EXPERIMENTAL STUDY

Changes in Gut Microbiome Structure and Function of Rats with Isoproterenol-Induced Heart Failure

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Summary
Recently, the potential role of gut microbiome (GM) in cardiovascular diseases has been revealed. Heart failure (HF) is one of the most prevalent cardiovascular diseases worldwide; however, whether GM dysbiosis participates in the development of HF remains largely unknown. This study aimed to investigate the specific changes in GM composition and function in isoproterenol (ISO)-induced HF in rats.

The rats were divided into C (control), 4w-HF (ISO, 2.5 mg/kg/day for 4 weeks, intraperitoneally), and 2w-HF (ISO, 2.5 mg/kg/day for 2 weeks, intraperitoneally) groups. The cardiac structure and function in rats were assessed, and metagenomic analyses were then performed. Compared with the healthy control group, we found that the Shannon diversity index and microbial gene count in the 4w-HF and 2w-HF groups was drastically decreased. High-throughput sequencing showed that the three groups differed in intestinal bacterial community composition. Overgrowth of bacteria, such as Prevotella, was observed in the 4w-HF group, with reduced growth of bacteria, such as Roseburia, Lactobacillus, and Butyrivibrio, associated with healthy status compared with the C group on the genus level. Concomitant with the alteration of GM composition, under-representation of health-linked microbial function was observed in both the 4w-HF and 2w-HF groups compared with the C group.

Iso-induced HF rats showed a significant decrease in the diversity and richness of the intestinal microbiome, with a downregulation of the key intestinal bacterial groups and overgrowth of bacteria considered to be involved in inflammatory responses as well as a decrease in health-linked microbial function. Our data indicated that altered GM may be a potential player in the pathogenesis and progression of HF.

(Key words: High-throughput sequencing, Community composition, Inflammation)

Methods

Animals: A total of 30 SPF SD male rats (body weight 200-220 g) were selected from 6-8 week-old rats and were purchased from Hunan SLAC Laboratory Animal Co., Ltd. (License No.: SCXK [Xiang] 2013-0004). All the rats were housed in a clean environment at the experi-
Establishment of the heart failure animal model: One week after the adaptive feeding, all the rats were randomly divided into three groups: control group (C group, n = 10); 2-week HF group (2w-HF group, n = 10); and 4-week HF group (4w-HF group, n = 10). In the 2w-HF group, rats were intraperitoneally injected with 2.5 mg/kg/day ISO (Shanghai Harvest Pharmaceutical Co., Ltd., Shanghai, China) for 2 weeks. In the 4w-HF group, rats were intraperitoneally injected with 2.5 mg/kg/day ISO for 4 weeks. In the C group, rats were injected with saline 2.5 mg/kg/day for 4 weeks.

Echocardiography assessment of cardiac function: Cardiac function in rats was assessed by measuring end-diastolic diameters (LVEDD) and end-systolic diameters (LVESD), ejection fraction of the left ventricle (LVEF), and interventricular septum thickness at end-diastole (IVSd). Ultrasonic detection of cardiac function indicators and interventricular septum thickness at end-diastole (IVSd). Ultrasonic detection of cardiac function indicators and interventricular septum thickness at end-diastole (IVSd). Ultrasonic detection of cardiac function indicators and interventricular septum thickness at end-diastole (IVSd). Ultrasonic detection of cardiac function indicators and interventricular septum thickness at end-diastole (IVSd). Ultrasonic detection of cardiac function indicators and interventricular septum thickness at end-diastole (IVSd). Ultrasonic detection of cardiac function indicators and interventricular septum thickness at end-diastole (IVSd). Ultrasonic detection of cardiac function indicators and interventricular septum thickness at end-diastole (IVSd). Ultrasonic detection of cardiac function indicators and interventricular septum thickness at end-diastole (IVSd). Ultrasonic detection of cardiac function indicators and interventricular septum thickness at end-diastole (IVSd). Ultrasonic detection of cardiac function indicators and interventricular septum thickness at end-diastole (IVSd). Ultrasonic detection of cardiac function indicators and interventricular septum thickness at end-diastole (IVSd). Ultrasonic detection of cardiac function indicators and interventricular septum thickness at end-diastole (IVSd). Ultrasonic detection of cardiac function indicators and interventricular septum thickness at end-diastole (IVSd).

Preparation of myocardial pathological sections: The rats were sacrificed via an intraperitoneal injection of 0.3% pentobarbital sodium (10 mL/kg). The chest was opened and the heart was completely exposed. The heart was cut open at the root of aorta and washed in cold saline. The surrounding connective tissues and blood vessels were then cut. The hearts were fixed with 10% formaldehyde solution and embedded in paraffin. Using conventional methods, the heart specimens were dehydrated and embedded in paraffin. Subsequently, hematoxylin and eosin (H&E) staining was performed on these specimens, and they were analyzed using optical microscopy.

Determination of cardiac weight index: The rats were weighed following the intraperitoneal injection of 0.3% pentobarbital sodium (10 mL/kg). The chest was opened, and the heart was completely exposed. The heart was cut open at the root of aorta and washed in cold saline. The surrounding connective tissues and blood vessels were cut, and water was filtered out of the organs. The extracted hearts were weighed, and the heart weight index was calculated for each rat as a ratio of heart to body weight.

Determination of serum BNP: Rat serum brain natriuretic peptide (BNP) was determined using an enzyme-linked immunosorbent assay with a B-type BNP kit (Wuhan Clone Technology Co., Ltd., product number CEA541 Ra), according to the manufacturer’s instructions. Absorbance was measured for samples and standards at 450 nm using an enzyme labeling instrument (Synergy HT; BioTek Instruments, Inc., Winooski, VT, USA). A standard curve (S-type) was plotted with known concentrations on the X-axis and measured optical density values on the Y-axis, on a semi-logarithmic scale. The quantities of BNP in the samples were derived from the standard curve.

Stool sample collection and DNA extraction: Freshly collected stool samples from each group were immediately frozen in a liquid nitrogen tank, then transported to the laboratory, and stored in a refrigerator at −80°C. Bacterial DNA was extracted at Novogene Bioinformatics Technology Co., Ltd. using the TIANGEN kit, according to the manufacturer’s recommendations.

Sequencing results pretreatment: Preprocessing the Raw Data obtained from the Illumina HiSeq sequencing platform using Readfq (V8) was conducted to acquire Clean Data for subsequent analysis. The specific processing steps were as follows: 1) removal of the reads that contained low-quality bases (default quality threshold value ≤ 38) above a certain portion (default length of 40 bp), 2) removal of the reads in which the N base had reached a certain percentage (default length of 10 bp); 3) removal of the reads that shared the overlap above a certain portion with Adapter (default length of 15 bp). Clean Data need to be blast to the host database which default using SoapAligner software to filter the reads that are of host origin (Version soap2.21, parameters: identity ≥ 90%, -I 30, -v 7 , -M 4, -m 200, -x 400).

Metagenome assembly: Clean Data were assembled and analyzed using the SOAPdenovo software (Version2.04, parameters: -d 1, -M 3, -R, -u, -F, -K 55), and the assembled Scaffigs from N connection were then interrupted and left without N. Clean Data of all the samples were compared with each Scaffold, respectively, using the SoapAligner software to acquire the PE reads that were not used (Version soap2.21, parameters: identity ≥ 90%, -m 200, -x 400). All the reads that were not used in the forward step of all the samples were combined and then the software of SOAPdenovo (Version 2.04) was used for mixed assembly with the parameters same as that for single assembly. The mixed assembled Scaffolds from N connection were broken, and the Scaffigs were obtained. Fragments shorter than 500 bp in all the Scaffigs were filtered for statistical analysis generated from both single and mixed assemblies.

Gene prediction and abundance analysis: The Scaffigs (≥ 500 bp) assembled from both single and mixed assemblies were predicted as ORF by MetaGeneMark (V2.10) software, and the length information shorter than 100 nt was filtered from the predicted result with default parameters. For ORF predicted, the CD-HIT software (V 4.5.8, parameters: -c 0.95, -G 0, -aS 0.9, -g 1, -d 0) was used for redundancy and to obtain a unique initial gene catalog (here the genes refer to the nucleotide sequences coded by unique and continuous genes). Clean Data of each sample was mapped to the initial gene catalog using SoapAligner, and the number of reads for which genes were mapped in each sample was obtained (soap2.21, parameters: -m 200, -x 400; identity ≥ 95%). The gene for which the number of reads was ≤ 2 in each sample was filtered, and the gene catalog (Unigenes) obtained was eventually used for subsequent analysis. Based on the number of mapped reads and length of gene, abundance information of each gene in each sample was indicated.
The basic information statistic, core-pan gene analysis, and analysis of number of genes were based on the abundance of each gene in each sample in the gene catalog.

α diversity and rarefaction curve: To estimate the genus richness of the samples, we calculated the within-sample(α) diversity using the Shannon diversity index. A high α diversity indicates a high richness within the sample. Rarefaction analysis was performed to assess reliability of the number of samples. For a given number of samples, we performed random sampling 100 times with replacement and estimated the total number of genes that could be identified from these samples. We constructed a rarefaction curve of the core and pan genes. The flattening of the core-pan rarefaction curve indicates that the number of samples is reasonable and that more samples may produce only a small number of new genes.

Taxonomic annotation and abundance profiling: The DIAMOND software (V0.7.9, parameters: blastp, -e 1e-5) was used to blast the Unigenes to the sequences of bacteria, fungi, archaea, and viruses that were extracted from the NCBI database (Version: 20161115). For the finally aligned results of each sequence, as each sequence may have multiple aligned results, the result was chosen of which the e value ≤ the smallest e value * 10^-10 took the LCA algorithm, which was applied to the system classification of MEGAN software to ensure the species annotation information of sequences. The table containing the number of genes and the abundance of information of each sample in the genus was obtained based on the LCA annotation result and gene abundance table. The abundance of a species in one sample was equal to the sum of the gene abundance annotated for the species, and the number of genes of a species in a sample was equal to the number of genes whose abundance was nonzero. Krnko analysis, the exhibition of generation situation of relative abundance, the exhibition of abundance cluster heat map, and principal component analysis (PCA) (R ade4 package, Version 2.15.3) decrease-dimension analysis were based on the abundance table of the genus. The more similar the species composition, the closer was the species in the PCA.

Functional annotations: The DIAMOND software (V 0.7.9, blastp, -e 1e-5) was used to blast the Unigenes to Kyoto Encyclopedia of Genes and Genomes (KEGG) database (Version 201609). For each sequence’s blast result, the best Blast Hit was used for subsequent analysis. Based on the results of the comparison, the abundance of the KEGG module was calculated by summing the abundance of genes annotated to the same feature. Based on the abundance table of the module level, a PCA dimensionality analysis was performed.

### Results

**General conditions and survival rate of rats:** Compared with the C group, rats in the 4w-HF group generally experienced decreased feeding and activity and slower weight gain and reported the appearance of asthma, whereas rats in the 2w-HF group showed no obvious signs. There were two deaths in the 4w-HF group, one in the 2w-HF group, and no deaths in the C group after 4 weeks.

**Echocardiographic data:** Compared with the C group, echocardiography data revealed that the left ventricular diameter was significantly enlarged and systolic function was decreased in the 4w-HF and 2w-HF groups (Table I). End-diastolic and end-systolic diameters and ejection fraction of the left ventricle were significantly deteriorated in the 4w-HF group compared with the 2w-HF group (Table I).

**HWH and serum concentration of BNP in rats:** The heart weight index, defined as the ratio of heart to body weight, was higher in the 2w-HF and 4w-HF groups than in the C group. As expected, the heart weight index was higher in the 4w-HF group than in the 2w-HF group. The serum level of BNP was significantly higher in the 4w-HF group than in the C and 2w-HF groups (Table II).

**Histopathological changes in myocardial tissues:** Compared with the C group, the myocardial cells of the 4w-HF group showed disorder and irregularity and exhibited overt hypertrophy, acidophilic degeneration, or necrosis of myocardial cells. Slight hypertrophy, degeneration, or necrosis of myocardial cells was noted in the 2w-HF group (Figure 1).

**Intestinal flora diversity:** As shown by the core-pan rarefaction analysis, the curve was near saturation, which suggested that the sequencing data were great enough with very few new genes undetected (Figure 2A and B). The number of genes in both the 2w-HF and 4w-HF groups was significantly decreased compared with that in the C group ($P = 0.032$, C versus 4w-HF; $P = 0.005$, C versus 2 w-HF; Figure 2C). Consistently, the Shannon diversity index was much lower in the 2w-HF and 4w-HF groups ($P = 0.011$, C versus 4w-HF; $P = 0.037$, C versus 2w-HF; Figure 2D), suggesting lower levels of gene richness and...
In addition, we observed four functional modules for the host and have been observed in healthy populations. These metabolic functions are essential for biosynthesis, citrate cycle, biotin biosynthesis, and galactose degradation. These metabolic functions are essential for the host and have been observed in healthy populations. In addition, we observed four functional modules elevated in the 4w-HF group, including the alphahemolysin/cyclolysin transport system, pertussis pathogenicity signature 2, and phospholipid transport system (Figure 4B). Thus, our observation indicates that isoproterenol-induced HF affects the gut bacterial function.

**Discussion**

To date, there are limited studies indicating a direct or indirect association between gut flora and HF, the so-called gut hypothesis of HF. The decreased cardiac output and elevated systemic congestion can result in intestinal ischemia, edema, and epithelial dysfunction, thus leading to increased bacterial translocation and/or increased circulating endotoxins, such as lipopolysaccharide (LPS). A high level of LPS in the blood is consistent with the intestinal translocation process in patients with heart failure. LPS levels in the blood of patients with decompensated HF is directly related to systemic inflammation, and it decreases after compensation. It is well-known that HF is associated with a chronic inflammatory state, which may be caused or aggravated by this pathological mechanism, thus indirectly affecting the function of cardiomyocytes.

At present, most studies on intestinal microbes and HF are mostly intra-population correlation studies. For researchers, it is necessary not only to exclude the individual difference of the population but also to separately analyze the structural changes of gut flora caused by HF, which makes the study very difficult, thus the establishment of HF model with animal provides a powerful tool for separate analyzing the effect of HF on intestinal flora structure. Catecholamine plays an important role in neuro-

| Table II. HWI and Serum Levels of BNP in Rats |
|------------------|-------|------------------|
| Group     | n   | HWI (mg/g)       | BNP (pg/mL)       |
| C         | 10  | 2.14 ± 0.10      | 63.98 ± 5.12      |
| 4w-HF     | 8   | 3.49 ± 0.14*     | 188.48 ± 21.64*   |
| 2w-HF     | 9   | 2.97 ± 0.13*     | 67.15 ± 5.27      |

Data were described as mean ± standard deviation. *P < 0.01, compared with the C group; †P < 0.01, compared with the 2w-HF group. C indicates control; 4w-HF, 4-week heart failure; 2w-HF, 2-week heart failure; HWI, heart weight index; and BNP, B-type brain natriuretic peptide.

![Figure 1. Myocardial biopsy of rat myocardial tissues stained with hematoxylin and eosin (H&E). A: control (C) group; B: 4w-HF group; and C: 2w-HF group](image-url)
humoral activation, cardiac output increases, and perfusion improvements in the peripheral tissues. However, excessive production of catecholamine or the use of exogenous catecholamine may be deleterious. Isoproterenol, which stimulates beta-receptors, causes myocardial damage and represents a well-established animal model of cardiac hypertrophy, left ventricular dysfunction, and HF. In this study, isoproterenol was used to induce HF in the rat models. Moreover, rats lived in the same environment, and there was no difference in the diets.

The changes in the structure and function of intestinal flora in different stages of HF were analyzed by macronome sequencing. The results of this study showed that isoproterenol-induced HF decreases the diversity and richness of intestinal flora in rats, which is consistent with previous reports from population studies suggesting possible deficiency of healthy microflora in patients with HF.

PCA showed that isoproterenol-induced HF alters gut microbiota community structure. Most importantly, the direct impact of HF on gut microbiota composition profile was notable from these studies. Bacteria such as Prevotella, Porphyromonas, and Anaerobiospirillum are increased in the HF groups. These microbes are considered to be involved in the inflammatory response of some diseases. For example, studies have found that Anaerobiospirillum can cause cholecystitis and fatal bacteremia, and Porphyromonas is considered to cause infection and periodontal disease pathogenic oral bacteria. Most notably, Prevotella originated from the mouth and vagina was abundant in our study cohort. The pathogenesis of periodontal diseases and rheumatoid arthritis is thought to be attributed to Prevotella. Researchers have previously suggested that Prevotella thrives in a pro-inflammatory environment of rheumatoid arthritis. Phosphoadenosine phosphosulphate reductase and superoxide reductase encoded
Figure 3. Isoproterenol-induced heart failure alters gut microbiota composition profile and community structure. A: The relative abundances of the top 10 bacteria at the genus level. B: The alteration of intestinal bacterial patterns at the genus level. The heatmap is color-based on row Z-scores. The rats with the highest and lowest bacterial levels are in red and blue, respectively. C: Principal component analysis was used to measure the shift of the intestinal bacterial composition profile.

Concomitant with the alterations in gut microbial composition, we observed a dysbiosis in bacterial gene functions. The genes associated with biosynthesis and transport of amino acids, such as lysine, threonine, and serine, which are essential for human health, were depleted in the metagenome of the study groups. Functional annotation also indicated a decrease in modules for citrate cycle and galactose degradation, suggesting an impaired capacity of energy production. Indeed, these metabolic functions are necessary for healthy populations. Because Lactobacillus can promote the metabolism of essential molecules such as amino acids, proteins, and nucleotides, the downregulation of these metabolic functions in this study may be related to the downregulation of Lactobacillus, indicating that Lactobacillus may promote the pathogenesis of HF by affecting metabolism in the body. Thus, bacterial gene functional dysbiosis may contribute to the susceptibility to HF.

Although, there is no direct evidence of correlation between gut microbial and HF to date, intestinal microflora can still be a potential target for the treatment of HF. Further research on the associated mechanisms to clarify whether gut bacterial metabolites contribute to the immune inflammatory system during the development of HF is underway. Thus, more attention should be given to gut bacteria in cases of HF.
**Figure 4.** Isoproterenol-induced heart failure alters function of gut microbiota. A: Principal component analysis was used to measure the shift of the intestinal bacterial function. B: The alteration of intestinal bacterial function based on KEGG module. The heatmap is color-based on row Z-scores. The rats with the highest and lowest bacterial level are in red and blue, respectively.

**Conclusion**

In summary, this study revealed changes in the structure and function of intestinal microflora caused by HF, but the specific mechanisms involved between microflora and HF are still unknown. Therefore, further research is needed to find a new treatment of HF and to provide a basis for interventional targets.

**Disclosures**

**Conflicts of interest:** The authors declare no conflict of interest.

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