Therapeutic effects of Clostridium butyricum on experimental colitis induced by oxazolone in rats

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AIM: To evaluate the therapeutic effects of a probiotic supplement (Clostridium butyricum, CGMCC0313) in a chemically-induced rat model of experimental colitis.

METHODS: An experimental ulcerative colitis model was established by rectal injection of oxazolone into the colon of 40 Wistar rats randomly divided into four groups. The positive control group was sacrificed 3 d after colitis onset. The remaining groups were fed daily with either 2 mL of C. butyricum (2.3 x 10^{11} CFU/L), 2 mL of mesalamine (100 g/L), or 1 mL of sodium butyrate (50 mmol/L) for 21 d. The animals' body weight, and wet colon mass index were measured and recorded, and serum levels of interleukin-23 (IL-23) and TNF-α were measured using ELISA. Expression of calcitonin gene-related peptide in colon tissue was measured by RT-PCR. Finally, changes in rat intestinal microflora status were measured in all groups.

RESULTS: We found that treatment with C. butyricum lowered the serum levels of both IL-23 and tumor necrosis factor-α (TNF-α) with similar or even better efficiency than that of mesalamine or sodium butyrate. The rat intestinal flora appeared to recover more quickly in the group treated with C. butyricum than in the mesalamine and sodium butyrate groups. Finally, we found that the expression level of calcitonin gene related peptide was elevated in colon tissue in the sodium butyrate treated group but not in the C. butyricum or mesalamine treated groups, indicating a sensitization of colon following sodium butyrate treatment.

CONCLUSION: In our experimental colitis model, treatment with C. butyricum CGMCC0313, a probiotic supplement, is at least as efficient as treatment with mesalamine.

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Key words: Clostridium butyricum; Interleukin-23; Tumor necrosis factor-α; Calcitonin gene related peptide; Colitis

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INTRODUCTION

Inflammatory bowel disease (IBD) includes ulcerative colitis (UC) and Crohn's disease (CD). The incidence rate of ulcerative colitis increases annually in China[1,2]. A number of recent studies have been published that focus on the role of cellular inflammatory factors and related immune mechanisms during the onset and progression of IBD. Furthermore, animal experiments show that inflammation of the bowel can be induced by dysbiosis
of, or immune tolerance deficiencies related to, the intestinal microbiological flora\textsuperscript{[3,4].}

Steroid hormones, immunosuppressive agents or salicylic acid derivatives are used to treat IBD with modest results and often with serious side effects. Several recent studies, however, on the microecological therapy of UC using \textit{Clostridium butyricum} preparations show promising results and have been drawing some attention in the biomedical research community\textsuperscript{[5-9].} Sodium butyrate, although it is a confirmed anti-inflammatory agent for the treatment of experimental colitis\textsuperscript{[10-18]}, can cause non-inflammatory colonic hypersensitivity\textsuperscript{[19].} Calcitonin gene related peptide (CGRP) may increase organ sensitivity, and sensory afferents are implicated in peritoneal irritation of organs involved in inflammation\textsuperscript{[16-18].}

Based on animal experiments and clinical applications, we found that treatment of ulcerative colitis with \textit{C. butyricum}, CGMCC0313.1 live bacterium, gives good results both in animal and human ulcerative colitis\textsuperscript{[19-22]}; but, the mechanisms are not yet fully understood. As a part of our effort to elucidate the therapeutic mechanisms of \textit{C. butyricum} CGMCC0313.1, we used an oxazolone induced rat model of experimental colitis to measure the effect of intrarectally administered \textit{C. butyricum} CGMCC0313.1 and two treatment controls on a set of UC relevant parameters.

Oxazolone is a chemical allergen and a sensitizing agent. Using oxazolone to induce colitis in rat constitutes a more satisfactory animal model of UC with a high degree of similarity to the histopathological characteristics and distribution of inflammation described in human UC\textsuperscript{[23,24].}

Thus, we compared \textit{C. butyricum} CGMCC0313.1 with sodium butyrate and mesalamine (5-aminosalicylic acid, one of the standard prescriptions for ulcerative colitis) to measure each compound’s effects on the repair of intestinal walls, on serum concentrations of interleukin-23 (IL-23) and tumor necrosis factor-\(\alpha\) (TNF-\(\alpha\)), on the level of CGRP-mRNA in rat colon tissue, and restoration of the balance of the intestinal microflora.

\section*{MATERIALS AND METHODS}

\textit{Wistar SPF} (specific-pathogen free) rats with body weight of 160-180 g were purchased from the Experimental Animal Center of Qingdao Institute for Drug Control, China. Oxazolone was purchased from Alfa Aesar (Great Britain). Mesalamine was purchased from Adepha Drug Group (France). Sodium butyrate was purchased from Sigma-Aldrich Chemical Company (St. Louis, Missouri, USA). \textit{C. butyricum}, CGMCC0313.1 powder or capsules, were kindly provided by East Sea Biological Engineering Co. Ltd, Shandong, China. Trizol was purchased from Invitrogen (USA). The transcription kit was purchased from Promega (USA). Primers were synthesized by Shanghai Biological Engineering Co., China. The EasyTek PCR Amplifier Kit was obtained from Shanghai Biological Engineering Co., China. The IL-23 ELISA Kit and TNF-\(\alpha\) Kit was purchased from Wuhan Boster Biological Engineering Co. Ltd, China.

\subsection*{Establishing an animal model of experimental colitis}

Our model was based on a method previously described by Lamprecht \textit{et al}\textsuperscript{[28]} and modified by us. Briefly, we used 40 animals for testing. A 2 cm \(\times\) 2 cm area on the back of each animal was shaved to expose the skin. Using a cotton ball, 300 \(\mu\)L Oxazolone (5% in absolute alcohol) was applied topically on the exposed area to induce an allergic reaction. After 5 and 7 d, 450 \(\mu\)L of 5% oxazolone in a 50% ethanol solution, was injected using 1 mm diameter rubber tubing inserted into the colon through the rectum to about 8 cm proximal to the anal verge. To ensure even distribution of the oxazolone solution throughout the entire colon and cecum, the animals were kept in a vertical position for 45 s by holding them up by their tails after injections.

\section*{Groups and treatment}

After the induction of colitis, the 40 treated animals were randomly divided into four groups. Thus, 10 animals belonged to the positive control (PC) group and received no treatment (three of these animals died during the study). Ten animals were assigned to the positive drug control (mesalamine, MA) group and received mesalamine treatment (two animals died during the study). Ten animals receiving probiotic treatment with \textit{C. butyricum} CGMCC313.1 (\textit{C. butyricum}), were part of the probiotic (PB) group. Finally, 10 animals received treatment with sodium butyrate (two of these animals died during the study). A group of eight animals were kept as negative (NC) controls and received no oxazolone, nor any drug treatments. All animals in the mesalamine, probiotic and sodium butyrate groups were treated for 21 d, once per day, by feeding with either 2 mL of \textit{C. butyricum} (2.3 \(\times\) 10\(^7\) CFU/L), 2 mL of mesalamine (100 g/L), or 1 mL of sodium butyrate (50 mmol/L) \textit{via} an orogastric tube. During the test period, animal behavior, bowel movements, and body weight was observed and recorded once per week. After 21 d of treatment, all animals were sacrificed by decapitation. The colons were cut longitudinally then cleaned with physiological saline. Excess water was removed with filtration paper before measuring the colon wet mass. Index of wet colon mass = colon wet mass (g)/body weight (kg). The colon tissue was subsequently perfused with a 10% formalin solution, gradually dehydrated with ethanol, embedded in paraffin and sliced into 5 \(\mu\)m sections. The sections were wet mounted on glass slides and subjected to hematoxylin-eosin staining. Pathological changes in the animal tissues were identified under light microscope.

\subsection*{Measurement of IL-23 and TNF-\(\alpha\) in rat serum}

Blood was collected after decapitation and incubated overnight at 4°C. Serum was recovered after centrifugation and aliquoted. The aliquots were stored at -20°C for later tests. The serum levels IL-23 and TNF-\(\alpha\) were measured using ELISA kits strictly following the manufacturer’s instructions.

\subsection*{Extraction of total RNA from colon tissue}

Colon tissue (100 mg) was ground to powder in liquid
nitrger and 1 mL Trizol was added. After thorough mixing, the suspension was transferred into 1.5 mL Eppendorf tubes, kept in a -20°C freezer for 1-2 h, then stored in liquid nitrogen for later use.

Frozen 1.5 mL Eppendorf tubes were taken out from the liquid nitrogen and allowed to warm to room temperature for 5 min. Chloroform (200 µL) was added and the mixture was shaken for 20 s, incubated for 5 min at room temperature and spun for 15 min at 4°C and 10 000 r/min. Isopropanol (500 µL) was thoroughly mixed into the supernatant recovered after centrifugation. The solution was incubated for 10 min at room temperature and spun for 10 min at 4°C 10 000 r/min. The supernatant was discarded and the pellet was washed once in 75% ethanol and allowed to air dry for 10 min at room temperature. Finally, the pellet was dissolved in 30 µL DEPC-water. Three 5 µL samples were taken from the solution; one for OD measurement (20 × dilution), one for RNA content analysis, and one for agarose gel electrophoresis. The rest of the solution was stored in liquid nitrogen.

**Confirmation of CGRP expression using RT-PCR**

cDNA was prepared using the AMV reverse transcriptase reaction. Reverse transcription solution contained 4 µL 25 mmol/L MgCl₂, 2 µL 10 × PCR buffer, 2 µL dNTP, 0.5 µL recombinant RNasin, 0.7 µL AMV reverse transcriptase, and 1 µL dT oligo solution. RNA (3 µg) was added in each 20 µL reaction mixture. Reaction parameters were as follows: pre-denaturation for at 70°C 10 min; elongation at 42°C for 15 min; denaturation at 95°C for 5 min, and hold at 4°C for 5 min. Products were stored at -20°C.

PCR reaction mixtures (50 µL) were prepared containing 15 µL dH₂O, 25 µL 2 × PCR buffer, 1 µL MgCl₂, 3 µL cDNA, and 3 µL of each primer (final concentration: 1 µmol/L) before mixing and spinning briefly.

For CGRP, the PCR reaction conditions were as follows: initialisation at 94°C for 5 min followed by 35 cycles of denaturation at 94°C for 30 s, annealing for 45 s at 56°C, and extension for 45 s at 72°C. The final extension was 10 min at 72°C. For β-actin, the same process was used, except the annealing temperature was adjusted to 50°C. After completion of the PCR-protocols, 5 µL of the reaction mixture was analyzed with agarose gel electrophoresis (1.5%). The bands were visualized under UV light.

The sequences of the primers were CGRP (sense AGGGTCGGAGTGTCTGTA and anti sense ATCCGGTGGGTTAGCAGAG) and β-actin (sense ATCATGTGAGGACGCCAC and antisense CATCTTGGTCGAAGTCCA).

**Analysis of intestinal flora**

Feces samples were collected under sterile conditions directly from the rat’s rectum before and after induction of colitis and after the treatments were given. After weighing, the freshly collected feces was suspended in physiological saline (10 × w/v) and mixed well. Aliquots (100 µL) of the mixtures were spread evenly on selective medium surfaces. After incubating 48 h at 37°C (aerobic bacteria) or 72 h at 37°C (anaerobic bacteria) live bacteria was counted (CFU/g). Intestinal content was cultured separately on EMB, BBL, MRS, LEVY, and FS media.

**Data and statistical analysis**

All data were given as mean ± SD. Data were analyzed in SPSS11.5, using one-way ANOVA to perform the comparisons among groups, then using least significant difference test (LSD-b) to perform the multi-comparisons among means, P < 0.05 was considered a significant difference.

**RESULTS**

**Lower body weight, loose or bloody stool, and higher index of wet colon mass in rat colitis induced with oxazolone**

The body weight decreased dramatically in the positive control group compared to the negative control group. The treatment groups also showed body weight loss; but, it was not as dramatic as the positive control group (Table 1). During the initiation phase of the UC model, most rats in the experimental group had loose, greasy or watery, sometimes bloody stools. Stools became normal in all treatment groups. Thus, the clinical symptoms of UC obviously improved upon treatment in this animal model. The wet colon mass index in the mesalamine group, the _C. butyricum_ group and sodium butyrate group was clearly lower than the positive control wet colon mass index (P < 0.05) (Table 1).

**Pathological changes in oxazolone-induced experimental colitis in rats**

Animals in the positive control group were sacrificed three days after the experimental model was successfully established. Most pathological changes were found in the middle and lower part of colon with hyperemia and dropsy in the distal colon. Pathological changes were continuously distributed throughout the affected parts of the colon. Microscopic inspection of the tissues under light revealed abscessions of the mucous membrane epithelial cells, erosion of the mucous membrane and ulcer formation. We also noticed a decrease in the number of goblet cells and a disappearance or atrophy of the intestinal glands. Inflammation was located in or beneath the mucous membrane, although, in some cases, the muscle layers also appeared to be affected. Tissues were infiltrated mainly by lymphocytes, oxyphilic cells and plasmocytes; and less with neutrophil granulocytes. After treatment, the hyperemia-like characteristic of the mucous membrane improved, the swelling receded and the erosion healed. A few small ulcers were still discernible under microscopy, but the inflammatory cell infiltration, when apparent, consisted mainly of lymphocytes and acidophilic granular cells (Figure 1).

**Serum levels of IL-23 and TNF-α in rat experimental colitis induced with oxazolone**

The serum levels of IL-23 and TNF-α in the positive
control group was remarkably higher than the negative control group ($P < 0.05$, Table 2). After treatment, rat serum levels of IL-23 in \textit{C. butyricum}, mesalamine and sodium butyrate groups became much lower than the positive control ($P < 0.01$). The levels of TNF-\(\alpha\) in the \textit{C. butyricum} group was clearly lower than the positive control, while there was no significant difference in the mesalamine and sodium butyrate groups (Table 2).

**Sodium butyrate treatment increases CGRP expression levels**

CGRP expression levels in the negative control, positive control, \textit{C. butyricum} and mesalamine groups were weak, whereas the expression of CGRP in the sodium butyrate group was remarkably enhanced (Figure 2).

**Effect on intestinal flora of rats with oxazolone-induced experimental colitis**

In normal rats, intestinal \textit{Colibacter}, \textit{Bifidobacterium}, \textit{Acidobacterium}, \textit{Fusobacterium}, and \textit{Clostridium} grew well (Table 3). After UC was established in this animal model, the number of intestinal \textit{Bifidobacterium} and \textit{Acidobacterium} in the positive control group decreased in comparison to the negative control ($P < 0.01$), whereas the number of \textit{Colibacter}, \textit{Fusobacterium} and \textit{Clostridium} increased ($P < 0.01$). After treatment with \textit{C. butyricum}, mesalamine or sodium butyrate, the amount of intestinal \textit{Bifidobacterium} and \textit{Acidobacterium} increased relative to the positive control, whereas the number of \textit{Colibacter}, \textit{Fusobacterium} and \textit{Clostridium} decreased ($P < 0.01$ and $P < 0.05$). Compared with the \textit{C. butyricum} group, the amount of \textit{Colibacter} increased ($P < 0.01$ and $P < 0.05$), and the number of \textit{Acidobacterium} clearly decreased (all $P < 0.01$) in the mesalamine and sodium butyrate groups. The amount of \textit{Clostridium} in the mesalamine group was significantly lower than in the other groups ($P < 0.01$). The remaining bacterial groups showed no significant differences between the treatment groups (Table 3).

**DISCUSSION**

In our rat model of experimental colitis, the inflammatory disease is induced by intrarectal administration of oxazolone. Symptoms of inflammation of the distal rat colon included reddening and swelling of the mucous membrane. The continuously distributed pathological

### Table 1: Effects on body weight and wet colon mass index in rat colitis induced with oxazolone (mean ± SD)

| Group | n | Dose | Body weight (g) | Wet colon mass index (g/kg) |
|-------|---|------|-----------------|-----------------------------|
| NC    | 8 | N/A  | 191.3 ± 24.2    | 5.3 ± 1.2                   |
| PC    | 7 | N/A  | 149.0 ± 15.9\(^{d}\) | 9.1 ± 2.5                   |
| PB    | 10| 2.3 × 10\(^{11}\) CFU/L | 175.9 ± 43.2 | 6.7 ± 1.3\(^{\ast}\) |
| MA    | 8 | 100 g/L | 179.8 ± 18.4    | 6.9 ± 2.3                   |
| SB    | 8 | 0.05 mol/L | 175.8 ± 23.0   | 6.5 ± 1.6\(^{\ast}\)       |

\(^{a}\) $P < 0.05$ vs positive control; \(^{b}\) $P < 0.01$ vs negative control; NC: Negative control; PC: Positive control; PB: Probiotic (\textit{C. butyricum}); MA: Mesalamine; SB: Sodium butyrate.

### Table 2: IL-23 and TNF-\(\alpha\) levels in rat serum in oxazolone-induced experimental colitis

| Group ($n = 7$) | Dose | IL-23 (ng/L) | TNF-\(\alpha\) (ng/L) |
|----------------|------|--------------|----------------------|
| NC            | N/A  | 5.75 ± 2.51  | 15.93 ± 11.36        |
| PC            | N/A  | 43.94 ± 20.36 | 28.17 ± 6.10\(^{c}\) |
| PB            | 2.3 × 10\(^{11}\) CFU/L | 5.99 ± 1.88\(^{b}\) | 16.05 ± 10.54\(^{d}\) |
| MA            | 100 g/L | 8.81 ± 3.78\(^{a}\) | 23.54 ± 11.03        |
| SB            | 0.05 mol/L | 8.38 ± 4.48\(^{b}\) | 23.18 ± 6.48         |

\(^{a}\) $P < 0.05$, \(^{b}\) $P < 0.01$ vs PC; \(^{c}\) $P < 0.05$, \(^{d}\) $P < 0.01$ vs NC. PC: Positive control; NC: Negative control; PB: Probiotic (\textit{C. butyricum}); MA: Mesalamine; SB: Sodium butyrate.
changes included the loss of epithelia cells, erosion of the epithelial mucosal layer, ulcers, a decrease in the number of goblet cells and a decrease in gland density. The inflammation appeared to be located in or beneath the epithelial mucous layer, although in some cases the muscle layer showed infiltration of inflammatory cells. The wet colon mass index increased in the positive control group, as did the serum concentrations the of cellular inflammation markers IL-23 and TNF-α.

After treatment with *C. butyricum*, mesalamine or sodium butyrate, the swelling and reddening of the colon mucous membrane improved. The mucous membrane was almost healed, and the wet colon mass index decreased significantly. The serum content of IL-23 and TNF-α was remarkably decreased, almost back to the normal levels, and the balance of intestinal flora was restored.

IL-23 is a cellular factor, a new member of the IL-12 family. Similar to IL-12, it is a heterodimer, sharing the p40 subunit with IL-12. The p40 and p19 subunits form a covalently linked heterodimer via a disulfide bond. In addition to the interleukin receptor subunit 12Rβ1 shared with IL-12, IL-23 has its own special receptor subunit IL23R. Activated dendritic cells, macrophages, T cells and blast cells all generate large amounts of p19 mRNA with Th1 cells expressing more p19 than Th2 cells. Among these cell types, only activated dendritic cells and macrophages produce both the p40[20] and p19 required for the formation of IL-23, which is then secreted by these cells. Thus, human and rat IL-23 is mainly produced by activated dendritic cells[21]. IL-23 can induce mononuclear cells and macrophages to express inflammatory factors IL-1, IL-6 and TNF-α[28,29]. A recent study indicated that IL-23 is a necessary factor for the induction of chronic congenital or immune-modulated bowel diseases[30]. Hue et al[31] demonstrated that TH17 cells play a key role in mediating chronic spontaneous inflammation reactions, and that IL-23, but not IL-12, is essential for the induction of chronic bowel diseases. Together with IL-1, IL-23 can directly stimulate T helper cells to form TH17 competent cells that secrete IL-17. Interleukin 17 can enhance tissue inflammation reactions with the associated immune responses.

Several recent studies confirm a link between the IL-23 receptor IL23R and inflammatory bowel disease, both in Crohn's disease and ulcerative colitis patients[12-37]. In our model, the serum levels of IL-23 rose significantly in the oxazolone treated animals, while the IL-23 levels dropped after treatment with mesalamine, *C. butyricum* or sodium butyrate. The decrease in serum IL-23 was greater in the group treated with *C. butyricum* than in both the mesalamine group and the sodium butyrate group.

TNF-α is mainly a product of macrophages that can induce widespread immune responses in many cell types. TNF-α can induce production of IEC prostaglandin and increase the expression of inner epithelial adherent molecule-1 further by promoting inflammation. It can also stimulate production of extracellular proteases and matrix metalloproteinases from fiber cell promotion. These proteases can degrade the matrix of the mucous membrane causing epithelial cell abscissions[38]. Furthermore, TNF-α can increase the permeability of the intestinal epithelium by decreasing the expression of transmembrane core proteins associated with tight junctions[39]. This is an important early pathological change in the mucous membrane in both IBS and IBD. Araki et al[40] found that feeding *C. butyricum* can reduce intestinal mucous membrane wounds and the frequency of bloody diarrhea in rat-UC induced with dextran sodium sulfate (DSS). Also, Lu et al[41] found a positive correlation between the severity of disease and the levels of IL-6 and TNF-α in patients with active ulcerative colitis during their clinical trials. Wan et al[42] demonstrated that the expression of TNF-α rose remarkably before and clearly dropped after *C. butyricum* treatment in a rat-UC model induced by immunological challenge using colonic mucosal membrane protein. They also found that the treatment effects were better when *C. butyricum* was combined with mesalamine than

![Figure 2: Effect on CGRP expression in treated or untreated rat experimental colitis induced with oxazolone.](image-url)
mesalamine alone. Our data indicate that the serum concentrations of TNF-α were significantly increased in the oxazolone-treated animals when compared with the negative control group. After treatment with a *C. butyricum* preparation, the TNF-α level decreased. Mesalamine and sodium butyrate had similar, but lesser effects on the TNF-α levels in our study.

Calcitonin Gene-Related Peptide (CGRP) consists of 37 amino acids and is widely distributed throughout the central nervous system (CNS), particularly in the accessory nerves, with a high concentration in the dorsal root ganglia (DRG) of the spinal cord. Retrograde labeling and IP Western blots confirmed that primary sensory neurons in the DRG contain CGRP, whereas sympathetic neurons in the lateral horn lack CGRP expression in spinal cord neurons in a dose dependent manner. According to our data, treatment with *C. butyricum* leads to the recovery of the balance of the intestinal microflora of the experimental animals (Table 3). Da-rong Zhang et al[43] gave *C. butyricum* to patients suffering from irritable bowel syndrome. They found that *C. butyricum* suppresses the proliferation of putrefactive and pathogenic bacteria, while it promotes the proliferation of intestinal *Bifidobacterium* and *Acidobacterium* and other beneficial microbes. The amount of beneficial microbes was significantly increased in mouse feces after treatment with *C. butyricum*. *C. butyricum* has been shown to suppress intestinal enterohemorrhagic *Escherichia coli*, *Shigella dysenteriae*, *Cholera salmonella*, and *Cholera bacillus in vitro*. Our data shows that the counts of intestinal *Bifidobacterium* and *Acidobacterium* rose dramatically and mesalamine, the control drug, lowered the prevalence of intestinal microbe balances, especially at decreasing *C. butyricum* almost returned to normal after the treatment with live *C. butyricum*. Our study also indicates that the *C. butyricum* preparation used in our study had a better effect than both mesalamine and sodium butyrate, both on the levels of the inflammatory effectors monitored in this study (IL-23 and TNF-α) and on restoring the balance of the intestinal microflora. The general idea that probiotics based on carefully selected microbes promotes a treatment for UC worthy of consideration is supported by results from clinical trials. Thus, a recent meta-analysis of the results from six published clinical trials concluded that probiotic treatment may reduce UC relapses better than placebo and equivalently to mesalamine treatment[44]. During our study on experimental colitis in rats, we found that treatment with a probiotic containing one well-characterized microorganism promoted the repair of the colon mucosa and recovery of intestinal flora. Thus, probiotics must continue to be a target for investigation both as a potential treatment for active UC and for the management of UC to prevent relapse.

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