Based on the special disease management of Crohn's disease diet studies

——a multicenter, randomized, controlled and open label study

Research Program

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1. Research background

Crohn's Disease (CD) is a chronic, segmental, full-thickness inflammatory bowel disease that involves the whole digestive tract, and is a type of inflammatory bowel Disease (IBD). Its clinical manifestations include diarrhea, hematochezia, abdominal pain and complications such as perforation, fistula formation and even cancer, which greatly affect the quality of life of patients[1]. CD patients need lifelong treatment and management, so the direct and indirect economic burden has become an unavoidable challenge for all countries in the world.

The pathogenesis of CD is unclear, which may be caused by environmental, genetic, infectious and immune factors[2]. Studies have shown the incidence of CD in Asia has increased since the 21st century. Considering the huge population base in China, it is predicted that the total number of IBD patients in China is likely to surpass that in North America in the near future[3]. In the studies related to environmental factors and CD, it has been found that farm life, exposure to animals in childhood[4], use of antibiotics in childhood[5] and other factors are related to the incidence of CD. **The reason may be that westernized diet and environment change intestinal flora, thus increasing the incidence of disease in susceptible population**[6].

Enteral nutrition plays an important role in the treatment of CD, which can not only reduce surgical complications but also improve patients' quality of life with a low incidence of side effects. Exclusive Enteral Nutrition (EEN) is the only established dietary treatment for children with CD. EEN has received particular attention in Europe, where 80% of patients have achieved clinical remission and even intestinal mucosal healing [7-9]。 Previous studies by our team at the IBD Center showed that EEN induced remission rate in adult patients with CD reached 67% [10]， However, the mechanism of action of EEN in treating IBD remains unclear [11-13]. So what effect does EEN have on the gut microbiota? Studies indicated that after EEN treatment, the levels of *Bacteroides* and *Firmicutes* in CD patients decreased, while the levels of *Gammaproteobacteria* and *Actinobacteria* and mucosa associated adhesive *Escherichia Coli* increased[14-17]. The number of anti-inflammatory *Faecalibacterium prausnitzii* has also decreased[18]， In addition, tailed phage viruses[19] were also increased[20]. **Changes in diet affect the amount of short-chain fatty acids and branched-chain fatty acids produced by changing the composition of intestinal flora, leading to changes in intestinal state.**

As CD is an incurable lifelong disease, how to maintain disease remission is an extremely important

part of disease management. Although EEN has a good effect on CD induced remission, due to the long treatment cycle and strict condition control, patients cannot eat other food except fixed nutrient solution, which leads to low compliance of patients, especially adults[10]. Therefore, the promotion of EEN in clinical work is limited, and it is difficult for EEN to be used as the preferred method of CD maintenance treatment.

How to develop a safe, effective and inexpensive treatment for CD patients in China is of great clinical and scientific significance. In recent years, a large number of adjuvant therapies related to CD diet have emerged, such as specific carbohydrate diet therapy[21] , Low Fodmap diet [22] , allergen-free diet [23], etc. But there has been no consistent or convincing evidence on the efficacy of these diets. In 2019, an important study published in the journal Gastroenterology offered a promising diet. The prospective use of half-dose enteral nutrition in combination with a diet-based personalized nutrition regimen (CD-Treat) showed favorable outcomes and high patient compliance in patients with CD [24]. But the diet in the study was Western-style, and it was a small sample research. Through the long-term clinical observation of our team, most patients with CD can achieve stable remission by removing the processed food and screening the corresponding food by combining the characteristics of Chinese diet with the food intolerance detection method. So, what kind of diet is suitable for the treatment of CD patients in China? We refer to the CD-Treat program, replacing the Western diet with Chinese diet, so as to design an individualized program CD-Chinese-food (CD-C-food) that is consistent with the eating habits of Chinese patients with CD. By comparing the effects of this individualized diet with EEN on inducing and maintaining remission in CD patients. In addition, we used intestinal microbiome, bacterial metabolites, intestinal metabolomics, inflammatory factors and other technical means to deeply analyze the therapeutic mechanism of this new diet regimen for CD patients.

To sum up, this project plans to develop a new diet therapy suitable for China -- CD-C-food. Based on the nutritional analysis of EEN, CD-C-Food is more in line with the common diet of Chinese patients' eating habits and economic conditions, and its expected therapeutic effect and influence on intestinal microorganism are similar to that of EEN. We will conduct a randomized control of adult subjects with a healthy CD-Chinese-food diet, treatment group of CD patients and animal model, using intestinal microbiome, intestinal metabolomics, bacterial metabolite analysis, inflammatory factors detection and other technical means. To explore the influence of intestinal microorganisms and their metabolites on the clinical remission effect and inflammatory response of patients with CD-C-Food CD, and to reveal the

possible internal mechanism; This study lays a foundation for further verification of larger samples in multi-centers across the country, and provides a new approach for the development of CD diet therapy with similar efficacy as EEN, but better taste and higher patient compliance.

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2. Research contents, research goals, and key scientific problems to be solved

Research contents:

In this study, CD-C-Food was developed to simulate the nutritional composition of EEN, and the efficacy of this diet treatment and the degree of replacement of EEN were tested in multiple populations including healthy adults, CD patients and animal models. Intestinal microbiome, intestinal metabonomics, bacterial metabolites analysis, inflammatory factors detection and other technical means were used to explore the influence of intestinal microbiome and its metabolites on the clinical relief effect and inflammatory response of patients with CD-C-Food, and to reveal the possible internal mechanism. Combined with the detection of inflammatory factors in the blood and inflammatory markers in the colon of patients with CD after treatment, the overall clinical effect was evaluated. In the case of certain affirmation of the treatment, a large sample of multi-center verification was conducted to complete the preliminary evaluation of the diet treatment program.

Research goals:

This study aims to obtain a diet therapy that can simulate the effect of EEN treatment but has a higher degree of compliance after scientific evaluation of the dietary therapy program CD-C-FOOD, so as to provide more effective approaches for CD treatment and improve the quality of life of patients to a large extent.

Key scientific problems to be solved:

1. To reveal the influence of CD-C-food on the inflammatory response and clinical treatment effect of patients with CD through intestinal microorganisms and metabolites, as well as its internal mechanism, which can be studied by 16S rRNA sequencing mass spectrometry for metabolite detection and pathological observation of animal models

2. To evaluate whether the therapeutic effect of CD-C-food on CD can achieve clinical remission effect. In the clinical pilot trial and subsequent large-sample verification of patients with CD, the study was conducted through observation of clinical inflammatory markers and comprehensive evaluation of clinical effect.

3. To reveal the effective mechanism of CD-C-FOOD and EEN, to conduct in-depth analysis of the process test products of the experimental group, and to conduct specific studies by using 16S rRNA sequencing mass spectrometry and pathological observation of animal models.

4. To evaluate whether the therapeutic effect of CD-C-food on CD can achieve clinical remission effect. In the clinical pilot trial and subsequent large-sample verification of patients with CD, the study was conducted through observation of clinical inflammatory markers and comprehensive evaluation of clinical effect.

3. Research protocol

1. Randomized controlled trials of healthy adults

(1) Specimen collection

A. Inclusion criteria:

a. 30 healthy adults over the age of 18;

b. Excluded subjects with any acute or chronic medical conditions (i.e., conditions requiring regular medical attention);

c. In the past month weight stable (plus or minus 2 kg);

d. There is no history of intestinal surgery;

e. No antibiotics or steroids have been used in the past 3 months.

B. Research procedure：

a. The participants were randomly divided into two groups, group 1 and group 2 with 15 people in each group. During the experiment, dietary diary was recorded;

b. During the first week, participants followed their usual eating habits；

c. In the second week, group 1 received EEN and group 2 received CD-C-food for 7 days. In the CD-C-food group, participants were first tested for blood FOOD intolerance IgG4 assay. The participants were given half a dose of enteral nutrient solution and were presented with a list of foods to choose from which they preferred, excluding intolerant foods and excluding wheat bran, lactose, alcohol, food additives and any worker's ingredients. Personalized meal plans provided by research nutritionists provide their daily energy needs. Energy needs were calculated from participants' estimated basal metabolic rate and self-reported levels of physical activity. EEN and CD-C-FOOD meals were provided free of charge to participants along with written preparation instructions.

d. The third and fourth week was a cleanup period, during which participants ate their usual diet and restored their gut microbiome to baseline, avoiding interference with contamination bias. In the third week, they followed their usual eating habits, and in the fourth week, they followed the diet of the first week and monitored their weight.

e. In the fifth week, the diet of the second week was repeated, but the diets of the two groups were switched, with group 1 receiving CD-C-Food and group 2 receiving EEN, for a total of 7 days

f. Appetite, gastrointestinal symptoms, and adherence to the experimental diet were assessed by questionnaire, and food adherence was further assessed by food surplus and food diary.

C. Sampling time: Fresh stool samples were collected at the end of the first, second, fourth, and fifth weeks. A total of four samples were provided by each participant.

(2) Detection of routine fecal indexes and study of intestinal microorganism：

A. The fresh Stool uses the Bristol Stool Form Scale to evaluate the shape and texture of the sample, and to measure the Stool water content, pH, and ammonia content.

B. Fecal microorganism detection: microbial diversity detection (16S rRNA second-generation sequencing) and quantitative microflora detection (qPCR quantitative detection).

(3) Detection of fecal bacterial metabolites

(4) Fecal metabolome study. Nontargeted metabolites of chloroform, methanol, and water (1:3:1) extracts were determined by high performance liquid chromatography-mass spectrometry (HPLC-MS).

2. Open label trial for CD patients

（1）specimen collection

A. Inclusion criteria：

a. 200 patients with CD recurrence aged 6-15 years;

b. Patients who had taken antibiotics within a month were excluded;

c. Patients who changed CD treatment strategy within 1 to 3 months were excluded;

d. The severity of the patient's illness required hospitalization;

e. Exclude patients with FOOD allergies from CD-C-Food.

B. Research procedure：

a. Participants were treated with CD-C-Food for 8 weeks. The FOOD supply was provided during the study period. The FOOD provided to CD patients was different from that of healthy volunteers, whose FOOD was directly packaged. Participants were asked to record their daily intake of food and drink.

b. Clinical examinations were conducted at two-week intervals, and no changes to the drug associated with CD therapy were allowed during the trial. If disease activity deteriorates at any time, or does not improve by 4 weeks, the patient discontinues the CD-C-Food treatment. Patients who responded clinically but did not enter clinical remission after 8 weeks had the option to continue CD-C-food treatment for 4 weeks.

c. Clinical response and clinical response were assessed every two weeks. Feces and blood were collected once at baseline, once after 4 weeks, again after 8 weeks, and again if a patient had completed 12 weeks of treatment.

d. The detailed procedures of intestinal microbiome study, bacterial metabolites and intestinal metabolomics of patients were referred to the previous experimental procedures.

e. Patients were tested for inflammatory cytokines in blood cells at all stages: The expression of TNF-α, interleukin (IL)-6, IL-10, IL-1B and CXCL-1 in the samples were detected by Milliplex (Merck) liquid-phase suspension chip technology.

（2）Clinical scores and clinical indicators:

CDAI (Crohn’s disease activity index) scores were performed every two weeks, feces collected every four

weeks were tested for fecal calprotectin, and blood was tested for serum albumin and C-reactive protein.

3. Animal experiment

（1）samples：

Adult (36-40 weeks of age) 12 Piebald Virol Glaxo (PVG) heterozygous HLA-B27 and 8 HLA-B7 transgenic rats were studied. B27 rats express the human major histocompatibility complex class I HLA-B27 gene and the related human β2-microglobulin gene and develop inflammation in their gastrointestinal tract (including ileum). They do not cause intestinal inflammation in a sterile environment, and antibiotic treatment can reduce the severity of ileitis. This model has been widely used in the study of inflammatory bowel disease, including with EEN intervention. The intestinal microbiota of B27 showed a similar phenotype to that of Cd, including an increase in proteobacteria and a decrease in sclerocytes. B7 rats did not have intestinal inflammation and were used as a control. Rats were placed in separate cages and raised under specific non-pathogenic conditions. Weight is monitored.

（2）procedures:

B27 rats was divided into three groups, which were CD-C-FOOD group, EEN group and conventional diet control group. B7 was divided into two groups, EEN group and normal diet control group. The net effect of EEN on their gut microbiota was studied in the B7 group setting without inflammation. There were 2 male rats and 2 female rats in each group, and there was no difference in body weight between groups.

4. Data analysis

The general linear model, Box-Cox transform and Fisher pair test were used for comparison between groups. The bacterial community diversity index among each group can be analyzed by alpha-diversity index. Community richness index (Chao and Ace) and Community diversity index (Shannon and Simpson) were calculated. The similarity and difference of microorganisms in different groups can be analyzed by Unifrac based PCoA. The differences between the groups were analyzed using NMDS (Nonmetric Multidimensional Scaling). PCA(Principal Component Analysis) principal component analysis can be used for grouping analysis between groups. Dietary pathology scores and metabolite markers were analyzed using SPSS, and fecal metabolites were analyzed using PCA, Unifrac based PCOA analysis, and permutation multivariate analysis of variance (PERMANOVA). Pearson test was used for correlation analysis. However, in metabolomic analysis, Spearman rank correlation test was used to compensate for the unbalanced influence of outliers, and Benjamin-Hochberg correction method was used to perform multiple

tests.