European Journal of Integrative Medicine 18 (2018) 1–7

Contents lists available at ScienceDirect

European Journal of Integrative Medicine

journal homepage: www.elsevier.com/locate/eujim

Clinical trial

The effects of sodium butyrate and high-performance inulin supplementation on the promotion of gut bacterium *Akkermansia muciniphila* growth and alterations in *miR-375* and *KLF5* expression in type 2 diabetic patients: A randomized, double-blind, placebo-controlled trial

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ARTICLE INFO

Keywords:
High performance inulin
Sodium butyrate
KLF5
miR-375
Akkermansia muciniphila
Randomised controlled trial

ABSTRACT

Introduction: The aim of this study is to evaluate the effect of sodium butyrate and High-Performance (HP) inulin supplementation on the promotion of gut bacterium *Akkermansia muciniphila* growth and alterations in microRNA-375 and Krüppel-like factor 5 (*KLF5*) expression in patients with T2D.

Methods: In this clinical trial, 60 patients with T2D were recruited and randomly allocated into four groups of equal size to receive 600 mg/day sodium butyrate (Group A), 10 g/day inulin powder (Group B), concomitant use of inulin and sodium butyrate (Group C), or placebo (Group D). Blood and stool samples were collected pre- and post-intervention. Quantitative real-time PCR analysis targeting the 16S rRNA gene of *A.muciniphila* was performed to determine its presence in the patient's stool. In addition, we assessed the *KLF5* mRNA expression and the plasmatic level of the microRNA-375 before and after the intervention.

Results: The results showed that *A. muciniphila* percent change increased significantly after supplementation with HP inulin ($p = 0.017$) and butyrate ($p = 0.036$). Also, supplementation with HP inulin significantly decreased the *KLF5* fold change after intervention (fold change 0.42 ± 0.15, $p = 0.037$). In particular, in comparison to the placebo group, an increased expression of *miR-375* was seen after butyrate and butyrate + inulin supplementation ($P = 0.003$ and $P = 0.007$ respectively).

Conclusions: We concluded that inulin and butyrate may potentially promote gut health and can be considered as novel therapeutic approache for the prevention and control of diabetes.

1. Introduction

The important role of intestinal microbiota in the control of metabolic diseases – including obesity, cancer, cardiovascular diseases, and diabetes – has recently been identified [1]. Recently, numerous studies have reported that gut microbiota dysbioses can be involved in the onset and amplification of insulin resistance and Type 2 diabetes (T2D) [2]. The gastrointestinal tract is colonized by a broad community of symbionts and commensal bacteria (e.g. *firmicutes, bacteroidetes*, *enterobacteriaceae*) which have a precise role in host metabolism, immune function, and other activities [3].

The analysis of intestinal microbiota shows that alterations in gut permeability are associated with insulin resistance and metabolic inflammation in patients with T2D [4]. An appropriate integrity of gut

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https://doi.org/10.1016/j.eujim.2017.12.011
Received 21 August 2017; Received in revised form 2 November 2017; Accepted 28 December 2017

barrier can modulate its permeability by controlling the thickness of the inner mucus layer [5]. This protective layer covers the intestinal tract and protects the underlying epithelium from the foreign pathogens, toxins, and bacterial invasion. It is also an important source of essential nutrients (e.g. carbon) and energy for commensal microbiota. Mucin – a major component of intestinal mucus – is a glycosylated protein with a peptide core rich in serine and threonine residues [6].

Most pioneering studies have found that *Akkermansia muciniphila,* which is related to the genera *Prosthecobacter* and *Verrucomicrobium,* is the sole representative of this phylum that is colonized in the human intestinal tract and is known as mucin-associated bacteria [7]. This gram-negative, strictly anaerobic bacterium is involved in mucus degradation and may constitute up to 3% of the intestinal microbiome community in healthy adults. A reduced presence of this bacterium was reported in diabetic mice and humans [8]. Degradation properties of *A. muciniphila* lead to the production of propionate and acetate, which can be used as an energy source for the host [9]. It has been demonstrated that prebiotic treatment increases the abundance of *A. muciniphila* in animal models [10].

According to the definition given by food and agriculture organization (FAO), " a prebiotic is a non-viable food component that confers a health benefit on the host associated with modulation of the microbiota" [11]. These low-digestible carbohydrates can ferment in the colon into short-chain fatty acids (SCFA: e.g. butyrate, propionate, and acetate), gases (e.g. carbon dioxide, hydrogen, and methane), and lactic acid [12]. The known prebiotic inulin is a mixture of fructo oligo- and polysaccharides, which belong to a class of compounds known as fructans [13]. High-performance (HP) inulin has a long-chain, high-molecular weight mix of inulin-type fructans, not including any fructan that has a degree of polymerization < 10 [4].

Previous studies have shown that SCFAs – particularly butyrate as a "postbiotics" product – may act directly or indirectly on intestinal epithelial cells. They can participate in the control of various metabolic processes by means of expression of some genes and microRNAs (miRNAs) [14].

MiRNAs, with 22–25 nucleotides are small non-coding RNAs that control gene expression via complementary base-pairing within the 3′-untranslated region (3′-UTR) of the protein-coding RNA (mRNA) [15]. Growing evidence suggests that miRNAs play an important role in the etiology and pathogenesis of some diseases such as diabetes [16]. MicroRNA-375 (*miR-375*) is one of the miRNAs involved in the control of the insulin gene expression in pancreatic β-cells. Analysis of miR-375 knockout mice showed hyperglycemia and glucose intolerance in these investigated animals. Also, a decrease in the number of β-cells and an increase in the number of α-cells were seen in these *miR-375* knockout mice [17].

Krüppel-like factor 5 (*KLF5*) is one of the predicted mRNA targets of *miR-375* [18]. *KLF5*, also known as *BTEB2* (Basic transcription element-binding protein 2) or *IKLF* (intestinal-enriched Krüppel-like factor), is a zinc-finger transcription factor that contributes to the regulation of differentiation, development, and cell cycle progression. It has an intense relationship with human health and disease [18,19]. The encoded protein of this gene is a transcriptional activator that binds to a specific recognition motif in the promoters of some target genes, such as peroxisome proliferator-activated gamma (*PPARγ*), *PPARα*, and CCAAT/enhancer binding proteins (C/EBPα), which have been implicated in adipogenesis and glucose homeostasis [19,20]. Numerous studies now report an intense association between *KLF5* overexpression and some metabolic disease like cardiovascular disease, colorectal cancer, and T2D [21,22].

It has been proved that *miR-375* expression is high in the human intestinal epithelium and its up- regulation is able to inhibit the translation of *KLF5* [18]. Based on this evidence, we aimed to investigate the effect of sodium butyrate (NaBut) and HP inulin supplementation on the promotion of gut bacterium *A.muciniphila* growth and alteration in *miR-375* and *KLF5* expression in T2D patients.

2. Methods

2.1. Study population

In this randomized, double-blind, placebo-controlled clinical trial, 60 subjects with T2DM participated voluntarily. They were recruited from the AZAR Cohort Study (Persian cohort, http://persiancohort.com) and Rohzendeh Health and Therapeutic Center in Shabestar (East Azerbaijan, Iran). The study took place between August 2016 and December 2016. The criteria for the diagnosis of diabetes were considered according to the American Diabetes Association criteria: fasting glucose ≥ 126 mg/dl or hemoglobin A1c ≥ 6.5% [23]. Inclusion criteria for the participants were: a history of DM > six months, consumption of anti-diabetic drugs, age range of 30–55 years, and body mass index (BMI) of 27–35 kg/m^2. The predefined exclusion criteria were a history of diagnosed gastrointestinal disease, coronary heart disease, renal failure, thyroid disease, liver or pancreatic illness, pregnancy or lactation, insulin therapy, consumption of pre-or probiotics, antibiotic or antacid drugs, and alcohol or tobacco use at the time of recruitment. The consumption of first line preventative drugs for the treatment of diabetes, hydroxy methyl glutaryl-coenzyme-A reductase inhibitors (statins) for the treatment of hypercholesterolemia, and ACE inhibitors for hypertension were not an exclusion criterion.

The Ethics Committee of Tabriz University of Medical Sciences approved this project (Ethical code: IR.TBZMED.1395.778). Signed informed consent was obtained from all participants. The current study is registered in the Iranian Registry of Clinical Trials (IRCT ID: IRCT201605262017N29) and trial protocol is available on the IRCT website.

The sample size of the study was estimated on the basis of prior data with confidence interval 95% and power 90%, based on fasting blood sugar [24]. This calculation determined a total sample size of 52 individuals, plus eight persons in case of withdrawals. Hence, a total of 60 patients were required. These 60 patients were allocated randomly (with allocation ratio 1:1) using randomized block procedure to one of four treatment orders (A, B, C, or D) by a computer-generated allocation schedule (Random Allocation Software) in which A was the butyrate group, B was the inulin group, C was the inulin plus butyrate group, and D was the placebo group. Each group consisted of 15 patients. Group A consumed 6 capsules of 100 mg sodium butyrate (BodyBio, USA) before and after each meal six times per day as recommended by the manufacturer and 10 g of starch powder as placebo for 45 days. Group B consumed 10 g/day of HP inulin supplement powder (Sensus, Borchwef 3, 4704 RG Roosendaal the Netherlands) as well as six 100 mg starch capsules as placebo. Group C underwent the concomitant use of NaBut capsules and HP inulin powder (10 g of inulin powder + six capsules of sodium butyrate) for 45 consecutive days, while Group D (control group) received six 100 mg starch capsules as well as 10 g of starch powder as placebo which were matched to study supplements in terms of color and size. Randomization of the participants between groups was done by a local physician. After the provision of supplements, the physician had no relationship with the study patients or involvement in other study processes. Participants were categorized by type of consumed medical drugs (glucose lowering and anti- hyperlipidemia drugs) and disease duration in this trial. All of the patients were requested to maintain their previous habitual lifestyle, physical activity, and dietary intake during the intervention. In order to minimize withdrawal and certify the consumption of the supplements, a health center staff member called on each patient weekly. All subjects were visited every 15 days during the trial period.

2.2. DNA isolation from fecal samples

The faeces of patients collected pre and post intervention and were stored at −80°C for bacterium analysis. DNA was extracted using a fecal DNA isolation kit (BioBasic, Canada) according to the