mice for fourteen days [7, 14]. Based on Cooper et al. [15], disease activity index (DAI) was scored including weight loss (%), stool consistency and bleeding.

Sample collection and H&E staining: All mice were sacrificed with intraperitoneal injection of 50 mg/kg pentobarbital sodium after Day 14. The distal colon was collected 1 cm from anus until ileocecal valve and then measured the colon length. About 1 cm length of colonic mucosa was frozen in liquid nitrogen and the

Table 4. DAI score of mice at different times in groups (mean ± SD)

Groups	Day 11	Day 12	Day 13	Day 14
Control group	0	0	0	0
DSS group	0.40±0.33*	1.13±0.95*	2.83±0.52*	3.33±3.06*
Low-dose (10^7 CFU/mice) CB group	0.33	1.04±0.35	1.94±0.25Δ	1.96±0.92Δ
Middle-dose (10^8 CFU/mice) CB group	0.22±0.24	0.89±0.65	1.53±0.50Δ	2.33±0.42Δ
High-dose (10^9 CFU/mice) CB group	0.06±0.14Δ	0.93±0.25	2.44±0.65	2.52±0.17

Notes: *$P<0.05$ between control group and DSS group, Δ$P<0.05$ between DSS group and different doses CB treated group.

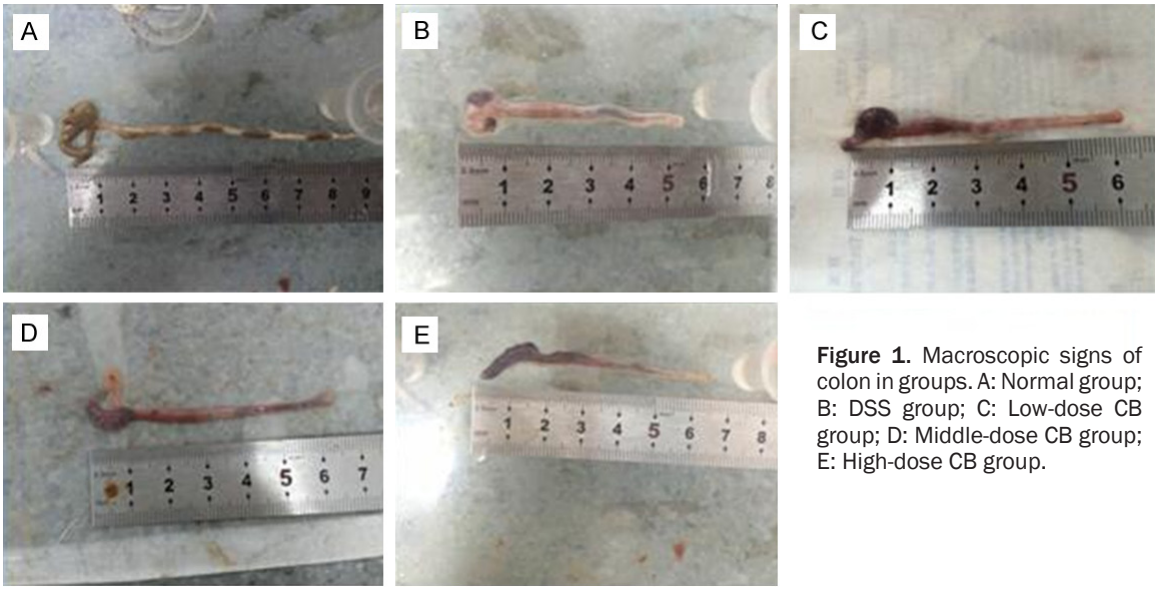

Figure 1. Macroscopic signs of colon in groups. A: Normal group; B: DSS group; C: Low-dose CB group; D: Middle-dose CB group; E: High-dose CB group.

Table 5. Colon length of mice in various groups (mean ± SD)

Groups	Colon length (cm)
Control group	6.85±0.76
DSS group	4.32±0.36*
Low-dose (10^7 CFU/mice) CB group	4.97±0.15Δ
Middle-dose (10^8 CFU/mice) CB group	5.41±0.3Δ
High-dose (10^9 CFU/mice) CB group	4.53±0.1

Notes: *P<0.05 between control group and DSS group, Δ$P<0.05$ between DSS group and different doses CB treated group.

other part was fixed with 4% paraformaldehyde for further paraffin embedding and slicing.

Histological damage scale of colon tissues in each group was assessed with H&E staining (**Table 1**) [15-18].

Immunohistochemistry (IHC) staining: Paraffin-embedded slices of colon tissues were deparaffinized, rehydrated, heat-induced antigen retrieval and hydrogen peroxidase blocked, thereafter, the sections blocked with appropriate bovine serum album (BSA) were treated with anti-E-cadherin (E-cad, 1:400) and anti-Vimentin (Vim, 1:100) overnight at 4°C and then incubated with secondary antibody kits (SP-9001, ZSGB-BIO) for 30 mins. Positive signals were visualized by DAB kit (ZLI-9017, ZSGB-BIO) and counter-stained with Mayer hematoxylin (G1080, Solarbio).

Real time qPCR for colon tissues: The total tissue RNA was extracted from fresh intestinal mucosa of mouse by RNAprep Pure Tissue Kit and then reversed into cDNA by using All-in-One™ First-Strand cDNA Synthesis Kit. The analysis of real time qPCR was used with All-in-one™ qPCR Mix by Step One Software V2.3PCR system (Thermo). The primer sequences were supplied by Sangon Biotech Shanghai Co.Ltd, with β-actin as reference gene and the other as target genes in **Table 2**.

Cell groups: IEC-6 cells were grown to 50% confluence and then incubated in fresh DMEM

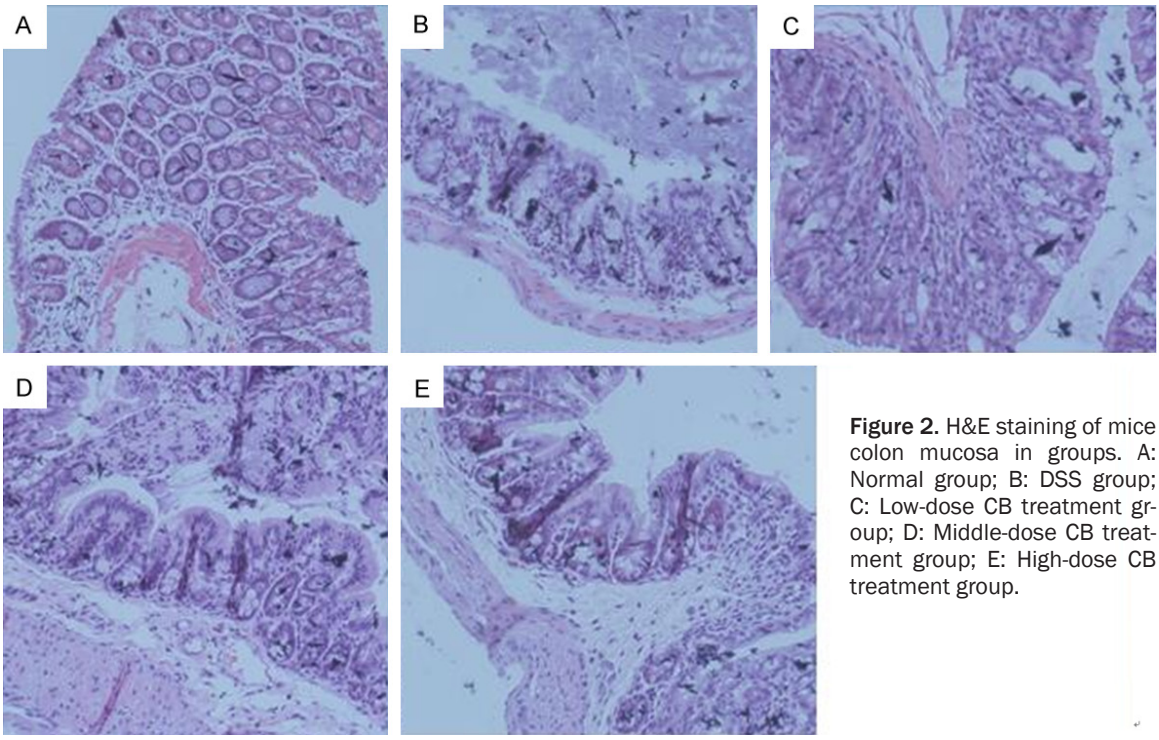

Figure 2. H&E staining of mice colon mucosa in groups. A: Normal group; B: DSS group; C: Low-dose CB treatment group; D: Middle-dose CB treatment group; E: High-dose CB treatment group.

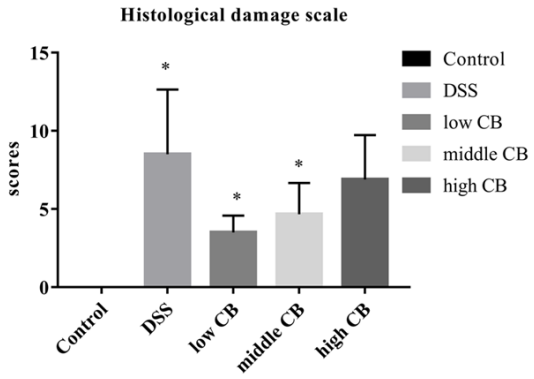

Figure 3. Histological damage scale of mice in the various groups, **P<0.05.*

without FBS and antibiotic overnight for further stimulation. The cells were divided into six groups, including the control group, 10 ng/ml TGF-β1-treated group, low-dose (10^6 CFU/ml) supernatant of CB treated group, high-dose (10^7 CFU/ml) supernatant of CB treated group, low-dose (10^6 CFU/ml) heat-killed CB treated group and the high-dose (10^7 CFU/ml) heat-killed CB treated group as in the previous studies [19, 20].

Real time qPCR for cells: The total RNA of cells in groups was extracted with RNAprep Pure Cell/Bacteria Kit and analyzed after being reversed into cDNA as the protocols of real time qPCR for tissues with the target genes in **Table 3**.

Western blotting: The cells of each group were rinsed in cold PBS and placed on ice with 80 μL lysis buffer per well (100:1 RIPA and PMSF). Cell lysates were centrifuged at 10,000 × rpm at 4°C for 15 minutes to collect the total cell protein. The heated protein was subjected to SDS-PAGE and electroblotted onto PVDF transfer membranes. The membranes were saturated for 2 hours at room temperature with 5% skim milk in 1 × TBST and incubated with mouse anti-human E-cadherin antibody (4A2. CST, 1:1000), rabbit anti-human Vimentin antibody (D21H3, CST, 1:1000) and monoclonal anti-β-actin antibody at 4°C overnight. Thereafter, the membranes were washed three times with 1 × TBST, and then incubated with 0.02% secondary antibodies in 5% nonfat milk. After three washes with 1 × TBST, the membranes were developed with ECL Western blotting detection reagents. Expression of E-cad and Vim protein in each group was valued by ImageJ software and Image Pro Plus6.0 software.

Statistical analysis: All data are presented as means ± standard deviation (SD) and under-

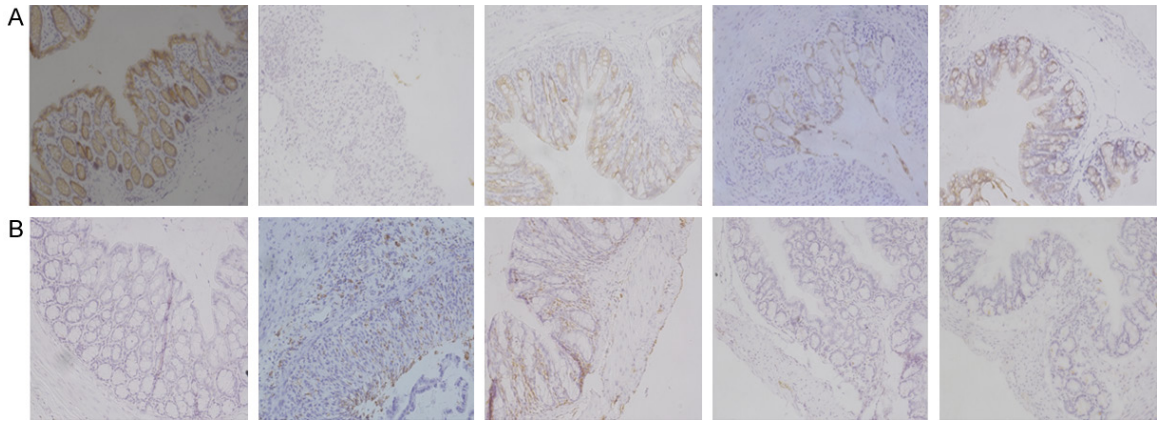

Figure 4. IHC staining of intestinal mucosa in groups (× 100). A: E-cad (normal group, DSS group, low-dose CB group, middle-dose CB group, high-dose CB group). B: Vim (normal group, DSS group, low-dose CB group, middle-dose CB group, high-dose CB group).

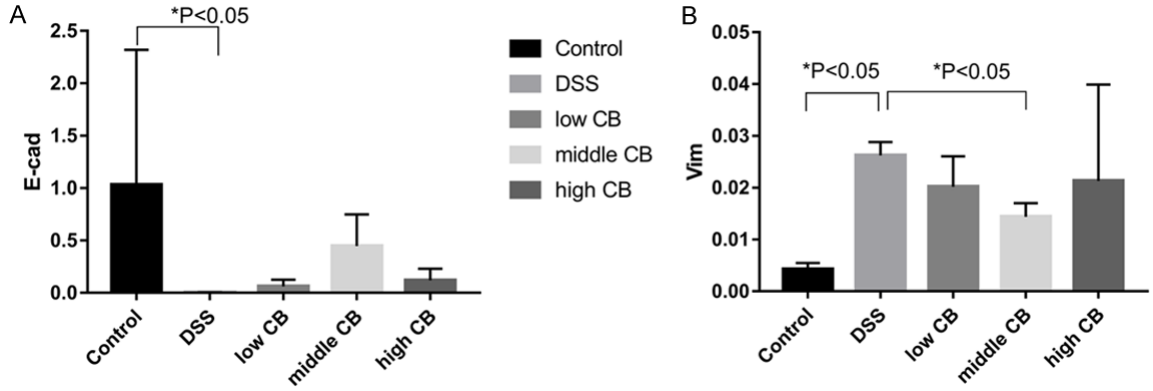

Figure 5. Expression of E-cad mRNA (A) and Vim mRNA (B) in colon tissue of various groups, **P<0.05.*

taken by SPSS 20.0 for statistical analysis. The statistical differences in groups for cells or mice were tested using one-way analysis of variance (ANOVA). Values of *P<0.05* were used as the criterion for statistical significance.

Results

DAI score and colon length

In our study, one mouse of middle-dose CB group died on Day 1 and one mouse of high-dose CB group died on Day 14. From Day 11, compared to the control group, the DAI of the DSS group became significantly increased (*P<0.05*). Compared to the colitis group, DAI of high-dose CB group significantly decreased on Day 11 (*P<0.05*) but this significant difference was instead by the low-dose and middle-dose CB group from Day 13 (*P<0.05*) in **Table 4**.

On Day 15, all mice were sacrificed to detect the macroscopic signs of colon tissue and measure the colon length. Mice in the control group had smooth surface of colonic mucosa and colon length was about 6.85±0.76 cm. However, for the DSS-induced group, the colonic mucosa became inflammatory edema with 4.32±0.36 cm colon length which was significantly eliminated compared to control group (*P<0.05*). After treatment with low-dose and middle-dose CB, the edema surface and the elimination of colon length was ameliorated compared to the DSS group (*P<0.05*). In the high-dose CB group, the mucosa surface of mice was still edema with 4.53±0.1 cm colon length (*P>0.05*), as in **Figure 1** and **Table 5**.

Histological features

In **Figure 2**, representative histological images of H&E stained colon sections from each group

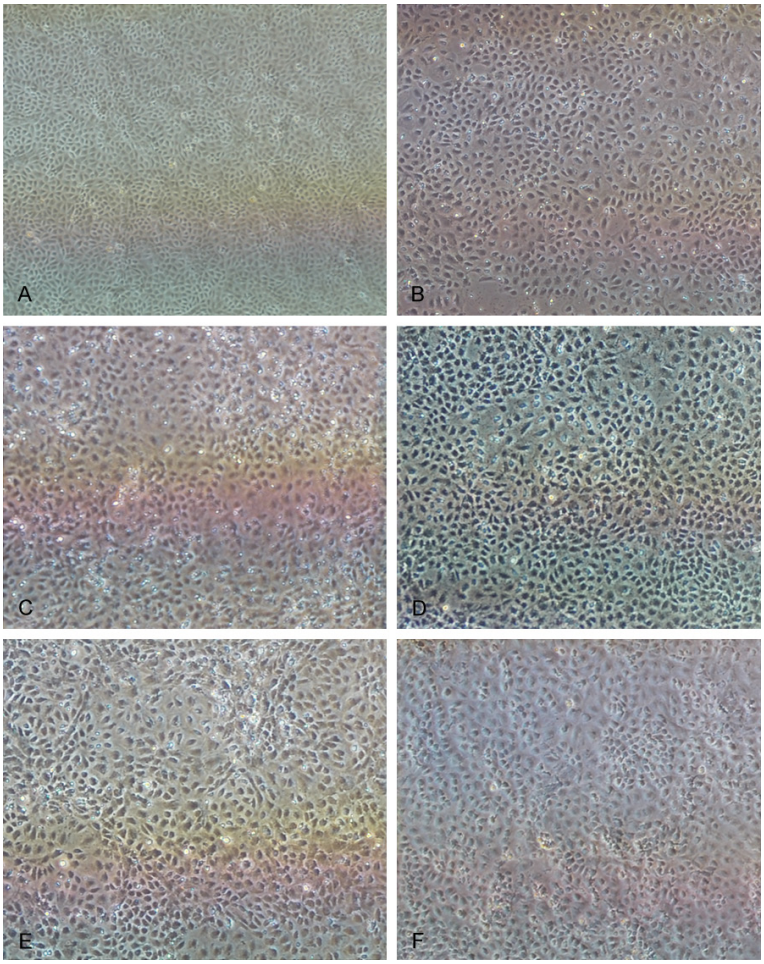

Figure 6. Morphology of cells in different treatment groups, × 200. A: Control group; B: TGF-β1-treated group; C: Low-dose supernatant of CB treated group; D: High-dose supernatant of CB treated group; E: low-dose heat-killed CB treated group; F: High-dose heat-killed CB treated group.

are shown. Compared to the control group (**Figure 2A**), the DSS group (**Figure 2B**) revealed that the entire crypt and epithelium was lost and remarkable infiltration of inflammatory cells was seen. After high-dose CB treated, the histology of tissues showed crypt regeneration but there was still remarkable infiltration of inflammatory cells (**Figure 2E**). In the low-dose and middle-dose CB group, crypt restoration and mild infiltration of inflammatory cells were observed (**Figure 2C** and **2D**) with significant elimination of histological damage scale ($P< 0.05$) in **Figure 3**.

E-cadherin (E-cad) and Vimentin (Vim) in intestinal tissue by IHC staining

For IHC staining in **Figure 4**, in contrast to the control group, expression of E-cad in intestinal epithelium was significantly down-regulated and Vim in mesenchyme was significantly up-regulated. In contrast to the DSS group, expression of E-cad was up-regulated in three CB groups, otherwise, Vim of middle-dose and high-dose CB group was significantly down-regulated.

E-cad mRNA and Vim mRNA in tissues

In **Figure 5**, compared with the control group, E-cad mRNA in the DSS group was significantly down-regulated and Vim mRNA was significantly up-regulated with $P<0.05$. In contrast to the DSS group, different doses CB treatment up-regulated expression of E-cad mRNA and down-regulated expression of Vim mRNA with significance in middle-dose CB group.

Cell morphology

IEC-6 cells in the control group were homogenous with a round-shaped appearance and displayed like cuboidal morphology with tight cell-junction. However, as TGF-β1-induced IEC-6 cells, they were uniformly spindle-shaped phenotype and loss of cell polarity and tight cell-junction. In contrast to the TGF-β1-induced IEC-6 group, both supernatant and the dead CB treated group could inhibit the EMT-like transformation of IEC-6 cells, especially treated by the high-dose heat-killed CB (**Figure 6**).

E-cad mRNA, Vim mRNA, and TGF-β1 mRNA of various cell groups by real time qPCR

In **Figure 7**, in contrast to control group, the expression of E-cad mRNA in TGF-β1-induced cell group was significantly decreased and the expression of Vim mRNA was significantly increased with $P<0.05$. The EMT-like cells after different doses supernatant and the dead CB group treatment showed expression of Vim

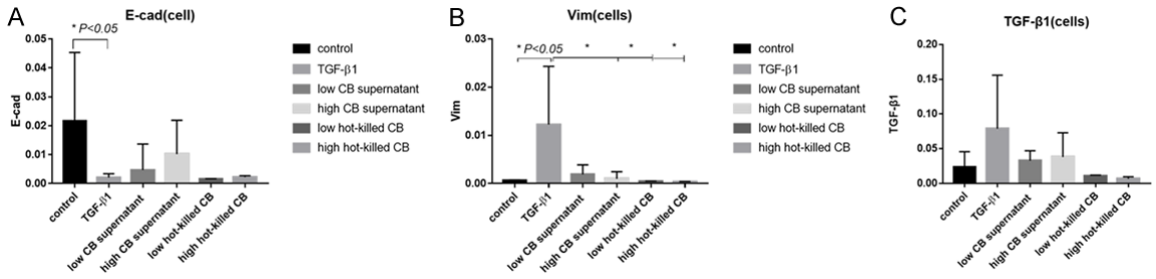

Figure 7. Expression of E-cad mRNA (A), Vim mRNA (B) and TGF-β1 mRNA (C) in various cell groups, **P<0.05.*

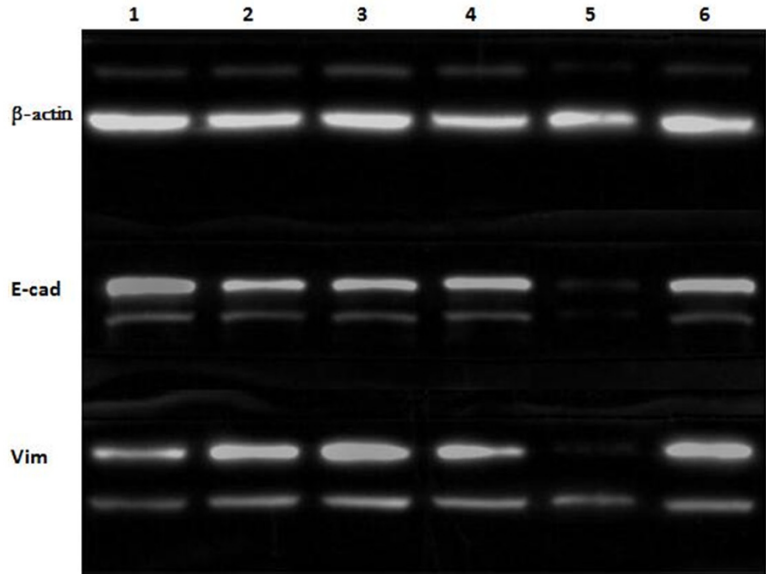

Figure 8. 1: Control group; 2: TGF-β1-treated group; 3: Low-dose supernatant of CB treated group; 4: High-dose supernatant of CB treated group; 5: Low-dose heat-killed CB treated group; 6: High-dose heat-killed CB treated group.

mRNA to be down-regulated significantly with *P<0.05*. For the effect on expression of E-cad mRNA of EMT-like cells, high-dose (10^7 CFU/ml) supernatant of CB showed the best, but the dead CB group had no effects.

E-cad and Vim of various cell groups by western blotting

In **Figure 8**, compared to the control group, protein expression of E-cad in the TGF-β1-treated group was decreased when Vim was increased. After different doses components treatment, the EMT-like cells in the high-dose CB group down-regulated expression of Vim and up-regulated expression of E-cad.

Discussions

Inflammatory bowel disease (IBD) is a group of intestinal inflammatory disorders characterized as loss of epithelial cells and tissue fibrosis. Although the precise etiology of this disease has not been elucidated, it has been suggested that environment, genetics, and immunity are involved in the pathogenesis accompanied with intestinal flora imbalance as an important factor [21, 22]. Clostridium butyricum, as one of intestinal flora, can produce high levels of short chain fatty acids (SCFAs) in anaerobic culture which can produce energy for intestinal epithelial cells and improve the proliferation of intestinal epithelium by infiltration into the intestinal mucosa barrier [23, 24]. Some studies report that butyric acids, as the major agent of SCFAs, mostly stay in the supernatant of bacteria and can active Treg cell of intestine mucosa, inhibit NF-κB signaling, and modulate TNF-α in order to induce apoptosis and make balance of intestinal immune environment [12, 25-27]. Moreover, butyric acids inhibit the inflammation of mucosa by down-regulating PU.1 and up-regulating TLR4 [28-31]. Some studies show that heat-killed bacteria can also protect the colitis by the correlation of bacteria peptide and pattern recognition receptors (PRRs) of intestinal epithelium to anti-inflammation and modulation of immune [32, 33].

In our study, we succeeded to induce experimental colitis model by 3.5% DSS and imitate the process of clinical UC. From the fourth day of DSS treated experiment, the mice began to appear loss of body weight, stool consistency, and occult blood or blood. DAI scores and the inflammatory damage scores increased fol-

lowed by the experimental days. After treatment by Clostridium butyricum (CB) in different doses, compared with the model group, the DAI score decreased as in the previous study. Moreover, in our study, we found that high-dose CB could significantly remedy the early experimental colitis on Day 11, but with the increasing of experimental days, the colitis remediation in low-dose and middle-dose CB groups became significant instead of the high-dose CB group ($P<0.05$). This may due to the different doses and PH of SCFAs induced by Clostridium butyricum in colon because the decent PH can give the intestinal epithelial cells the profitable environment and make them best metabolism.

Intestinal epithelial cells (IECs) deficiency and tissue fibrosis are the characteristics for IBDs, which are similar to the process of EMT [34]. EMT is a process of transition from epithelial cells to mesenchymal cells, including loss of epithelial cells polarity and tight cell-cell junction, down-regulation of expression of E-cadherin, and having fibrosis cells traits. *In vitro*, TGF-β and TNF are usually used to induce IECs EMT-like transformation [35-37]. *In vivo*, Clostridium butyricum in different doses could inhibit EMT in the C57BL/6 mice colitis model, including increasing the expression of E-cadherin and decreasing the expression of Vimentin. For the level of mRNA, 10^8 CFU/ml Clostridium butyricum could make the best therapeutic efficiency. As for the level of protein, 10^7-10^9 CFU/ml Clostridium butyricum could increase expression of E-cadherin and keep the intestinal epithelium intact. However, for inhibiting the process of fibrosis, the higher dose (10^8-10^9 CFU/ml) of Clostridium butyricum is better than the low-dose group. Furthermore, we also succeeded in inducing IEC-6 EMT-like transformation by 10 ng/ml TGF-β1 *in vitro*. Furthermore, we also found that components of Clostridium butyricum in different doses could keep the cell-cell junction intact, which is best for 10^7 CFU/ml heat-killed Clostridium butyricum. Moreover, 10^7 CFU/ml heat-killed Clostridium butyricum could increase expression of E-cadherin similar to the effect of live bacteria on the amelioration of epithelium damage. However, for real time qPCR, only the supernatant of bacteria, not the heat-killed bacteria, increased expression of E-cadherin mRNA and this effect was dependent on the bacteria dose which may be caused by SCFAs induced by the bacteria. Additionally, the higher dose of supernatant can make better in inhibiting the fibrosis of intestine including decreasing the expression of vimentin mRNA which may be caused by TGF-β1 signal pathways.

In conclusion, Clostridium butyricum can significantly ameliorate the experimental colitis and inhibit tissue damage and fibrosis, which is related to inhibition of EMT by potential components of Clostridium butyricum dependent on dose.

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