Letter to the Editor

Short-term Chronic Intermittent Hypobaric Hypoxia Alters Gut Microbiota Composition in Rats*

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Chronic intermittent hypobaric hypoxia (CIHH) is a treatment of moderate hypoxia that simulates high altitude interrupted by normoxia. Growing evidence shows that CIHH has multiple beneficial effects on the body[1]. Our previous studies showed that CIHH (simulating 5,000-m altitude for 28 days, 6 hours daily) protects the heart against ischemic/reperfusion-induced arrhythmias and damage through increased coronary flow and myocardial capillary angiogenesis, enhanced cardiac anti-oxidation, induction of heat shock protein 70, inhibition of endoplasmic reticulum stress, activation of ATP-sensitive potassium channels, and inhibition of mitochondrial permeability transition pores[2]. In addition, CIHH decreases high blood pressure by facilitating the baroreceptor reflex and enhancing arterial relaxation in renal vascular hypertensive rats. CIHH also improves metabolic dysfunction in rats with diabetes and metabolic syndrome. However, the mechanism is not fully understood.

The gastrointestinal tract harbors 1,000-5,000 species of bacteria, and the number of microbial cells within the gut lumen is about 10 times higher than the number of eukaryotic cells in the human body. These bacteria are essential to physiology, as they break down indigestible dietary components and serve as a natural defense against pathogens. The gut microbiota is commonly referred to as an essential organ because its composition and richness are constantly adapting to the challenges presented by the environment or by the host, such as age, diet, lifestyle modifications, and disease states[3]. Thus, the gut microbiota exerts a fundamental influence on systemic immunity and metabolism, and a healthy gut microbiota is largely responsible for the overall health of the host.

Recent studies have highlighted that changes in the composition of commensal bacterial populations are linked to multiple metabolic and inflammatory diseases in humans, including inflammatory bowel disease, obesity, type 2 diabetes, cardiovascular diseases, allergy, and colorectal cancer[3-5]. For example, the gut microbiota participates in the development, regulation, and treatment of hypertension and gut microbiota-associated dysbiosis contributes to the development of hypertension[5]. This suggests that a favorable change in the gut microbiota results in a beneficial effect on hypertension. We hypothesized that the beneficial effects of CIHH, such as anti-hypertension and anti-diabetes effects, might be carried out by altering the composition of the gut microbiota. The aim of present study is to explore the effect of CIHH on gut microbiota composition.

Adult male Sprague-Dawley rats 8-10 weeks old were randomly divided into two groups: the CIHH group (CIHH, n = 6) and a control group (Control, n = 6). All rats were housed and fed in a temperature-controlled (22 ± 1) °C clean-grade animal room with a 12 h/12 h light/dark cycle and free access to water and food. The clean water and standard rat food were sterilized by autoclaving to avoid contamination. CIHH rats were placed in a hypobaric chamber for the hypobaric hypoxia treatment simulating a 5,000-m altitude (P_B = 404 mmHg, Po_2 = 84 mmHg) for 28 days, 6 h per day (excluding the ascending and descending times). All experiments were conducted in compliance with the Guide for the Care and Use of Laboratory Animals (National Research Council, 1996), and all techniques and procedures were reviewed and approved by the Hebei Medical University Institutional Animal Care and Use Committee.

Total genomic DNA from samples was extracted using the CTAB/SDS method. DNA concentration and purity were monitored by 1% agarose gel

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electrophoresis. DNA was diluted to 1 ng/μL using sterile water.

The V4 region of the bacterial 16S rDNA gene from each DNA sample was amplified using the reverse primers 515f/806r (515f: 5'-GTGCCAGCMG CCGCGGTAA-3', 806r: 5'-GGACTACHVGGGTWTCTA AT-3'). The polymerase chain reaction (PCR) reaction was performed using the high-fidelity PCR Mastermix (New England Biolabs, Ipswich, MA, USA) with the following cycling parameters: 94 °C for 3 min (1 cycle), 94 °C for 45 s/50 °C for 60 s/72 °C for 90 s (35 cycles), and a last step of 72 °C for 10 min. The PCR products were mixed in equally dense ratios. Then, the mixture of PCR products was purified with the Qiagen Gel Extraction Kit (Qiagen, Hilden, Germany).

Sequencing libraries were generated using the TruSeq DNA PCR-Free Sample Preparation Kit (Illumina, San Diego, CA, USA) following the manufacturer's protocol, and index codes were added. Library quality was assessed on the Qubit 2.0 Fluorometer (Thermo Scientific, Waltham, MA, USA) and the Agilent Bioanalyzer 2100 system (Agilent Technologies, Palo Alto, CA, USA). The library was sequenced on an Illumina HiSeq2500 platform, and 250 bp paired-end reads were generated.

Paired-end reads were merged using FLASH (V 1.2.7). The tags were compared with the reference database using the UCHIME algorithm to remove chimeric sequences, and the effective tags were finally obtained. The operational taxonomic units (OTUs) were picked using the *de novo* OTU picking protocol with a 97% similarity threshold. The GreenGene Database was used based on the RDP classifier (Version 2.2) algorithm to annotate taxonomic information of each representative sequence. The alpha diversity analysis included the Shannon and Chao1 indices. All indices in our samples were calculated with QIIME (Version 1.7.0) and displayed with R software (Version 2.15.3).

Statistical analysis was performed with Prism version 7 (GraphPad Software Inc., La Jolla, CA, USA). All data are expressed as mean ± SEM. Data were compared by two-sided Student's *t*-test. *P*-values < 0.05 were considered significant.

It was observed that totals of 57,823 ± 1,785 and 58,091 ± 6,229 raw reeds were obtained from fecal samples of the Control and CIHH rats, respectively. After stitching and filtration, 54,415 ± 1,627 reads were received in Control rats and 54,340 ± 5,569 reads were received in CIHH rats. The total number of OTUs at the 97% similarity level was 1,141 ± 12 in Control rats and 1,186 ± 56 in CIHH rats (Table 1).

The estimators of community richness (Chao1) and evenness (Shannon index) are summarized in Table 1. No difference in the community richness estimator was observed between the two groups ($P > 0.05$, Table 1). The Shannon index was higher in the CIHH than the Control group ($P < 0.01$, Table 1), indicating an increase in evenness of the species distribution in stool samples from CIHH rats compared to Control rats. This finding suggests that the abundance of dominant species decreased and that several minor members of the community increased in response to CIHH exposure, while the main species in the community remained unaltered.

As shown in Figure 1, the most predominant phyla were Firmicutes (72.79% in Control *vs.* 64.26% in CIHH, $P < 0.01$), Bacteroidetes (15.32% in Control *vs.* 20.42% in CIHH, $P < 0.05$), Proteobacteria (5.18% in Control *vs.* 9.31% in CIHH, $P > 0.05$), and Tenericutes (4.08% in Control *vs.* 4.11% in CIHH, $P > 0.05$) followed by Cyanobacteria (0.53% in Control *vs.* 0.25% in CIHH, $P < 0.05$). In addition, the Firmicutes/Bacteroidetes (F/B) ratio was significantly lower in CIHH rats than that in Control rats ($P < 0.01$, Figure 1B). The remainder of the bacterial population belonged to other phyla that had relative low abundances in the samples.

Some significant differences were found in the microbial composition at the genus level between the CIHH and Control rats (Figure 2). Abundance of the top 10 genera was determined among the rats, with four significantly different genera between CIHH and Control rats (Figure 2). *Lactobacillus*, *Prevotella*,

Table 1. Summary of Pyrosequencing Data

Group	Reads	OTUs	Chao1	Shannon
Control	54,415 ± 1,627	1,141 ± 12	1,243 ± 12	7.57 ± 0.06
CIHH	54,340 ± 5,569	1,186 ± 56	1,233 ± 75	8.01 ± 0.05**

Note. The number of reads, operational taxonomic units (OTUs), and Chao1 and Shannon indices. Control: control group, CIHH: CIHH group; all data are expressed as mean ± SEM, $n = 6$ for each group, $^{**}P < 0.01$ *vs.* Control.

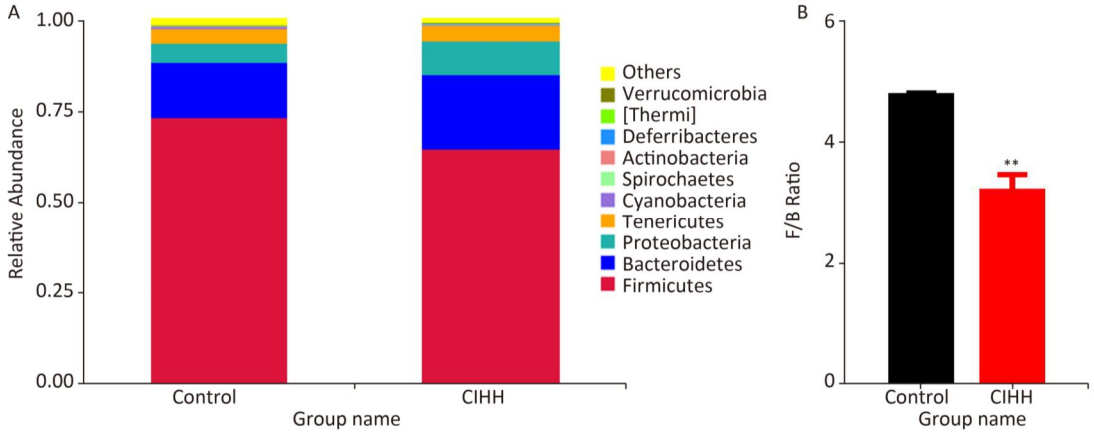

Figure 1. Effect of exposure to chronic intermittent hypobaric hypoxia (CIHH) on the relative abundance of bacteria at the phylum level in the fecal microbiota (A) and the Firmicutes/Bacteroidetes ratio (B). Control: control group, CIHH: CIHH exposure group; all data are mean ± SEM, n = 6/group. $^{**}P < 0.01$ *vs.* Control.

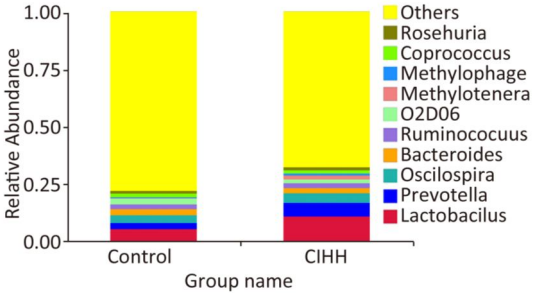

Figure 2. Effect of exposure to chronic intermittent hypobaric hypoxia (CIHH) on the relative abundance of bacteria at the generic level in the fecal microbiota. Control: control group, CIHH: CIHH exposure group; n = 6/group.

and *Methylotenera* were higher, and 02d06 was lower in CIHH rats than those in Control rats.

Recent studies have shown that gut microbial dysbiosis is associated with many chronic diseases, such as obesity, diabetes and hypertension[3-5]. An increase in the Firmicutes/Bacteroidetes (F/B) ratio, caused by an increase in the abundance of Firmicutes or a decrease in Bacteroidetes, is considered a signature of gut dysbiosis associated with obesity and hypertension[5,6]. Ley et al. reported that the relative proportion of Bacteroidetes decreases in obese subjects compared with lean subjects, and that this proportion increases with weight loss in response to two types of low-calorie diets[6]. Notably, the F/B ratio is well validated in rodent and human samples[7]. WKY rats gavaged with SHR microbiota result in increased blood pressure as well as F/B ratio[8]. Our results indicate that the F/B ratio decreased in the CIHH group compared to the unexposed control, which may explain the anti-hypertensive and improving glucose and lipid metabolic effects in response to CIHH.

A growing body of evidence indicates that the gut microbiota exerts important influences on the physiological homeostasis of their mammalian hosts by signaling through metabolic byproducts such as short-chain fatty acids (SCFAs)[9]. SCFAs produced by the gut microbiota have may beneficial functions for the host, such as protection against diet-induced obesity, improved insulin sensitivity in type 2 diabetes, and decreased blood pressure. In our study, we also found a significant increase in the gut microbiota at the generic level, such as *Lactobacillus* and *Prevotella*, in CIHH-treated rats compared to the unexposed control. These genera are all associated with the production of SCFAs[10]. This may be another reason for the anti-hypertensive and glucose and lipid metabolism improving effects in response to the CIHH treatment.

Some limitations of this study should be discussed. The baseline microbiota should have been measured in stool to compare data before and after CIHH exposure. In addition the number of animals should be increased. Thus, further study is needed.

In summary, our exploratory study showed for the first time that short-term CIHH, simulating high altitude hypoxia, altered gut microbiota composition and the F/B ratio, which might be related to the beneficial effects of CIHH on the body.

No conflicts of interest, financial or otherwise, are declared by the authors.

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