Title: Modulations of gut microbiota and metabolites by dietary intervention with heat-stabilised rice bran in adults of high risk colorectal cancer - a double blinded randomized controlled study

Research Plan and Methodology

Study design

This pilot study will be a double-blind randomized controlled trial with 40 patients at high risk for the development of CRC will be recruited from Prince of Wales Hospital or community source. Participants will be allocated to either the intervention or control groups in a 1:1 ratio by randomisation. Written informed consent will be obtained from participants before they are enrolled in **t**he study. Ethical approval will be sought from the joint Chinese University– and New Territories East Hospital Cluster clinical research ethics committee.

Subject recruitment

Inclusion criteria for the participants:

1. Chinese aged 50 or above
2. Categorised as a high risk group for the development of CRC (score 4-7) by the Asian-Pacific Colorectal Screening tool (APCS)
3. No gastrointestinal symptoms suggestive of colorectal cancer
4. Negative fecal occult blood test or negative colonoscopy findings for CRC within 1 year
5. No history of food allergy
6. No dietary restriction
7. Not currently taking cholesterol-lowering medications
8. Not currently taking non-steroidal anti-inflammatory drugs (NSAIDs)
9. No probiotic and prebiotics use for the past 3 months
10. No history of gallstones
11. No Chinese medicine use (except traditional Chinese soup) for the past 3 months

12. No antibiotic taking for the past 3 months

Exclusion criteria for the participants:

1. Having lower gastrointestinal symptoms in the past week
2. Currently pregnant unless sterilization was done or in menopause or lactating (self-reported by the participants)
3. Having diabetes mellitus

Participants will be recruited in the health talks organized by the Chinese University of Hong Kong or community source in Hong Kong. All potential subjects will be screened by our research staff for eligibility according to the above criteria, and all those eligible will be given detailed information about the study. Demographic characteristics that were known to affect the gut microbiota, including age, gender, body mass index, dietary habits (intake of dietary fats, fruit and vegetables, dietary fibre, white and red meat, and dairy products), perceived stress levels, smoking pattern and amount of alcohol drinking (1-3), will be collected. Written informed consent will be obtained from participants before they are enrolled in the study.

Randomisation

After written consent is obtained from participants, they will be allocated to either the intervention or control groups in a 1:1 ratio by block randomisation with a block size of 10 to maintain a good balance of participants between the two groups throughout the subject recruitment period. A sequence of grouping identifiers (I=intervention group and C=control group) will be prepared in advance by an independent statistician using computer-generated random codes. The patients and outcome assessors will be blinded to the group allocation.

Intervention

The intervention group will be given packets of heat-stabilised rice bran, of which they will be instructed to take 30 grams per day, at 24-hour intervals, during the 24-week period. The control group will be provided with a rice flour placebo, similar in appearance to the intervention group's

rice bran, and will also be instructed to consume 30 grams of the product per day at 24-hour intervals during the intervention. The nutritional content of rice bran and rice flour are shown in Appendix 1 and Appendix 2, respectively. Both groups will also be instructed not to change their normal daily routines, including their dietary habits and levels of physical activity, throughout the entire course of the 24-week intervention.

Faecal diary

A self-reported faecal diary will be used to assess changes in bowel habits. Participants will be asked to record the frequency and amount of stool and its consistency using Bristol stool scale (4). In addition, they will be asked to document any abdominal discomfort, pain, bloating or other adverse effects after consumption of rice bran or rice flour. Research staff will collect the data at the baseline in face-to-face interviews, after which participants will be instructed to make a daily entry in the faecal diary provided until the end of the intervention.

Stool sample collection

Participants will be instructed to collect two stool samples, weighing 3-4 g, in two separate tubes using a sterile collection brush before intervention (T1) and at 6 weeks (T1), 12 weeks (T2), 24 weeks (T3) after intervention. Not more than seven days delay for each time point is allowed. The samples will be used for analysis of microbiome and fecal calprotectin, respectively. Samples will be transported to the laboratory at 4 °C within 24 hours after defecation and immediately stored at -80 °C in a freezer before analysis.

Venous blood collection

Venous blood (10 ml) will be obtained from the antecubital vein at each follow-up visit. The sample will be used for hs-CRP analysis. Serum samples will be saved at -80 °C until the laboratory analysis.

Stool DNA extraction

Approximately 200 mg of each stool sample will be used for DNA extraction using the QIAamp DNA Stool Mini Kit (QIAGEN Inc, Germantown, MD, USA), with some modifications. Stool samples will be lysed in 1.5 ml buffer, vortexed for 15 sec and incubated at 55-65°C on a rotator

overnight. The lysed samples will be centrifuged at 13,300 rpm for 3 minutes to pellet the debris. 1.5 ml of supernatant will then be transferred to a fresh 2.0 ml reaction tube followed by the addition of one InhibitEX tablet. The tubes will be vortexed for 1 minute to dissolve the tablet and the suspension will be incubated for 1 minute at room temperature to remove the stool inhibitors. After Proteinase K digestion at 70°C for 15 minutes and subsequent washing with 500 µl of buffer AW1 and AW2, the DNA will be eluted in 100 µl of low TE buffer. Extracted DNA will be quantified using the Nanodrop-2000c spectrophotometer (Thermo Fisher Scientific Inc., Wilmington, DE, USA) and normalised to 20 ng/µl. Extracted DNA will then be stored immediately at -20°C before high fidelity PCR.

16S metagenomic sequencing

A metagenomic library will be constructed using the PCR primers flanking the V4 region of the 16S ribosomal gene, with PCR reactions performed in duplicate for each sample using the high fidelity PCR master mix at 94°C for 1 minute followed by 30 cycles at 94°C for 40 seconds, 50°C for 40 seconds and 72°C for 1 minute. PCR products will be purified using the QIAquick Gel Extraction Kit (QIAGEN Inc, Germantown, MD, USA) followed by quantification by the Bioanalyser 2100 (Agilent) and *Qubit*™ 3.0 spectrophotometer (Thermo Fisher Scientific Inc., Wilmington, DE, USA). The final metagenomic libraries will be normalised and pooled for next generation sequencing using the IonTorent PGM platform (Invitrogen).

hs CRP analysis

The serum hs-CRP level, a factor known to exhibit positive correlation with CRC risks (5), will be measured by solid-phase enzyme-linked immunosorbent assay using the human hs-CRP ELISA kit (Hycult Biotech, Netherlands). Serum will be prepared from the freshly collected blood and then diluted 1:1,000 in sample dilution buffer. Serum samples or standards of 100 µl in triplicates will be added to the reaction well and incubated at 20-25°C for 30 minutes. After washing the wells three times with wash buffer and subsequent incubation for 30 minutes with 100 µl of peroxidase conjugated antibody at 20-25°C, 100 µl substrate tetramethybenzidine (TMB) will be added to the well for colour development at 20-25°C for 10 minutes. The enzymatic reaction will be stopped by adding 50 µl of 0.5M sulfuric acid followed by spectrophotometric measurement at 450 nm. A standard curve will be prepared and the sample hs-CRP concentration will be

determined accordingly. The mean value of the triplicates' readings will be calculated and used for data analysis.

Fecal Calprotectin analysis

Stool samples will be collected in screw-capped plastic containers, sent to the laboratory and stored at -80 °C. Fecal calprotectin will be extracted and analysed by sandwich ELISA according to the manufacturer's instructions (EK-CAL; Buhlmann Lab. AG, Schönenbuch, Switzerland). The 50 mg stool sample will be placed into a preweighted tube with the inoculation loop. 2.45ml of 1 x B-CAL-EX buffer will be added into each tube and the samples vortexed vigorously for 30 minutes. 1 ml of the homogenate will be transferred to a new 1.5 ml Eppendorf and centrifuged at 3,000 *g* for 5 minutes. The supernatant will be transferred into a new tube for ELISA. Calprotectin ELISA plate (EK-CAL; Buhlmann Lab. AG, Schönenbuch, Switzerland) will be washed twice with 300 µl wash buffer followed by the addition of 100 µl of standards or 1:50 diluted stool extract in triplicates. After incubation at 25°C for 30 minutes at 450 rpm on a plate rotator, the well will be washed three times with 300 µl of wash buffer followed by incubation with 100 µl of enzyme label at 25°C for 30 minutes. After incubation, the wells will be washed five times using 300 µl of wash buffer and incubated with 100 µl of TMB substrate for 15 minutes at 25°C. Finally, the enzymatic reactions will be stopped with 100 µl stop solution and spectrophotometric measurement carried out at 450 nm by means of the microplate reader.

Data collection procedures

After receiving informed consent from the eligible subjects and their randomization into groups, they will be asked to complete a short questionnaire concerned with demographic data and usual bowel habits and abdominal discomfort as described above. A blood sample will also be taken on this occasion. They will be provided with two stool containers and detailed instructions for the collection of stool samples before the intervention (T0), at six (T1), twelve (T2) and twenty-four (T3) weeks thereafter. Subjects will be arranged to come back to a nurse-led clinic for compliance and adverse event assessment and blood sample collection at T1, T2 and T3. Compliance will be assessed in terms of number of times taken the study product divided by the total period. Participants will also be provided with a log-book, for them to record the time of rice bran / rice flour consumption on each day, as well as any instances of consumption of medications listed in

the inclusion criteria during the intervention. Further, they will be instructed to record their bowel habits and any adverse events throughout the intervention. Our research staff will contact the study subjects by phone and remind them to bring the stool sample, the log-book and the faecal diary at the follow-up visits. Participants will be given HK$ 200 coupon, HK$50 for each visit, as a token of our appreciation of their support for the study.

Data analysis

Data will be summarized and presented in a suitable form of descriptive statistics. Normality of variables with continuous data will be assessed using skewness and kurtosis statistics and normal probability plot. Appropriate transformations will be made to skewed variables before subjected to statistical analysis. Baseline demographic and clinical characteristics between the intervention and control groups will be compared by independent t, chi-square or Fisher's exact tests, as appropriate, in order to ensure that the confounding factors have not affected the outcomes involved in the study. For measures of physical activity, collected data will be converted to metabolic equivalents (MET) and analysed according to the Global Physical Activity Questionnaire Analysis Guide (6). Generalized estimating equations (GEE) model will be used to compare the differential changes on the outcome variables across time and between the two groups with adjustment for potential confounding variables so as to improve the precision of intervention effect estimations. Baseline characteristics with p values <0.25 for between-group differences and other variables that may confound the comparison results of the outcomes based on clinical judgment will be considered as potential confounding variables. GEE models can fit different types of outcome variables with the use of appropriate link-functions and account for intra-correlated repeated measures data. It can also produce unbiased estimates for missing data caused by incomplete visits or dropout cases, provided the data are missing at completely random. All statistical analyses will be performed using IBM SPSS (IBM Crop. Armonk, NY). All statistical tests will be two-sided with level of significance set at 5%.

Metagenomic data analysis

The metagenomic data set will be analysed by means of the mothur, a widely used bioinformatics software for the analysis of 16S rRNA gene sequences in bacteria. An analysis of molecular variance (AMOVA) using a p-value threshold of 0.01 will be used to determine variation in the

sample community. PcoA loadings will be generated in mothur. Differentially abundant bacterial taxa in the stool before and after taking rice bran will be studied using the METASTATS function in mothur. Bacterial diversity of the samples will also be analysed using the mothur, where the level of significance in the differences of alpha and beta diversity of samples at various time points will be determined using t-test.

Ethical considerations

Ethical approval will be sought from the joint Chinese University– and New Territories East Hospital Cluster clinical research ethics committee. The study is in compliance with the Declaration of Helsinki and ICH GCP. Before participants are enrolled, they will be given details of the project, including data collection procedures, by means of an information sheet. They will be told of their rights to withdraw from the study at any time without penalty, and assured that all responses they provide in questionnaires will be kept anonymous and confidential. There will also be sufficient time for questions before they are asked to give their informed consent to participate.

References

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