

Regular Article

Zhengganxifeng Decoction Affects Gut Microbiota and Reduces Blood Pressure *via* Renin–Angiotensin System

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Received January 22, 2019; accepted May 22, 2019

Zhengganxifeng decoction (ZGXFD) is a traditional Chinese medicinal formula, from “Medical Zhong parameter West recorded” by Xichun Zhang, which has been applied to the treatment of clinical essential hypertension. Besides its effect in blood pressure reduction, ZGXFD is also known to be a radical therapy with little or no side effects. Compared with western medicines, Chinese medicinal formulas have the advantage of simultaneously attacking multiple targets. However, such a property brings trouble to the pharmacological studies of Chinese medicines. This study investigated the composition of gut microbiota in spontaneously hypertensive rats (SHR) treated with ZGXFD. ZGXFD was shown to cause similar effects in the treatment group as benazepril: both were able to reduce in SHR the microbial diversity, Firmicutes to Bacteroidetes (F/B) ratio and coccus to bacillus (C/B) ratio. Meanwhile, ZGXFD can maintain the integrity of intestinal mechanistic barrier and elevate the percentage of bacteria producing short chain fatty acids (SCFA). By investigating renin–angiotensin system (RAS) system, we found that ZGXFD can decrease the expression of angiotensin-converting-enzyme (ACE) in lungs, which in turn causes a increase in AngI produces angiotensin1–7 (Ang1–7) and decrease in AngII. ZGXFD regulate blood pressure in SHR *via* RAS.

Key words Zhengganxifeng decoction; spontaneously hypertensive rat; blood pressure; gut microbial composition

INTRODUCTION

By virtue of improved sanitation and ever-developing medical technology, the incidence and lethality of infectious diseases are steadily declining. However, population aging, urbanization and a shift in life and dietary style, noncommunicable diseases have become the major threat to human health, including cancer, diabetes and cardiovascular disease. As a type of cardiovascular disease, hypertension can lead to sudden death, heart disease, cerebral hemorrhage, renal failure and apoplexy, *etc.*^{1–3} Developing protocols for early diagnosis and effective treatment is a burning issue and demands cooperative work from scientists all over the world.⁴ As a relatively new substitute of modern medicine, Chinese medicine becomes more and more often accepted as a complementary or even alternative treatment for cardiovascular disease.^{5–7}

As a traditional Chinese medicinal formula, Zhengganxifeng decoction (ZGXFD) contains 12 ingredients and has been used for the treatment of clinical idiopathic hypertension.⁸ Because of the heterogeneous composition and synergy among different components, Chinese medicines are able to simultaneously attack multiple targets. Yet the complex composition makes it extremely difficult, if not impossible, to study and interpret their pharmacological mechanisms.

More than 1×10^{14} microbes, mostly bacteria, reside in our intestinal tracts, which are generally called gut microbiota.⁹ Mainly belong to four phyla: Firmicutes, Bacteroidetes, Actinobacteria and Proteobacteria. A theory called metagenomics has been recently proposed that two sets of genomes exist in human bodies, one from our parents and the other from

microbes residing inside us. The genomic interactions are reflected in the extensive involvement of gut microbes in each of our physiological activities.¹⁰

The maintenance of diversity and balance of gut microbiota is critical for a healthy immune system and physiological homeostasis.^{11,12} Because the composition and richness of gut microbiota depend on the age, dietary habit, lifestyle and physical condition of the host, some researchers proposed to regard it as an acquired organ.^{13,14} It has been reported that essential hypertension is always accompanied with negative changes in gut microbial composition.¹⁵ When inflammation occurs in the gastrointestinal tract, damaged immunologic barrier can lead to dysbiosis in gut microbiota: harmful bacteria inhibited in normal conditions massively proliferate. Therefore, we hypothesized that ZGXFD may have an influence on gut microbiota, which contributes to its effect on blood pressure reduction.

MATERIALS AND METHODS

Animals All rats in the study are male and 14-week-old. Thirty spontaneously hypertensive rats (SHR) and 6 Wistar-Kyoto (WKY) rats were used, each weighed 350 ± 20 g (the two strains share the same genetic background). Both strains were purchased from Beijing Vital River Laboratory Animal Technology Co., Ltd. (China) and kept in a SPF laboratory with constant temperature ($22 \pm 2^\circ\text{C}$), humidity ($55 \pm 5\%$) and a 12 light/12 dark cycle. All rats were fed with standard fodder according to GB14924.3-2010 and sterile water.

Medication The composition and doses of ZGXFD re-

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ferred to “Medical Zhong parameter West recorded” by Xi-chun Zhang: achyranthes root 30g (Guanlan Pharmaceutical Co., Ltd., Gansu, China), ruddle 30g (Guanlan Pharmaceutical Co., Ltd.), dragon bone 15g (Guanlan Pharmaceutical Co., Ltd.), oyster shell 15g (Guanlan Pharmaceutical Co., Ltd.), plastrum testudinis 15g (Guanlan Pharmaceutical Co., Ltd.), white peony root 15g (Guanlan Pharmaceutical Co., Ltd.), radix scrophulariae 15g (Guanlan Pharmaceutical Co., Ltd.), radix asparagi 15g (Guanlan Pharmaceutical Co., Ltd.), fructus toosendan 6g (Guanlan Pharmaceutical Co., Ltd.), raw malt 6g (Guanlan Pharmaceutical Co., Ltd.), artemisia capillaris Thunb 6g (Guanlan Pharmaceutical Co., Ltd.), and glycyrrhiza 4.5g (Guanlan Pharmaceutical Co., Ltd.). Ruddle, dragon bone, oyster shell and plastrum testudinis were decocted for 2h and then decocted with remaining ingredients for 3 times. Distilled water was used and the volume was ten times of all other ingredients each time. The medicine was then filtrated, concentrated to 1.5, 3.0 and 6.0g/mL, then stored in the 4°C refrigerator of Gansu University of Traditional Chinese Medicine. Benazepril Hydrochloride Tablets were purchased from Beijing Novartis Pharma Co., Ltd., Beijing, China.

Doses The low-dose, medium-dose and high-dose groups were drenched with $15\text{ g}\cdot\text{kg}^{-1}\cdot\text{d}^{-1}$ (concentration: 1.5g/mL), $30\text{ g}\cdot\text{kg}^{-1}\cdot\text{d}^{-1}$ (concentration: 3.0g/mL) and $60\text{ g}\cdot\text{kg}^{-1}\cdot\text{d}^{-1}$ (concentration: 6.0g/mL), respectively. The dose for the benazepril-treated group was $0.90\text{ mg}\cdot\text{kg}^{-1}\cdot\text{d}^{-1}$. Corresponding volumes of distilled water were drenched to rats in the control and WKY groups. (All doses referred to Experimental Methodology of Pharmacology (the 4th version) by Wei Wei, Ximei Wu and Yuanjian Li.)

Measurement of Blood Pressure and Heart Rates of Rats After 8 weeks of medicine treatment, the arterial pressure on the tails of rats was measured in the early morning by BP-97A, Sofcron. Each rat was measure 5 times and the average was calculated. When measuring the blood pressure, researchers waited 3min for rats to get calmed down. Data are representative of three independent experiments ($n = 6$). Student’s *t*-test was used to assess statistical significance.

Fecal Sampling and 16S Ribosomal DNA (rDNA) Sequencing Fecal samples from the rectum were collected in a sterile tube immediately after euthanasia and stored at -80°C until processing. DNA was extracted using cetyl trimethyl ammonium bromide/sodium dodecyl sulfate (CTAB/SDS) method as described previously. Hypervariable region V4 of 16S ribosomal RNA (rRNA) genes was amplified using forward primer 515F (GTGCCAGCMGCCGCGGTAA) and reverse primer 806R (GGACTCHVGGGTWTCTAAT). PCR amplicons were sequenced with Illumina HiSeq2500 platform. Raw sequence data were filtered, processed and analyzed according to the QIIME (V1.7.0) quality controlled process. Sequences with $\geq 97\%$ similarity were assigned to the same operational taxonomic units (OTUs). Taxonomic annotation was made using RDP classifier algorithm and the GreenGene Database. 16S rRNA gene sequences were analyzed with the QIIME (Version1.7.0) software package and displayed with R software (Version 2.15.3). Data are representative of three independent experiments ($n = 6$). Student’s *t*-test was used to assess statistical significance.

Measurement of Coccus to Bacillus (C/B) Ratios Feces were sectioned into $1.5\times 2\text{ cm}^2$ pieces and naturally dehydrated. Gram staining was conducted after fixing the samples with

the flame of an alcohol stove. Bacteria in evenly stained parts were counted under an oil immersion lens and C/B ratios were then calculated. Data are representative of three independent experiments ($n = 6$). Student’s *t*-test was used to assess statistical significance.

Extraction and Detection of Short Chain Fatty Acids (SCFAs) 0.3g feces were dissolved with 15mL 100mmol/L 2-ethyl butyrate and 50mL 5mmol/L HCl at room temperature for 10min. After 15000rpm, 10min of centrifugation, the supernate was filtered and loaded onto DBFFAP column ($30\text{ m}\times 250\mu\text{ m}\times 0.25\mu\text{ m}$) for GC analysis (carrier gas: 99.999% N_2 ; flow rate: 0.8mL/min; auxiliary gas: 99.999% H_2 ; FID and injection temperature: 280 and 250°C, respectively; split ratio: 50:1; injection volume: 1 μL ; temperature programming: starting from 60°C, increasing at 20°C/min until 220°C and staying for 1 min). Data are representative of three independent experiments ($n = 6$). Student’s *t*-test was used to assess statistical significance.

Enzyme-Lined Immunosorbent Assay (ELISA) of Plasma Five milliliter arterial blood was collected and anticoagulated by ethylenediaminetetraacetic acid (EDTA). After 1000r/min of centrifugation for 10min in room temperature, supernate was collected and store in -80 – -20°C refrigerator. Following steps were performed according to the kit instruction (MEIMIAN, Jingsu Fiya Biological Technology Co., Ltd., China). Data are representative of three independent experiments ($n = 6$). Student’s *t*-test was used to assess statistical significance.

Statistics All numerical results were processed by SPSS 21.0 and represented as mean \pm deviation. $p < 0.05$ indicated the existence of statistical significance.

RESULTS

ZGXFD Can Effectively Inhibit Blood Pressure Increase in SHR In the present study, we investigated the effect of ZFXFD on spontaneously hypertensive rats (SHR), while using Wistar Kyoto rats (WKY) as the control group. Benazepril, an angiotensin-converting-enzyme (ACE) inhibitor, is known to be highly effect, low cost and have minor side effects, and thus is widely used in modern medicine for treating hypertension.^{16–18)} To compare the effect of blood pressure reduction induced by benazepril and ZGXFD, we administered the medicines to two groups of mice for 8 weeks. It was found that although ZGXFD was not as effective as benazepril on blood pressure reduction, the systolic blood pressure (SBP) and diastolic blood pressure (DBP) of the group of mice treated with ZGXFD significantly decreased, compared untreated group (Figs. 1A, B). Blood pressure closely correlated with HR(heart rates): a rise in blood pressure frequently accompanies with an increased heart rate. It was observed that ZGXFD caused the exact same effect on heart rate. It indicated that ZGXFD was able to reduce both blood pressure and heart rate of SHR (Fig. 1C).

For western pharmaceuticals, medicines are developed to attack various pathways after a thorough study of the pathogenesis of a certain disease. Yet traditional Chinese medicine (TCM) makes the prescription according to the symptom that patients exhibit. ZGXFD was describe to cure headache, dizziness, visual impairment, tinnitus and palpitation, all of which are just the clinical manifestation of hypertension.

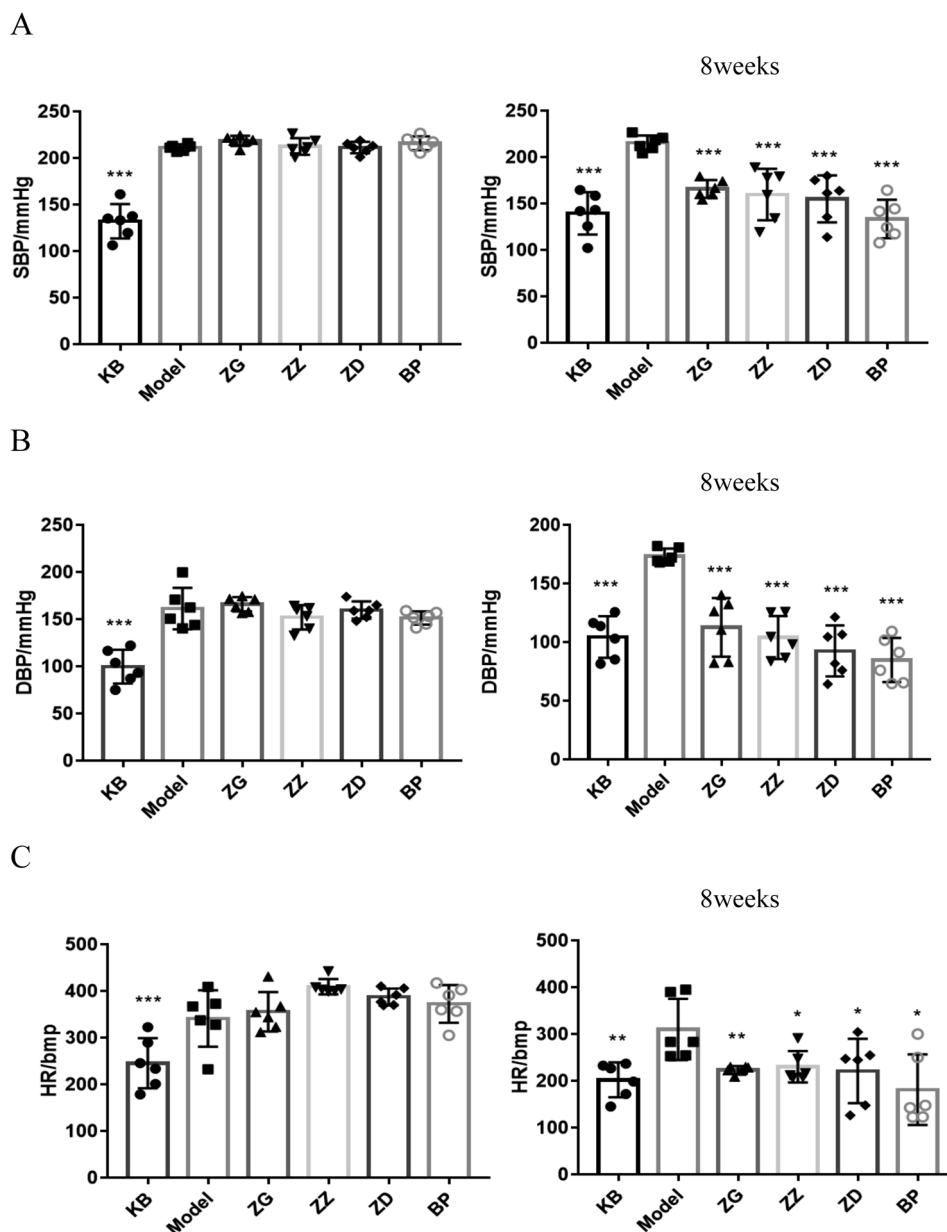


Fig. 1. The Influence of ZGXFD on Blood Pressure in SHR

A. SBP of rats before and 8 weeks after the treatment of ZGXFD. B. DBP of rats before and 8 weeks after the treatment of ZGXFD. C. HR of rats before and 8 weeks after the treatment of ZGXFD. Data are expressed as the mean \pm standard deviation (S.D.) ($n = 6$), *** $p < 0.005$, ** $p < 0.01$, * $p < 0.05$ vs. Model group. Each group contains 6 rats. KB, Model represent the WKY group and untreated SHR group, respectively. ZG, ZZ, ZD represent high-dose, medium-dose and low-dose ZGXFD treated SHR groups. BP represents the group of rats treated with benazepril. Data are representative of three independent experiments ($n = 6$). Student's t -test was used to assess statistical significance.

However, how ZGXFD worked remained unexplored. Attention has recently been drawn to the connection between gut microbiota and hypertension.^{19,20} It was reported that essential hypertension can cause the change in the structure of gut flora, which was why ZGXFD was hypothesized to impose an effect on gut microbiota as well. We use DNA sequencing to analyze the gut microbial composition of SHR.

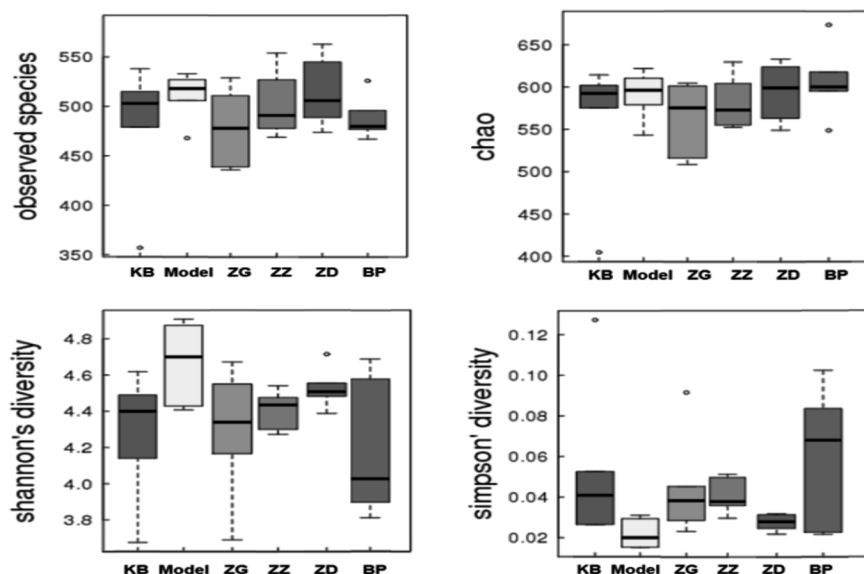
Hypertension Is Linked to Gut Microbial Composition of SHR Gut microbiota were collected from feces and analyzed by 16S rDNA sequencing. Big differences of microbial composition was detected after mice treated with ZGXFD and benazepril. We used observed species composition, Chao richness, Shannon diversity and Simpson diversity to evaluate the diversity of gut microbiota. We found that the gut microbial

diversity of SHR was higher than that of WKY and got lower after the treatment of ZGXFD and benazepril. Also, the bacterial diversity in the group treated with ZGXFD was negatively correlated with the doses (Fig. 2A).

We investigated bacterial composition in each group of rats by principal component analysis (PCA), a statistical procedure for analyzing and simplifying data sets, in which a close distance between two samples indicates a similar composition. The results showed that the bacterial composition was significantly different among the model group and groups treated with ZGXFD or benazepril, suggesting an influence ZGXFD imposed on the gut microbiota of SHR (Fig. 2B).

ZGXFD Contributes to Gut Microbial Homeostasis and Integrity of Intestinal Mechanical Barrier The amount

A



B

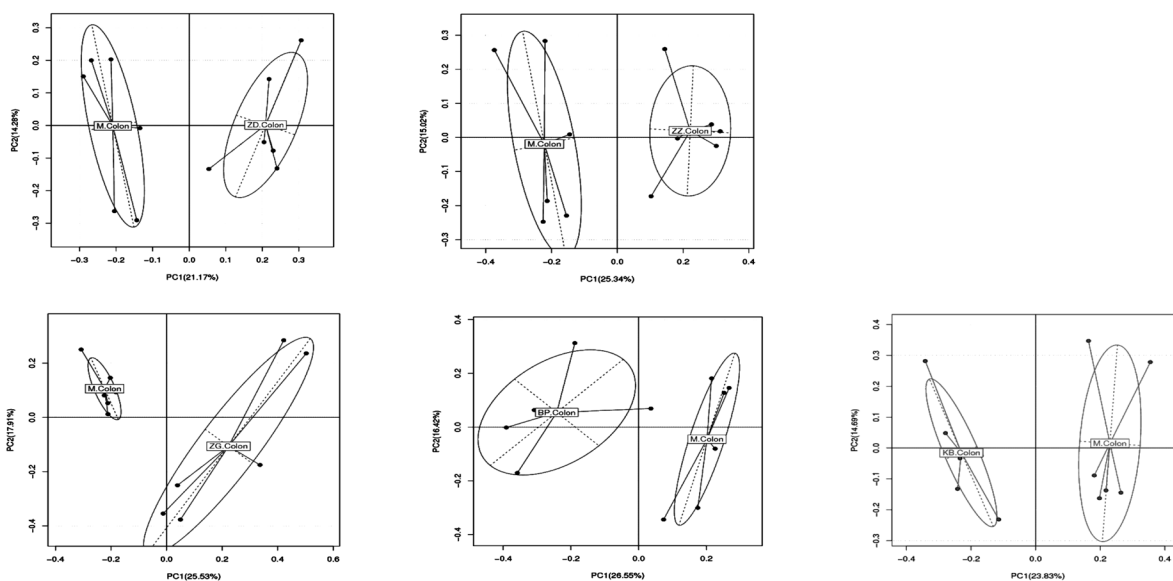


Fig. 2. The Changes in Gut Microbial Composition of SHR after Drug Treatment

A. ZGXF D can reduce the diversity of gut microbiota in SHR. Rat feces were processed for 16s rDNA sequencing. Species composition, Chao richness, Shannon diversity and Simpson diversity were used to evaluate gut microbial diversity. Each group contains 6 rats. B. PCA analysis are run to analyze the main and auxiliary microbial composition in each group. The horizontal and vertical axes represent the main and auxiliary composition, respectively. The differences are represented as the distance between any two points. KB, Model represent the WKY group and untreated SHR group, respectively. ZG, ZZ, ZD represent high-dose, medium-dose and low-dose ZGXF D treated SHR groups. BP represents the group of rats treated with benazepril. Data are representative of three independent experiments ($n = 6$). Student's *t*-test was used to assess statistical significance.

of gut microbiota per adult animal researches up to trillions. They are highly diverse, but most of them belong to four phyla: Firmicutes, Bacteroidetes, Proteobacteria and Actinobacteria. Firmicutes to Bacteroidetes (F/B) ratio was considered as an important indicator of gut microbial homeostasis.²¹⁾ Either an increase in the abundance of Firmicutes or a decrease in the abundance of Bacteroidetes leads to a rise in F/B ratio, which indicates gut dysbiosis. *Coccus* to *Bacillus* (C/B) ratio is also commonly used as an indicator of gut bacterial homeostasis, whose normal range is between 0.05 and 0.1.^{22–24)} We used these two indicators to evaluate the influence ZGXF D exerted on gut microbial homeostasis. According to 16S rDNA

sequencing, the F/B ratio of the untreated SHR group was found to be 60% higher than the WKY group, and 20–50% higher than the groups treated with ZGXF D and benazepril (Fig. 3A). It was also found that the C/B ratio of the untreated SHR group was significantly higher than other groups (Fig. 3B). Gut microbes reside on the surface of intestinal epithelial cells and provide the intestinal mucosal tissue with a layer of barrier from potential exogenous pathogens. Gut dysbiosis damages the intestinal mechanical barrier. D-Lactic acid is produced by bacterial metabolism and degradation of dead bacteria.^{25,26)} Diamine oxidase (DAO) is highly active in intestinal villi. When the damage barrier leads to increased

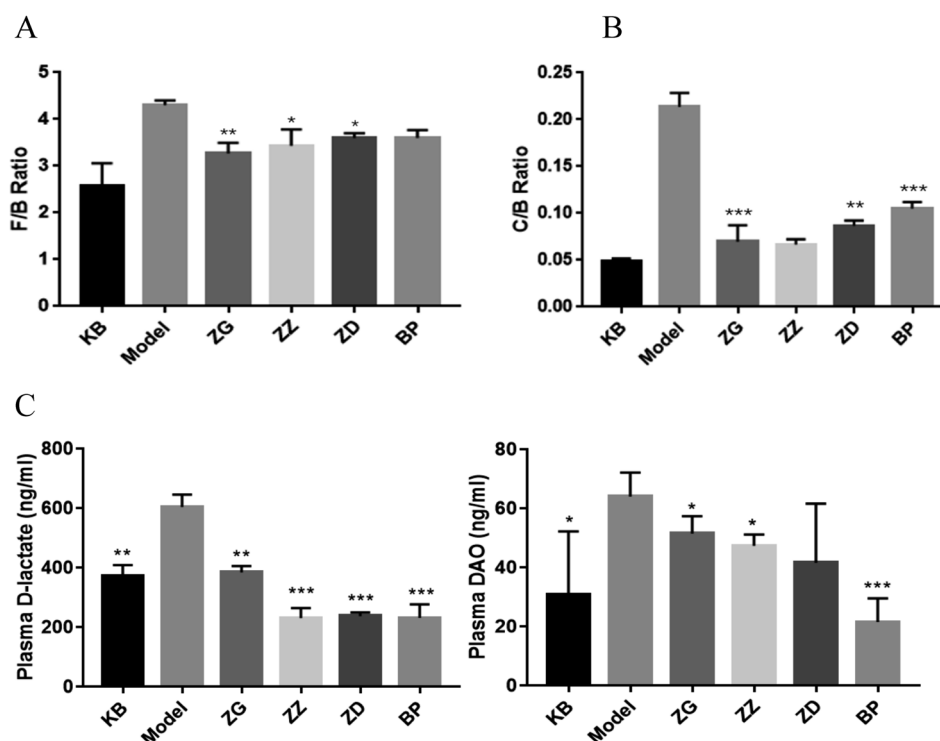


Fig. 3. ZGXF maintains gut microbial homeostasis

A. F/B ratio of feces was analyzed by 16S rDNA sequencing B. C/B ratio of feces was analyzed by smear tests C. The concentration of D-lactate and DAO in plasma. Data are expressed as the mean \pm S.D. ($n = 6$), *** $p < 0.005$, ** $p < 0.01$, * $p < 0.05$ vs. Model group. Each group contains 6 rats. KB, Model represent the WKY group and untreated SHR group, respectively. ZG, ZZ, ZD represent high-dose, medium-dose and low-dose ZGXF treated SHR groups. BP represents the group of rats treated with benazepril. Data are representative of three independent experiments ($n = 6$). Student's t -test was used to assess statistical significance.

intestinal permeability, D-lactic acid and DAO enter blood.^{27,28}) Therefore, their content in blood is a marker for the integrity of intestinal barrier. ELISA was used to evaluate the content of plasma D-lactic acid and DAO. Results showed that the content of plasma D-lactic acid and DAO in SHR was significantly higher than that of WKY and got reduced after the treatment of ZGXF (Fig. 3C).

We determined through 16S rDNA sequencing that both ZGXF and benazepril led to a drop in the gut bacterial diversity and the F/B and C/B ratios, which may suggest a similar mechanism in reducing blood pressure. As a prodrug, benazepril is converted by hydrolysis in the liver to the active form, benazeprilat. The latter is an inhibitor for angiotensin converting enzyme (ACE), which can inhibit the conversion of ACE1 to ACE2 and thus reduce blood pressure through the renin-angiotensin system (RAS).²⁹) Therefore, we then investigated the influence ZGXF might impose on RAS system.

ZGXF Promotes the Production of SCFA Short-chain fatty acids (SCFA) are produced during intestinal microbial metabolism, including acetic acid, propionic acid and butyric acid. They provide energy for intestinal epithelial cells, affect the metabolism, proliferation and differentiation of intestinal cells, and repress inflammation by regulating T cells. Thus, SCFAs are crucial for the maintenance of normal intestinal functioning. It was observed that the population of bacteria producing SCFAs was three times lower in SHR than that in WKY (Fig. 4A). ZGXF reconstructed the gut microbial composition and increased the fecal SCFA content in SHR (Fig. 4B).

ZGXF Increases Angiotensin1-7 (Ang1-7) and Decreases AngiotensinII in Blood RAS regulates cardio-

vascular functions. Plasma renin, primarily produced by the kidney, converts angiotensinogen into angiotensin I (AngI), which is subsequently converted into angiotensin II (AngII). AngII then combines with AngII receptor to function as a vasoconstrictor — to cause constriction of blood vessels.³⁰⁻³³) On the other hand, AngI produces Ang1-7 to inhibit the activity of AngII and therefore to reduce blood pressure.³⁴⁻³⁶) But it was not found that ZGXF had an effect on the level of plasma renin (Fig. 5A). So we hypothesized that ZGXF might regulate blood pressure by influencing the conversion of AngI into AngII, as well other downstream factors. To confirm this, we analyzed the levels of AngII and Ang1-7 in the blood of SHR treated with ZGXF. We observed a significant decline in the level of AngII but a rise in the level of Ang1-7 (Figs. 5B, C). This suggested that increased Ang1-7 caused by ZGXF inhibited AngII, which finally led to the reduction of blood pressure.

DISCUSSION

The present study explained the functional mechanism of the traditional Chinese medicinal formula, ZGXF. The main discoveries were: (1) ZGXF can effectively reduce the blood pressure and heart rates of SHR; (2) Significant differences in gut microbial composition exist among WKY, SHR treated with ZGXF, and SHR without treatment; (3) ZGXF can cause reductions in F/B and C/B ratios, indicating an effect on maintaining gut microbial homeostasis; (4) ZGXF helps maintain the integrity of gut mechanistic barrier and increase the percentage of microbes producing SCFAs. (5) ZGXF increase blood Ang1-7 and decrease AngII, thus reducing blood

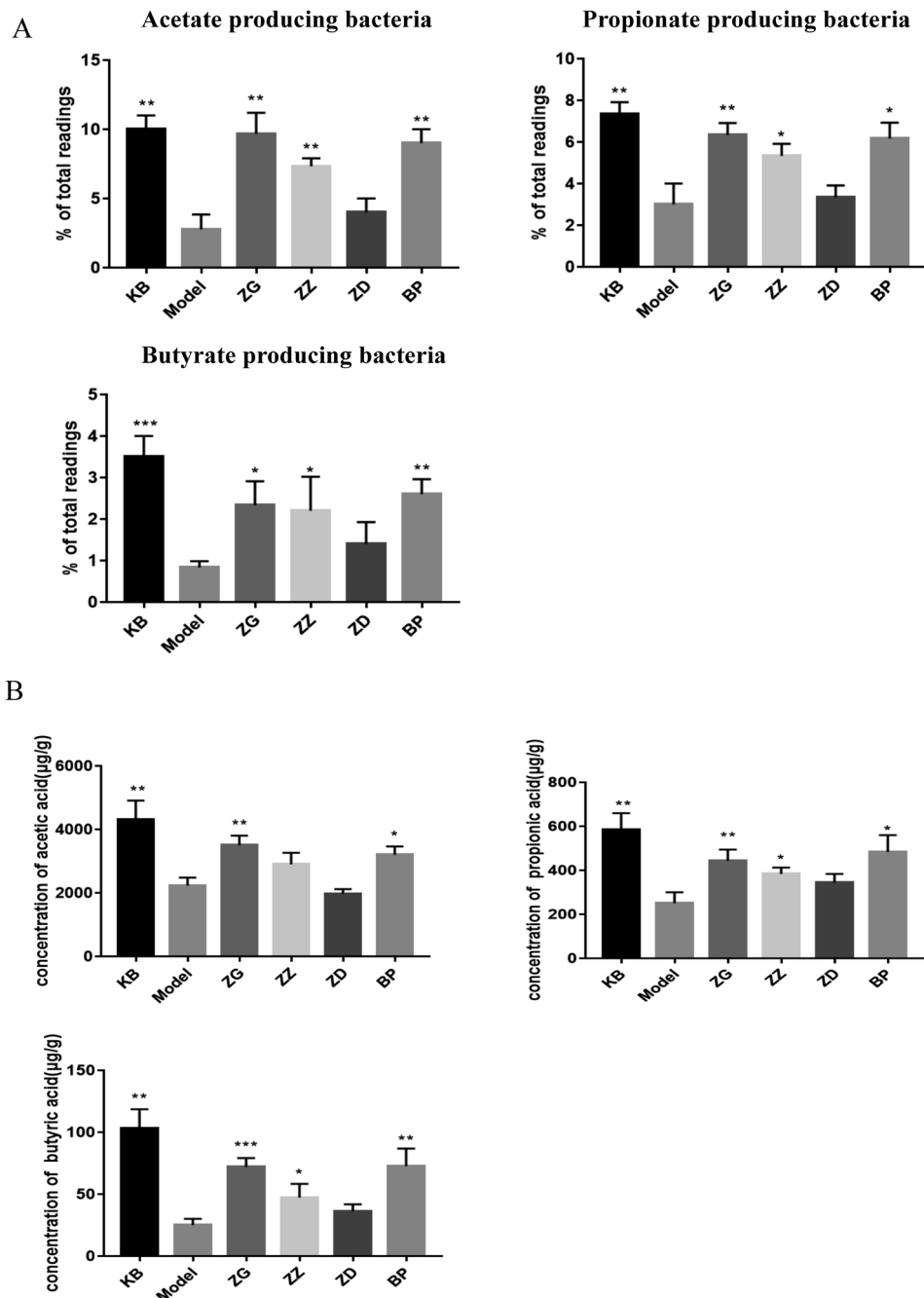


Fig. 4. ZGXFD Promotes SCFA Production

A. The relative proportions of acetate, propionate and butyrate producing bacteria in the gut microbiota. B. The content of acetate, propionate and butyrate in rat feces. The end metabolites of each assigned bacterial genus were used to analyze and sort the sequencing data. Bacteria were considered belong to more than one group if they were found to be producers of multiple end products. Since equol, histamine, hydrogen and propionate producing genera made up a small part of the population, they were excluded from our analysis. Data are expressed as the mean \pm S.D. ($n=6$), *** $p < 0.005$, ** $p < 0.01$, * $p < 0.05$ vs. Model group. Each group contains 6 rats. KB, Model represent the WKY group and untreated SHR group, respectively. ZG, ZZ, ZD represent high-dose, medium-dose and low-dose ZGXFD treated SHR groups. BP represents the group of rats treated with benazepril. Data are representative of three independent experiments ($n=6$). Student's *t*-test was used to assess statistical significance.

pressure *via* RAS system.

Chinese medicinal formulas have been used in clinical treatment since ancient time. But back then, a lack of diagnostic accuracy and sparseness of pathogenetic studies, doctors determined the medicinal formulas only according to symptoms. For instance, ZGXFD was described in the pharmacopoeia to treat symptoms, including treat headache, dizziness, visual impairment, tinnitus and palpitation. With the development of medical science and diagnostic techniques, it has been recognized that these are symptoms related with

high blood pressure and that ZGXFD can be used in the treatment of hypertension. When studying pharmacologic action of Chinese medical formulas, two frequent problems in front of researchers are: (1) the complex multi-target activities of Chinese medicinal formulas; (2) the complicated interaction of ingredients. Consequently, most research of ZGXFD focused on how to apply ZGXFD to treat clinical essential hypertension and how to improve its effect by combined treatment. Studies about how it functioned were substantially less. Our study started with the influence of ZGXFD on gut microbial

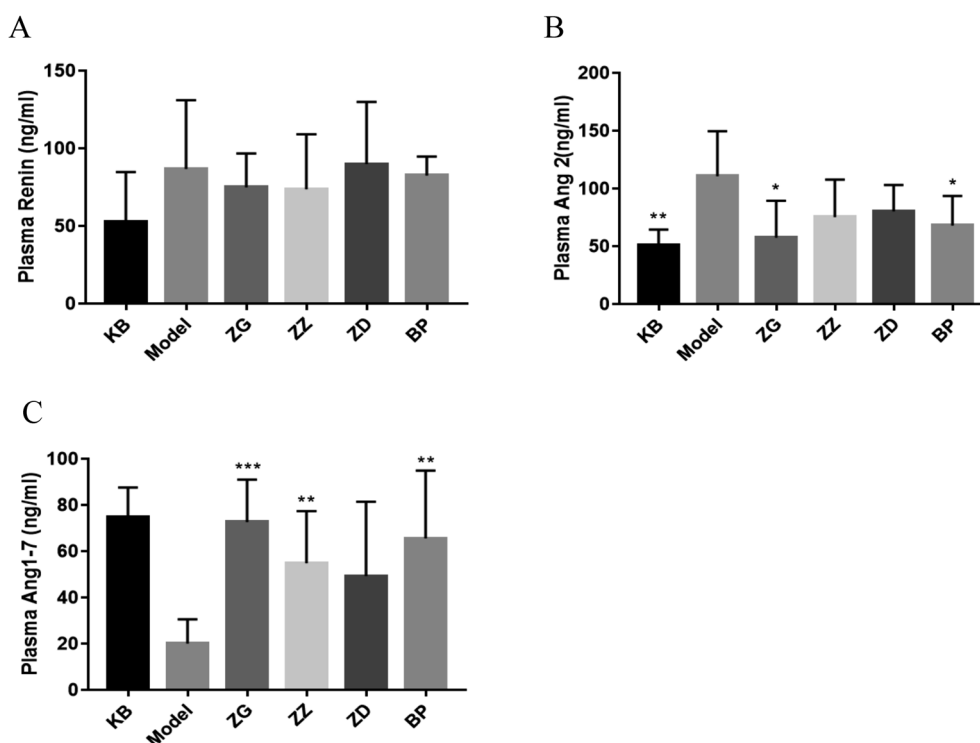


Fig. 5. The Influence of ZGXFD on RAS Pathway

A. Renin concentration in plasma; B. AngII concentration in plasma; C. The concentration of Ang1-7 in plasma. Data are expressed as the mean \pm S.D. ($n=8$), *** $p<0.005$, ** $p<0.01$, * $p<0.05$ vs. Model group. Each group contains 6 rats. KB, Model represent the WKY group and untreated SHR group, respectively. ZG, ZZ, ZD represent high-dose, medium-dose and low-dose ZGXFD treated SHR groups. BP represents the group of rats treated with benazepril. Data are representative of three independent experiments ($n=6$). Student's t -test was used to assess statistical significance.

composition and identified its regulation on blood pressure via the RAS system, providing future clinical application of ZGXFD with theoretical foundation.

Gut microbial homeostatic imbalance is directly related with such chronic diseases like obesity, diabetes, hypertension and cardiac dysfunction.³⁷⁻³⁹) Recent studies showed that marked differences existed between gut microbial composition of SHR and WKY.⁴⁰) Much attention has been drawn to the correlation between gut microbiota and hypertension. We used metrics, including species composition, Chao richness, Shannon diversity and Simpson diversity, to analyze the connection between gut microbial diversity and hypertension and the effect of ZGXFD on enteric bacterial composition. It is found that the gut microbial diversity of untreated SHR group was significantly higher than other groups. The reason for this might be that hypertension influenced the gastrointestinal tract of SHR and damaged the balance between probiotics and other bacteria. Probiotic lost their edge, leading to the mass propagation of neutral and harmful bacteria. To confirm the hypothesis, we utilized the F/B and B/C rates to evaluate the maintenance of gut microbial homeostasis: the increase in F/B and B/C rates is a sign for imbalanced gut microbiota, which is in turn closely related to cardiovascular disease, diabetes and obesity. ZGXFD and benazepril are both conducive to the maintenance of gut microbial homeostasis.

The metabolites of gut microbiota are regarded as essential actors in the metabolic process of the host organism. SCFAs are main products of gut microbes and signaling molecules involved in metabolism, immunity and infection. They include formic acid, acetic acid, propionic acid, butyric acid, and valeric acid and the latter three acids compose 95% of the total

mass. SCFAs play an important role in the pathogenesis of hypertension; these acids influence blood pressure by regulating vasodilatation. They act on the G protein-coupled receptor expressed on the vascular endothelial cells, thus loosening the tension of these cells and reducing blood pressure.

RAS is one of the most important systems regulating blood pressure. Famous anti-hypertensives, such as benazepril, captopril, enalapril and losartan, all reduce blood pressure through RAS system.⁴¹⁻⁴⁴) In the present study, the influence of ZGXFD on gut microbial composition was similar to that of benazepril, thus making us consider that ZGXFD might affect blood pressure by RAS system as well. Our results provide evidence for the hypothesis. However, the link between RAS system and gut microbial diversity remains obscure. In other words, it is unclear whether ZGXFD influences RAS system and thus leads to the change in gut microbiota, or ZGXFD changes gut microbiota first, which then poses an effect on the RAS system. Further studies about the dynamic interaction among the RAS system, gut microbiota and ZGXFD will provide us more information about the use of the time-honored medicine in treating hypertension.

Acknowledgments This work was supported by the National Natural Science Foundation of China (No. 81560716, 81860832).

Conflict of Interest The authors declare no conflict of interest.

Supplementary Materials The online version of this article contains supplementary materials.

REFERENCES

- 1) Tereshchenko LG, Soliman EZ, Davis BR, Oparil S. Risk stratification of sudden cardiac death in hypertension. *J. Electrocardiol.*, **50**, 798–801 (2017).
- 2) Kokubo Y, Matsumoto C. Hypertension is a risk factor for several types of heart disease: review of prospective studies. *Adv. Exp. Med. Biol.*, **956**, 419–426 (2016).
- 3) Cai J, Jang JY, Kim J, Shin K, Kim KS, Park D, Kim TS, Lee SP, Ahn B, Choi EK, Lee J, Kim YB. Comparative effects of plant oils on the cerebral hemorrhage in stroke-prone spontaneously hypertensive rats. *Nutr. Neurosci.*, **19**, 318–326 (2016).
- 4) Mendizábal Y, Llorens S, Nava E. Hypertension in metabolic syndrome: vascular pathophysiology. *Int. J. Hypertens.*, **2013**, 230868 (2013).
- 5) Chen KJ, Hui KK, Lee MS, Xu H. The potential benefit of complementary/alternative medicine in cardiovascular diseases. *Evid. Based Complement. Alternat. Med.*, **2012**, 125029 (2012).
- 6) Xu H, Chen K. Integrative medicine: the experience from China. *J. Altern. Complement. Med.*, **14**, 3–7 (2008).
- 7) Xiong XJ, Chu FY, Li HX, He QY. Clinical application of the TCM classic formulae for treating chronic bronchitis. *J. Tradit. Chin. Med.*, **31**, 69–72 (2011).
- 8) Xiong X, Yang X, Feng B, Liu W, Duan L, Gao A, Li H, Ma J, Du X, Li N, Wang P, Su K, Chu F, Zhang G, Li X, Wang J. Zhen gan xi feng decoction, a traditional chinese herbal formula, for the treatment of essential hypertension: a systematic review of randomized controlled trials. *Evid. Based Complement. Alternat. Med.*, **2013**, 982380 (2013).
- 9) Tomasello G, Bellavia M, Palumbo VD, Gioviale MC, Damiani P, Lo Monte AI. From gut microflora imbalance to mycobacteria infection: is there a relationship with chronic intestinal inflammatory diseases? *Ann. Ital. Chir.*, **82**, 361–368 (2011).
- 10) Human Microbiome Project Consortium. A framework for human microbiome research. *Nature*, **486**, 215–221 (2012).
- 11) Chow J, Lee SM, Shen Y, Khosravi A, Mazmanian SK. Host-bacterial symbiosis in health and disease. *Adv. Immunol.*, **107**, 243–274 (2010).
- 12) McDermott AJ, Huffnagle GB. The microbiome and regulation of mucosal immunity. *Immunology*, **142**, 24–31 (2014).
- 13) Mariat D, Firmesse O, Levenez F, Guimaraes V, Sokol H, Dore J, Corthier G, Furet JP. The Firmicutes/Bacteroidetes ratio of the human microbiota changes with age. *BMC Microbiol.*, **9**, 123 (2009).
- 14) Shen TD. Diet and gut microbiota in health and disease. *Nestle Nutr. Inst. Workshop Ser.*, **88**, 117–126 (2017).
- 15) Li J, Zhao F, Wang Y, Chen J, Tao J, Tian G, Wu S, Liu W, Cui Q, Geng B, Zhang W, Weldon R, Auguste K, Yang L, Liu X, Chen L, Yang X, Zhu B, Cai J. Gut microbiota dysbiosis contributes to the development of hypertension. *Microbiome*, **5**, 14 (2017).
- 16) Jamerson K, Weber MA, Bakris GL, Dahlof B, Pitt B, Shi V, Hester A, Gupte J, Gatlin M, Velazquez EJ. Benazepril plus amlodipine or hydrochlorothiazide for hypertension in high-risk patients. *N. Engl. J. Med.*, **359**, 2417–2428 (2008).
- 17) Gismondi RA, Oigman W, Bedirian R, Pozzobon CR, Ladeira MC, Neves MF. Comparison of benazepril and losartan on endothelial function and vascular stiffness in patients with Type 2 diabetes mellitus and hypertension: A randomized controlled trial. *J. Renin Angiotensin Aldosterone Syst.*, **16**, 967–974 (2015).
- 18) Jiang S, Pan M, Wu S, Venners SA, Zhong G, Hsu YH, Weinstock J, Wang B, Tang G, Liu D, Xu X. Elevation in Total Homocysteine Levels in Chinese Patients With Essential Hypertension Treated With Antihypertensive Benazepril. *Clin. Appl. Thromb. Hemost.*, **22**, 191–198 (2016).
- 19) Richards EM, Pepine CJ, Raizada MK, Kim S. The Gut, Its Microbiome, and Hypertension. *Curr. Hypertens. Rep.*, **19**, 36 (2017).
- 20) Kang Y, Cai Y. Gut microbiota and hypertension: From pathogenesis to new therapeutic strategies. *Clinics and Research in Hepatology and Gastroenterology*, **42**, 110–117 (2018).
- 21) Turnbaugh PJ, Ley RE, Hamady M, Fraser-Liggett CM, Knight R, Gordon JI. The human microbiome project. *Nature*, **449**, 804–810 (2007).
- 22) Watanabe IS, Ogawa K, Cury DP, Dias FJ, Sosthenes MC, Issa JP, Iyomasa MM. Fine structure of bacterial adhesion to the epithelial cell membranes of the filiform papillae of tongue and palatine mucosa of rodents: a morphometric, TEM, and HRSEM study. *Microsc. Res. Tech.*, **76**, 1226–1233 (2013).
- 23) Ishaq HM, Mohammad IS, Guo H, Shahzad M, Hou YJ, Ma C, Naseem Z, Wu X, Shi P, Xu J. Molecular estimation of alteration in intestinal microbial composition in Hashimoto's thyroiditis patients. *Biomedicine & Pharmacotherapy*, **95**, 865–874 (2017).
- 24) Wan Q, Li J, Ye Q, Zhou J. [Manifestation, distribution of pathogen, and resistance of bloodstream infections after renal transplantation: clinical analysis of 71 patients]. *J. Cent. South Univ. (Med. Sci.)*, **38**, 938–943 (2013).
- 25) Uribarri J, Oh MS, Carroll HJ. D-lactic acidosis. A review of clinical presentation, biochemical features, and pathophysiologic mechanisms. *Medicine*, **77**, 73–82 (1998).
- 26) Yilmaz B, Schibli S, Macpherson AJ, Sokollik C. D-Lactic acidosis: successful suppression of D-lactate-producing *Lactobacillus* by probiotics. *Pediatrics*, **142**, e20180337 (2018).
- 27) Luk GD, Bayless TM, Baylin SB. Diamine oxidase (histaminase). A circulating marker for rat intestinal mucosal maturation and integrity. *J. Clin. Invest.*, **66**, 66–70 (1980).
- 28) Zhao L, Luo L, Jia W, Xiao J, Huang G, Tian G, Li J, Xiao Y. Serum diamine oxidase as a hemorrhagic shock biomarker in a rabbit model. *PLOS ONE*, **9**, e102285 (2014).
- 29) MacNab M, Mallows S. Safety profile of benazepril in essential hypertension. *Clin. Cardiol.*, **14** (Suppl. 4), IV33–IV37, discussion, IV51–IV55 (1991).
- 30) Patel S, Rauf A, Khan H, Abu-Izneid T. Renin-angiotensin-aldosterone (RAAS): The ubiquitous system for homeostasis and pathologies. *Biomedicine & Pharmacotherapy*, **94**, 317–325 (2017).
- 31) Zhang F, Liu H, Liu D, Liu Y, Li H, Tan X, Liu F, Peng Y, Zhang H. Effects of RAAS Inhibitors in Patients with Kidney Disease. *Curr. Hypertens. Rep.*, **19**, 72 (2017).
- 32) Ghazi L, Drawz P. Advances in understanding the renin-angiotensin-aldosterone system (RAAS) in blood pressure control and recent pivotal trials of RAAS blockade in heart failure and diabetic nephropathy. *F1000 Res.*, **6**, 297 (2017).
- 33) Feldman DL, Jin L, Xuan H, Contrebas A, Zhou Y, Webb RL, Mueller DN, Feldt S, Cumin F, Maniara W, Persohn E, Schuetz H, Jan Danser AH, Nguyen G. Effects of aliskiren on blood pressure, albuminuria, and (pro)renin receptor expression in diabetic TG (mRen-2)27 rats. *Hypertension*, **52**, 130–136 (2008).
- 34) Abuhashish HM, Ahmed MM, Sabry D, Khattab MM, Al-Rejaie SS. ACE-2/Ang1-7/Mas cascade mediates ACE inhibitor, captopril, protective effects in estrogen-deficient osteoporotic rats. *Biomedicine & Pharmacotherapy*, **92**, 58–68 (2017).
- 35) Kuczeriszka M, Kompanowska-Jeziarska E, Sadowski J, Prieto MC, Navar LG. Modulating role of Ang1-7 in control of blood pressure and renal function in AngII-infused hypertensive rats. *Am. J. Hypertens.*, **31**, 504–511 (2018).
- 36) Meng W, Zhao W, Zhao T, Liu C, Chen Y, Liu H, Sun Y. Autocrine and paracrine function of Angiotensin 1-7 in tissue repair during hypertension. *Am. J. Hypertens.*, **27**, 775–782 (2014).
- 37) Patterson E, Ryan PM, Cryan JF, Dinan TG, Ross RP, Fitzgerald GF, Stanton C. Gut microbiota, obesity and diabetes. *Postgrad. Med. J.*, **92**, 286–300 (2016).
- 38) Howitt MR, Garrett WS. A complex microworld in the gut: gut microbiota and cardiovascular disease connectivity. *Nat. Med.*, **18**, 1188–1189 (2012).
- 39) Sanz Y, Moya-Perez A. Microbiota, inflammation and obesity. *Adv.*

- Exp. Med. Biol.*, **817**, 291–317 (2014).
- 40) Yang T, Santisteban MM, Rodriguez V, Li E, Ahmari N, Carvajal JM, Zadeh M, Gong M, Qi Y, Zubcevic J, Sahay B, Pepine CJ, Raizada MK, Mohamadzadeh M. Gut dysbiosis is linked to hypertension. *Hypertension*, **65**, 1331–1340 (2015).
- 41) Simko F, Pechanova O, Repova K, Aziriova S, Krajcirovicova K, Celec P, Tothova L, Vrankova S, Balazova L, Zorad S, Adamcova M. Lactacystin-induced model of hypertension in rats: effects of melatonin and captopril. *Int. J. Mol. Sci.*, **18**, 1612 (2017).
- 42) Zhou Y, Zhao L, Zhang Z, Lu X. Protective effect of enalapril against methionine-enriched diet-induced hypertension: role of endoplasmic reticulum and oxidative stress. *Biomed. Res. Int.*, **2015**, 724876 (2015).
- 43) He DH, Lin JX, Zhang LM, Xu CS, Xie Q. Early treatment with losartan effectively ameliorates hypertension and improves vascular remodeling and function in a prehypertensive rat model. *Life Sci.*, **173**, 20–27 (2017).
- 44) Matsumura K, Arima H, Tominaga M, Ohtsubo T, Sasaguri T, Fujii K, Fukuhara M, Uezono K, Morinaga Y, Ohta Y, Otonari T, Kawasaki J, Kato I, Tsuchihashi T. Effect of losartan on serum uric acid in hypertension treated with a diuretic: the COMFORT study. *Clin. Exp. Hypertens.*, **37**, 192–196 (2015).