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Original Research Article

Ganjiang Huangqin Huanglian Renshen Decoction improves insulin sensitivity by regulating intestinal flora in rats with Type 2 diabetes mellitus

Zhiping Peng, Yuanjun Zheng*
Department of Geratology, Hangzhou TCM Hospital Affiliated to Zhejiang Chinese Medical University, No. 453 Tiyuchang Road, Hangzhou, Zhejiang 310007, China

For correspondence:* ***Email:* *13003617778@163.com;* ***Tel:*** *+86-0571-85827720*

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Abstract

Purpose: *To study the effect of Ganjiang Huangqin Huanglian Renshen Decoction (GHHRD) on intestinal microflora and insulin resistance (IR) in type 2 diabetes mellitus (T2DM) rats.*
Methods: *Streptozotocin (STZ) injection was used to establish a rat model of T2DM. The T2DM rats were divided into 5 groups: control, T2DM, T2DM + low-dose traditional Chinese medicine (TCM), T2DM + high-dose TCM, and T2DM + metformin groups. Total DNA kit was used for extraction of total DNA from fecal microorganisms from the T2DM model rats. High-throughput 16S rDNA sequencing was carried out to identify the intestinal flora of T2DM model rats. Fasting plasma glucose (FPG) and fasting serum insulin (FINS) levels were determined by a biochemical analyzer.*
Results: *The 16S rDNA sequencing analysis showed that after GHHRD treatment, the levels of Ruminococcus, Prevotellaceae, Escherichia-Shigella and Bacteroidota in the intestinal tract of T2DM model rats exhibited significant changes. Furthermore, GHHRD decreased the Homeostasis Model Assessment-Insulin Resistance (HOMA-IR) index and IR in T2DM model rats.*
Conclusion: *GHHRD mitigates IR in T2DM model rats through alterations in the intestinal flora of the rats. This provides a theoretical basis for the potential use of GHHRD in the clinical management of T2DM.*

Keywords: *Type 2 diabetes mellitus (T2DM), Ganjiang Huangqin Huanglian Renshen Decoction (GHHRD), Intestinal microflora, Escherichia-Shigella, Insulin resistance*

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INTRODUCTION

Type 2 diabetes mellitus (T2DM), a global pandemic with high incidence and prevalence, affects the health of high percentage of people in the age range of 35-40 worldwide(1). It is a metabolic disease manifested by insulin resistance (IR) and β-cell dysfunction(2). Recent studies have shown that the development of T2DM is influenced by the composition and function of intestinal flora(3). Therefore, intestinal flora may be regarded as a new therapeutic angle for reducing the symptoms of T2DM.

Ganjiang Huangqin Huanglian Renshen Decoction (GHHRD) is derived from *Shang Han*

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Lun (*Treatise on Exogenous Febrile Disease*), and it is thought to be effectively reduce blood glucose levels in T2DM rats. Furthermore, GHHRD has been proven to effectively reduce the clinical symptoms and blood glucose levels in people with T2DM. However, the mechanism underlying the effect of GHHRD on T2DM has not yet been elucidated.

This study aimed to investigate the mechanism of GHHRD in the treatment of T2DM. Firstly, the relationship between GHHRD and intestinal flora in T2DM rats was determined. Then, analysis of correlation between intestinal flora and IR in T2DM was done. It was finally found that GHHRD exerted its effects on T2DM rats through modulation of the intestinal flora. Therefore, GHHRD may be regarded as a new and effective treatment for T2DM.

EXPERIMENTAL

Materials

4-week-old healthy male SPF Sprague Dawley rats of mean weight 100 ±10 g were purchased from Beijing Huafukang Biotechnology Co. Ltd. (SCXK, Beijing 2009-0007). Streptozotocin, metformin and fecal genomic DNA extraction kits were purchased from Beijing Solarbio Science & Technology Co. Ltd. TransStart Fastpfu DNA polymerase was bought from Beijing TransGen Biotech Co. Ltd, while Biochemical Analyzer was purchased from the HITACHI Company, Japan.

Ethical consideration

This animal study protocol used was reviewed and approved by the Institutional Animal Care and Use Committee (IACUC), ZJCLA (batch no. ZJCLA-IACUC-20020068); and the research was conducted in line with the guidelines of NIH guidelines(4).

Establishment of T2DM rat model and rat grouping

The T2DM rat model was established using low-dose streptozotocin (STZ) injection and high-fat diet. After 72 h of STZ injection, blood glucose level > 16.7mmol/L denoted successful establishment of T2DM Model. The required concentration of GHHRD was prepared using 9 g of Ganjiang (*Rhizoma zingiberis*), 30 g of Huangqin (*Scutellaria baicalensis* Georgi), 15 g of Huanglian (*Coptis chinensis* Franch.), 30 g of *Anemarrhena asphodeloides* Bunge, and 6 g of American ginseng.

The rats were divided into control group (NC), T2DM group, T2DM + low-dose TCM (0.9 g/mL) group (low-dose TCM); T2DM + high-dose TCM (1.8 g/mL) group (high-dose TCM) and T2DM + metformin (0.3 g/mL) group (T2DM + EJ), with 6 rats in each group. All rats were given gastric perfusion twice a day for 12 weeks.

Identification of intestinal flora in T2DM

Total DNA was extracted from microorganisms in the faeces of T2DM model rats using DNA extraction kit. The quality of DNA was measured with agarose gel electrophoresis, and ultraviolet spectrophotometer was used for DNA quantification. Next, the 16S rDNA V3-V4 fragments were amplified, the reaction system: denaturation at 98 ^{o}C for 30 sec, denaturation at 98 ^{o}C for 10 sec, annealing at 54 ^{o}C for 30 sec, 35 cycles of extension at 72 ^{o}C for 45 sec, and final extension at 72 ^{o}C for 10 min. The sequences of primers used were: 341 forward primer (F): 5'-CCTACGGGNGGCWGCAG-3'; 805 reverse primer (R): 5'-GACTACHVGGGTATCTAATCC-3'. The data obtained were analyzed with Agilent 2100 Bioanalyzer (Agilent, USA) and Library Quantification Kit for Illumina Platform (Kapa Biosciences, Woburn, MA, USA).

The qualified sequencing libraries were subjected to gradient dilution, mixed in appropriate proportion based on the required amount of sequencing, and denatured to single strand with NaOH. Then, 2×250 bp paired-end sequencing was performed using NovaSeq 6000 sequencer. Data splitting, overlap removal, splicing and filtering were performed after obtaining the paired-end sequencing data, and DADA2 of qiime dada2 denoise-paired was applied for length filtering and denoising to obtain AVS-signature sequences and AVS-abundance forms. Alpha diversity analysis, beta diversity analysis, species annotation, differential analysis and advanced analysis were performed based on the obtained AVS-signature sequences and AVS-abundance forms.

Blood sample collection and testing

Rat caudal vein blood samples were collected. Fasting plasma glucose (FPG) and fasting serum insulin (FINS) were determined by biochemical analyzer. Homeostasis Model Assessment-Insulin Resistance (HOMA-IR) was used as an index for the evaluation of IR in each rat, the value of which was calculated as in Eq 1.

$$HOMA\text{-}IR = \{(FBG \times FINS)/22.5\} \quad \ldots\ldots\ldots (1)$$

Statistical analysis

The experimental data were processed using R language and GraphPad Prism. $P < 0.05$ indicated significant differences.

RESULTS

GHHRD regulated the intestinal flora structure of T2DM rats

In order to investigate changes in intestinal microflora of T2DM rats treated with GHHRD, 16S rDNA V3-V4 fragments in rat fecal samples were amplified and subjected to Illumina sequencing. Analyses of results from principal component analysis (PCA), non-metric multidimensional scaling (NMDS) and unweighted pair group method with arithmetic mean (UPGMA) showed that samples with large deviations in each group were removed (biological replicates >3 in each group). The alpha diversity index of intestinal flora in rats under different treatment conditions is shown in Table 1. The Shannon index, observed otus index, Simpson index, Chao1 index and pielou index of intestinal flora in rats in the T2DM group were lower than the corresponding values in rats in the NC group. These results indicate that the alpha diversity index of intestinal flora in the T2DM group was relatively decreased. However, when compared with the T2DM group, the alpha diversity index of T2DM + low-dose TCM group was increased, but it didn't reach the level alpha diversity index in the NC group, while it was increased significantly in T2DM + EJ and T2DM + high-dose TCM groups. These data demonstrate that GHHRD and metformin enhanced the richness and evenness of intestinal microflora in T2DM rats, to some extent. Principal coordinate analysis (PCoA) for optimal eigenvalue based on the distance matrix (any type of distance), revealed that high-dose GHHRD and metformin markedly changed the structure of intestinal flora (Figure 1 A).

Figure 1 B shows the column stacking diagram of composition of species in each group at the phylum level. The rat intestinal tract contains 19 phyla of microorganisms, with *Firmicutes* and *Bacteroidota* as the most dominant. Figure 1C presents a column stacking diagram of species composition in each group at the genus level, with 30 genera included. When exposed to STZ, there were higher levels of intestinal *Proteobacteria, Enterococcus, Ruminococcus* and *Prevotellaceae* in the T2DM group than in the NC group, while intestinal *Firmicutes, Ligilactobacillus, Escherichia-Shigella and Bacteroidota* were lower than those in the healthy samples in the NC group. However, treatment of the T2DM model rats with GHHRD resulted in lower intestinal levels of *Ruminococcus* and *Prevotellaceae* in the T2DM+ TCM groups than in the T2DM group, while the levels of *Firmicutes*, *Ligilactobacillus* and *Escherichia-Shigella* were higher than those in the T2DM group. These research results indicate that GHHRD changed the composition of intestinal flora in the rat model of T2DM.

GHHRD reduced IR in T2DM rats

HOMA-IR is an indicator used to evaluate IR levels. As the level of IR increases, HOMA-IR index increases. It was observed that the HOMA-IR index was elevated in rats in the T2DM group, when compared with the NC group. Moreover, the HOMA-IR index of rats in T2DM + low-dose TCM, T2DM + high-dose TCM group, and T2DM + EJ group were reduced significantly, when compared with those in the T2DM group (Table 2 and Figure 2). These results suggest that the HOMA-IR index was reduced and IR was mitigated in T2DM rats administered GHHRD.

Analysis of correlation between intestinal flora and HOMA-IR index

The results of this analysis showed that *Proteobacteria, Enterococcus, Ruminococcus, Firmicutes, Escherichia-Shigella,* and *Bacteroidota* were not significantly associated with HOMA-IR index (Table 3). However, *Prevotellaceae* was positively correlated with HOMA-IR index, while *Ligilactobacillus* was negatively correlated with HOMA-IR index (Figure 3). These results reveal that GHHRD reduced IR in T2DM model rats by changing the composition of intestinal microflora.

Table 1: Alpha diversity index of each group

Group	observed_otus	shannon	simpson	chao1	pielou_e
NC	777.00±152.72	7.72±0.65	0.98±0.01	780.05±154.08	0.81±0.04
T2DM	597.60±232.83	6.74±0.71	0.96±0.02	602.83±238.26	0.74±0.06
low-dose TCM	598.50±115.94	7.23±0.15	0.98±0.00	599.59±116.42	0.79±0.03
high-dose TCM	696.25±72.42	7.05±0.49	0.96±0.02	698.87±75.41	0.75±0.05
T2DM + EJ	732.83±159.78	7.20±0.63	0.97±0.02	736.40±161.69	0.76±0.04

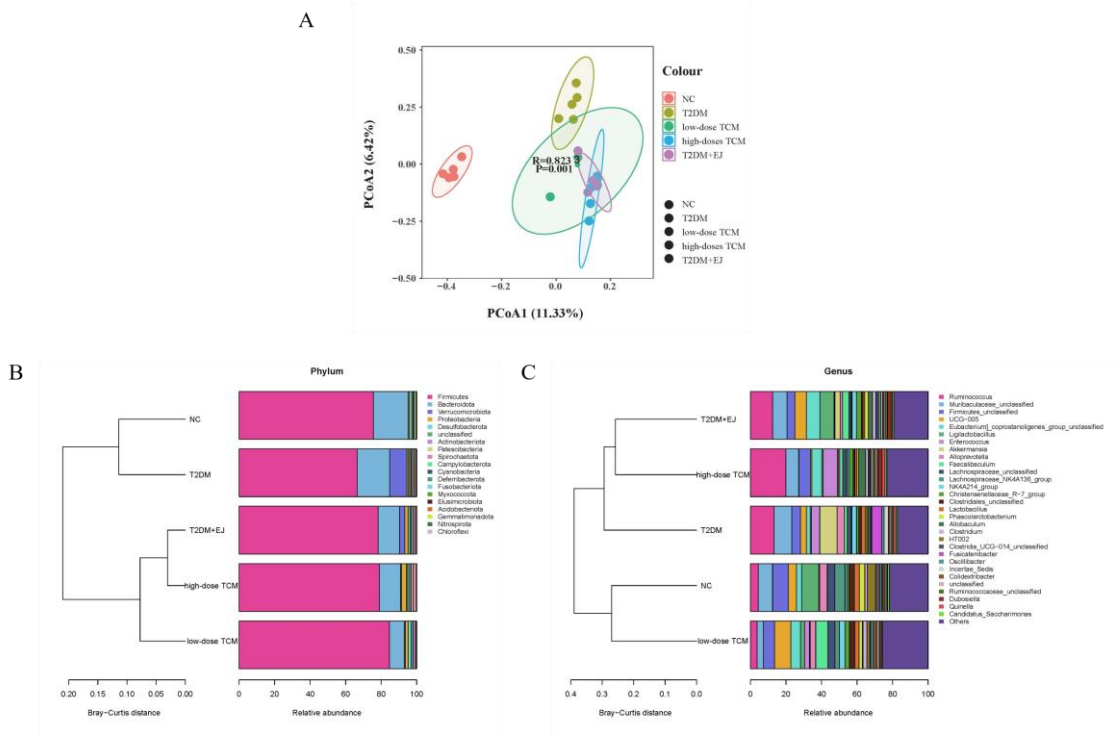

Figure 1: Beta diversity analysis of each group. A. PCoA; B. column stacking diagram based on the species classification at the phylum level; C. column stacking diagram based on the species classification at the genus level

Table 2: Comparison of HOMA-IR levels amongst the five groups

Group	Number of cases	HOMA-IR
NC	5	1.06±0.03
T2DM	5	2.82±0.38
low-dose TCM	4	1.92±0.50
high-dose TCM	4	1.71±0.34
T2DM + EJ	6	1.35±0.33

Table 3: Correlation between intestinal flora and HOMA-IR

Intestinal flora	rho	*P*-value	Correlation
Proteobacteria	-0.070135732	0.744687265	Negative
Enterococcus	0.366907905	0.077795978	Positive
Ruminococcus	0.226098584	0.288074079	Positive
Prevotellaceae	0.48609397	0.016024306	Positive
Firmicutes	-0.222479687	0.29606566	Negative
Ligilactobacillus	-0.511262585	0.010668355	Negative
Escherichia-Shigella	-0.294963951	0.161740411	Negative
Bacteroidota	-0.278530214	0.187526588	Negative

Rho: pearson correlation coefficient; correlation: trends in changes between intestinal flora and HOMA-IR; negative: negative correlation; positive: positive correlation

DISCUSSION

T2DM is associated with inflammation caused by overnutrition and other environmental factors (5). IR may be improved by intestinal flora via its influence on inflammatory response (6). It has been reported that intestinal flora plays significant roles in the occurrence and progression of T2DM by affecting the pathways of short-chain fatty acid metabolism, inflammatory response, and bile acid metabolism (7). Studies have shown that precise supplementation of probiotics and fecal transplant may be used for the prevention and treatment of T2DM via changes in composition of intestinal microorganisms (8). Furthermore, a study showed that pathogenic bacteria isolated from the intestinal of obese patients with diabetes mellitus induced obesity and IR in germ-free mice (9).

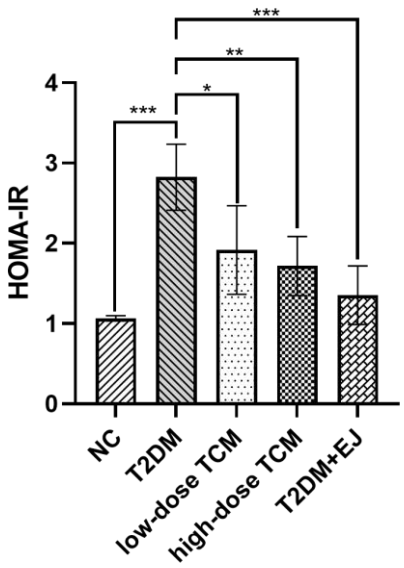

Figure 2: Comparison of HOMA-IR levels amongst the five groups (*P < 0.05, **p < 0.01, ***p < 0.001)

According to existing experiments, the diversity and abundance of intestinal flora are affected by exercise, thereby affecting diabetes. Researchers have found that exercise improves insulin sensitivity by regulating levels of short-chain fatty acids (metabolites of intestinal microflora), thereby improving the health conditions of patients with diabetes mellitus (10).

These findings suggest that intestinal flora may ameliorate the symptoms of T2DM and IR. In addition, metformin is a common drug used for the treatment of T2DM, and evidence has suggested that the hypoglycemic effect of metformin is associated with changes in intestinal flora(11). The current study revealed that GHHRD alters the composition of intestinal flora in a rat model of T2DM. This result is consistent with the findings of previous research.

The intestine is the largest immune organ in the body. It is an essential prerequisite that in order to exert immune function, the intestinal tract should maintain homeostasis in the types and contents of intestinal flora. The intestinal flora refers to the microorganisms located on the surface of intestinal mucosa. This microflora is considered as an environmental factor involved in processes such as human growth, development, physiological activities and diseases (12). Studies on metagenomics have confirmed that intestinal flora composition of T2DM patients is significantly different from that in the normal population (13). Studies have revealed that intestinal microorganisms are participated in the occurrence and development of T2DM by affecting the metabolism of bile acids, the integrity of the intestinal barrier, and the production of short-chain fatty acids (14).

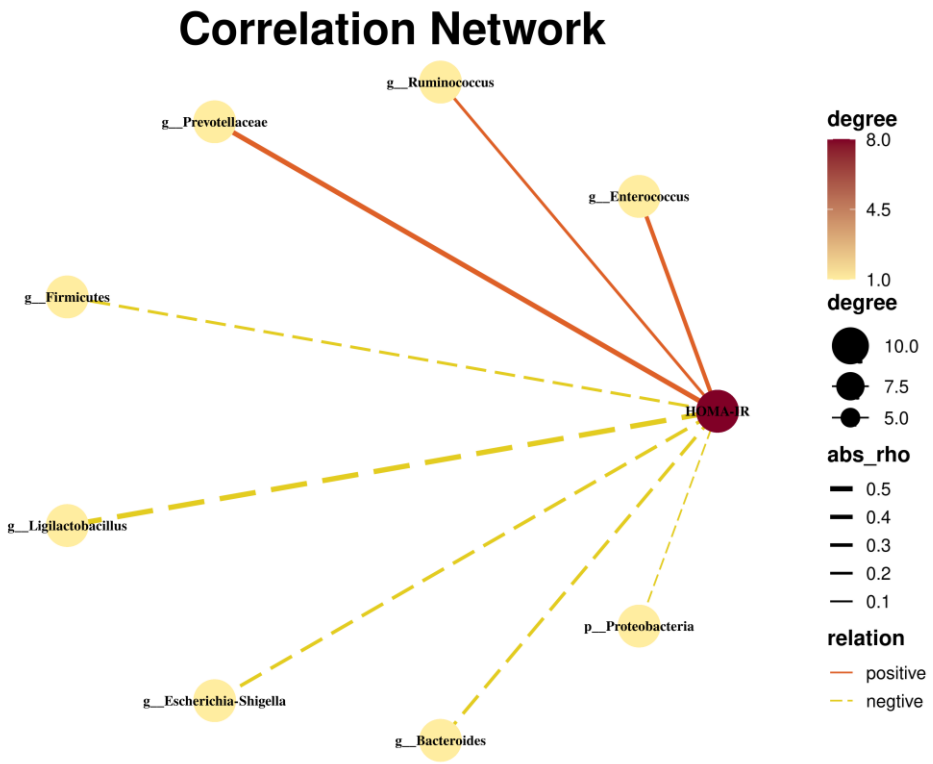

Figure 3: Correlation between intestinal flora and HOMA-IR

Traditional Chinese medicine (TCM) reduces the inflammation, hyperglycemia and imbalance in intestinal flora in T2DM patients by altering levels of microorganisms such as *Staphylococcus, Aerococcus* and *Corynebacterium* (15). Animal studies have revealed that Rhubarb anthraquinone glycosides (RAGs) regulate IR by improving intestinal flora (16). Therefore, we studied the effect of GHHRD on the structure of intestinal flora in T2DM model rats was investigated. Results from 16S sequencing showed marked increases in the number of opportunistic pathogens such as *Proteobacteria, Enterococcus, Ruminococcus* and *Prevotellaceae* in the intestinal flora of T2DM patients. Moreover, there were decreases in the number of probiotics, including *Firmicutes, Ligilactobacillus, Escherichia-Shigella* and *Bacteroidota*. Metformin is the most common drug for the management of diabetes mellitus, but it produces adverse effects such as symptoms of blurred vision, body fatigue, abdominal distension, diarrhea, nausea and retching (17). Interestingly, a study has reported that after administration of GHHRD for 3 months, patients with T2DM had no adverse effects, most likely due to *Ligilactobacillus* (18). In this study, it was found that after metformin treatment, *Ligilactobacillus* count was reduced significantly, when compared to that of the T2DM model, resulting in intestinal discomfort. In contrast, the content of *Ligilactobacillus* was gradually elevated in the T2DM + TCM group with increased dose of TCM, thereby reducing the symptoms of intestinal discomfort. These results suggest that GHHRD reduced the occurrence of adverse effects by regulating the intestinal flora in the rat model of T2DM. In addition, HOMA-IR is regarded as a major indicator of IR in people with T2DM, and it is used to predict development of the disease. Through experiments in this study, it was found out that HOMA-IR index was reduced in T2DM rats administered GHHRD, indicating that IR of T2DM rats was decreased.

CONCLUSION

This study has shown that GHHRD significantly improves insulin sensitivity in T2DM rats by changing the composition of intestinal flora. This finding may provide a new therapeutic strategy for the management of T2DM.

DECLARATIONS

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Funding

None provided.

Ethical approval

None provided.

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Conflict of Interest

No conflict of interest associated with this work.

Contribution of Authors

We declare that this work was done by the authors named in this article, and all liabilities pertaining to claims relating to the content of this article will be borne by the authors.

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