Therapeutic Effect of San-Zi-Yang-Qin Decoction on Obese Pre-hypertension: A Non-targeted Combined with Pseudo-targeted Metabolomics

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Abstract

Background
Evaluation of the intervention effect of SZD on high-fat and high-salt induced obese Pre-hypertension rats from the perspective of metabolomics, and to explore the metabolic mechanism of SZD for the treatment of obese Pre-hypertension

Methods
In this study, the efficacy of SZD was evaluated by blood pressure, body weight, Lee's index, and biochemical indexes, and the mechanism of SZD in the treatment of obese prehypertensive rats induced by high-salt the high-fat mode was explored through non-targeted metabolomics combined with pseudo-targeted metabolomics.

Results
SZD intervention reduced systolic blood pressure(SBP), diastolic blood pressure(DBP), mean arterial pressure, and reduced body weight and Lee's index in rats, which had some improvement effect on obesity. It also reduced triglyceride(TG), total cholesterol(TC), Low-density lipoprotein cholesterol(LDL),Hypersensitivity C-reactive protein(Hs-CRP) levels in rats, but had no significant elevation effect on High-density lipoprotein cholesterol(HDL). Analysis of 35 biomarkers in the model and 31 biomarkers in the SZD intervention by non-targeted metabolomics revealed that SZD interfered with 7 of these metabolites (estradiol, sphingosine, TXB2, LysoPC (20:2), LysoPE (22:0), LysoPC (22:5), LysoPC (20:0)). The further content analysis of 7 metabolites by pseudo-targeted metabolomics revealed an increase in estradiol and sphingomyelin, and a decrease in LysoPC (20:2), LysoPE (22:0), LysoPC (22:5), and LysoPC (20:0) after SZD intervention, which involved in glycerophospholipid metabolism, sphingolipid metabolism, linoleic acid metabolism, and arachidic acid metabolism.

Conclusions
SZD was finally found to improve obesity and decreased blood pressure. The preliminary investigation of the efficacy mechanism of SZD intervention in obese Pre-hypertension enriched the pharmacological effects of SZD.

1 Background
Pre-hypertension is a transitional stage from normotension to hypertension. Obesity, a risk factor for Pre-hypertension, not only leads to increased blood pressure but also promotes the development of metabolic syndrome. Metabolic syndrome, also known as insulin resistance syndrome, is a common condition that includes insulin resistance, abdominal obesity, hypertension, and dyslipidemia (significantly elevated total cholesterol(TC), low-density lipoprotein cholesterol(LDL), and reduced high-density lipoprotein cholesterol(HDL) levels)(McCracken, Monaghan et al., 2018). Pre-hypertension and metabolic syndrome are lifestyle diseases exacerbated by obesity, as both disease processes are strongly associated with body weight (Kachur, Morera et al., 2018, Kokubo & Kamide, 2009). An elevated visceral obesity index leads to increased blood pressure (Leite, Cota et al., 2021). Especially, the obese or have metabolic syndrome have higher vasoconstriction, which increases blood pressure via endothelin receptor A (Rocha, Templeton et al., 2014). The prevalence of metabolic syndrome among young adults in the United States is increasing yearly (Hirode & Wong, 2020). Therefore, the treatment of obesity and Pre-hypertension is of great significance.

Obesity is a risk factor for the development of metabolic syndrome in humans (Sumner, Khalil et al., 2012), and the adipose tissue, is central to the development and progression of metabolic syndrome, contributing to the development of hypertension (Katsimardou, Imprialos et al., 2020, Kotsis, Stabouli et al., 2010, Kumari, Kumar et al., 2019). As overnutrition leads to adipocyte hypertrophy or proliferation, hypertrophic adipocytes grow beyond their blood supply, inducing a hypoxic state (Halberg, Wernstedt-Asterholm et al., 2008) could further lead to cellular necrosis, producing pro-inflammatory factors, and increasing blood pressure. Increased serum TG levels exacerbate insulin resistance, which activates the sympathetic nervous system, upregulates angiotensin II receptors, and reduces nitric oxide synthesis, leading to increased heart rate and blood pressure (Mancia, Bousquet et al., 2007, Tziomalos, Athyros et al., 2010). Free fatty acids associated with obesity also contribute to elevated blood pressure (Iannello, Milazzo et al., 2007). Triglycerides(TG) are the most abundant lipids in the body, contributing to the development and progression of hypertension by forming large amounts of free fatty acids, disrupting the integrity of vascular endothelial cells, inactivating nitric oxide oxidation, and causing endothelial dysfunction (Lundman, Eriksson et al., 1997). Studies showed that diglycerides have important functions in lowering blood lipids, reducing visceral fat, and inhibiting weight gain by inhibiting the accumulation of TC in the body (Nagao, Watanabe et al., 2000).

TCM is widely used in China and Asia, and many herbs or herbal extracts are effective in controlling blood pressure and lowering blood lipids (Tian, Zhang et al., 2020, Zhang, Liu et al., 2020). The TCM prescription San-Zi-Yang-Qin Decoction (SZD) originated from “Han Shi Yi
Tongtong comprises three traditional Chinese medicines: *Semen Raphani* (dried ripe seeds of *Raphanus sativus L.*), *Perilla frutescens* (L.) Britt. (dried ripe fruits of the plant *Perilla*), and *Sinapis alba L.* (dried ripe seeds of *Sinapis alba L.*). As the most used drug in prescription, modern pharmacological studies have shown that *Semen Raphani* contains a variety of active compounds with antihypertensive effects, such as alkaloids and sinapine (Gao, Wang et al., 2022). The alkaloids in the aqueous extract of *Semen Raphani* were found to have significant antihypertensive effects, improving vascular endothelial damage in hypertensive rats (Li, Jiang et al., 2015b). In addition, the water-soluble alkaloids showed significant protective effects against oxidative damage by reducing serum malondialdehyde levels and enhancing superoxide dismutase activity in vivo (Sham, Yuen et al., 2013, Tian, Jiang et al., 2018, Xu, Li et al., 2017). As compositional indicators and pharmacologically active components (Li, Yang et al., 2015a), sinapine is present in the form of sinapine thiocyanate (ST) in *Semen Raphani* and *Semen Sinapis* (Guan, Lin et al., 2022). ST has been shown to have clear antihypertensive effects (Guan et al., 2022) and inhibit vascular inflammation by inhibiting the secretion of adhesion factors by the vascular endothelium (Li et al., 2015a). In addition, ST reduced the expression of coagulation-related factors in vascular endothelial cells, thereby inhibiting the thrombotic state caused by endothelial inflammatory injury (Li, Zhang et al., 2017) and improving hypertensive endothelial dysfunction by inhibiting the activation of nucleotide-bound leucine-rich repeat receptor inflammasome (Liu, Yin et al., 2020). *Perilla frutescens* seeds are a good source of fatty acid components such as oleic, linoleic, and linolenic acids (Ahmed, 2018), which are expected to have various health benefits in humans as lowering serum cholesterol and TC levels, and preventing excessive visceral adipose tissue growth (Asif, 2011, Yu, Qiu et al., 2017). In this study, SZD emphasized the dosage of *Semen Raphani*in the proportion of drugs to increase the amount of ST, improving the antihypertensive effect of SZD. TC has long been used in China for the treatment of various diseases with good results (Guo, Luo et al., 2020, Wu, Zhang et al., 2019), whereas the efficacy and therapeutic goals of SZD for obesity Pre-hypertension are unclear due to the lack of modern scientific and technological support.

In recent years, metabolomics has been increasingly applied to reveal the mechanism of efficacy of herbal prescriptions for the treatment of diseases, especially the untargeted and targeted metabolomics approaches based on liquid chromatography-mass spectrometry. Non-targeted metabolomics is the most comprehensive coverage of metabolites possible (Patti, Yanes et al., 2012), providing a larger number of metabolites (Wang, Chen et al., 2017, Yang & Lao, 2019, Yin & Xu, 2014), however, the reproducibility and linear range of metabolites are limited (Zhang, Wu et al., 2020, Zheng, Zhao et al., 2020). Targeted metabolomics is the absolute quantification of specific metabolites (Griffiths, Koal et al., 2010). It can provide a wide linear range, high reproducibility, and sensitivity compared to non-targeting, but low efficiency and narrow coverage of metabolite detection (Wei, Li et al., 2010). Therefore, a pseudo-targeted metabolomics strategy is proposed to combine the advantages of non-targeting and targeting to establish an alternative to non-targeting methods with high sensitivity, high specificity, and excellent quantitative capabilities (Chen, Kong et al., 2013, Li, Ruan et al., 2012). Rich metabolite information from untargeted metabolic profiles based on UPLC-Q-Orbitrap-MS ensures high coverage (Zheng et al., 2020), expanding the linear range of target metabolites for selected metabolites, improving detection accuracy and accurate identification of potential biomarkers (Xu, Li et al., 2019).

This study constructed a high-salt + high-fat rat model of obese Pre-hypertension. Then, the therapeutic effect of SZD on obese Pre-hypertension was evaluated by combining blood pressure, body weight, Lee's index, and biochemical parameters in serum. Finally, a non-targeted metabolomics approach was used to analyze the metabolites associated with SZD intervention in the obese Pre-hypertension rat model, and a pseudo-targeted metabolomics approach was used to analyze the changes in metabolite levels associated with SZD intervention and to characterize the specific metabolic pathways associated with SZD intervention.

**2 Materials and Methods**

**2.1 Reagents and materials**

Salt was purchased from Shandong Daiyue Salt Manufacturing Co., Ltd. (Shanghai, PR. China). High-fat feed was purchased from Research Diets, Inc. (New Brunswick, USA). TC, TG, HDL, LDL, and Hs-CRP were purchased from Medical Electronics Co., Ltd. (Guilin, PR. China). HPLC-grade methanol and acetonitrile were purchased from (Merck, China), and HPLC-grade formic acid was purchased from (Thermos Fisher, USA). Ultrapure water was purchased from China Watsons Co., Ltd. (Guangdong, PR. China).

**2.2 Plant materials and formula compositions**

*Semen Raphani* is the dried and mature seeds of the cruciferous radish *Raphanus sativus L.* (batch number: 210502) purchased from Shandong Baiweitang TCMCo., Ltd. *Perilla frutescens* seeds are the dry ripe fruit of *Perilla frutescens* (L.) Britt. (Lot No.: 2105300552), *Semen Sinapis* is the dry ripe seed of the cruciferous plant *Sinapis alba L.* (Lot No.: 2106200122) in Huqiao Pharmaceutical Co., Ltd., Haozhou City, Anhui Province. It was also identified by Professor Liu Hongyan of Chinese Medicine Appraisal of the Shanzhong University of TCM. SZD is made of *Semen Raphani*, *Perilla frutescens* seeds, and *Semen Sinapis* in a weight ratio of 5:2:2 (Jingtong, 2018, Jiuyuan, 2019).

**2.3 Drug preparation**

Put the three samples into a grinder and grind them, add water to soak for 1 h, heat for 40 min, filter to obtain a decoction, add water and heat for 40 min after filtration, filter again to obtain a decoction, mix the decoction twice and use the rotary Evaporator concentrated at 55°C, freeze-dried,
placed at -20°C, and dissolved in water before use.

### 2.4 Animal preparation

This study was conducted by the Regulations on the Administration of Laboratory Animals promulgated by the National Science and Technology Commission of the People's Republic of China. All procedures and care of rats were following institutional guidelines for the use of animals in research. Male WKY rats, 100 to 120 g, were purchased from Beijing Charles River Experimental Animal Technology Co., Ltd. (Certificate of conformity: SCXX (Beijing) 2016 - 0006), all rats were fed with standard chow and kept in SPF facilities at a temperature of 23 ± 1°C, relative humidity environment of 55% ± 5%, standard 12h/12h (light/dark) cycle, adaptive feeding for 7 days, and humane care according to the 3R principles used in laboratory animals. The experimental protocol and process were approved by the Animal Ethics Committee of the Laboratory Animal Center of Shandong University of Traditional Chinese Medicine (Approval no. SDUTCM20201019003).

Rats were tested according to their respective body surface area, and the animals were randomly divided into three groups: normal control group (Control, n = 10), model group (Model, n = 10) and SZD treat group (SZD-treat, n = 30). The normal control group was given 0.9% normal saline by gavage at a dose of 1mL/100g, once a day, and fed normal chow; the model group and the SZD-treat group were given at a dose of 1mL/100g, rats were given 6% saline by gavage, once a day, and fed a high-fat diet at the same time. At 14 weeks, the SZD-treat group was divided into high-dose group (1.125g/kg, n = 10), medium-dose group (0.625g/kg, n = 10) and low-dose group (0.3125g/kg, n = 10) according to the dose administered, and the normal and model groups were given the same volume of saline once a day until 24 weeks, SZD intervention rats were continued to be fed high fat diet at the same time.

### 2.5 Blood pressure, body weight, and Lee's index in rats

The indexes associated with Pre-hypertension were blood pressure and Hs-CRP and the indicators related to obesity, weight, Lee's index, and blood lipid content. In the process of model establishment, the indicators of the model group were significantly different from the normal group for model success. The blood pressure of the rat in a quiet state was detected by the non-invasive tail artery method, the power was turned on, a quiet, unmanned, and warm environment was selected, the temperature was adjusted to 38°C and the sensitivity was set to 2, the preheating was performed for 5 min, and the cage was controlled during the measurement, keeping the temperature unchanged, the front half of the rat was netted with a rat net and placed in a thermal insulation cylinder, wrapped with a ratbag, the tail was exposed outside the ratbag, and the tail was passed through the pressure induction device, and the tip of the pressure sensor logo consistent with the direction of the tip of the tail of the rat, place the pressure sensor at the base of the rat's tail. After the rat is stable for 5~10 min, click the blood pressure measurement. Record the data when the image fluctuation is stable, and measure the blood pressure three times at the same time every week to take the average value.

Body weight was measured weekly. The calculation formula of Lee's index = body weight (g)^(1/3) × 10/body length × 10^3 (cm), where body length is defined as the distance from the tip of the rat's nose to the anus.

### 2.6 Sample collection and preparation

The rats in the three groups were fasted and watered before dissection, and 1.5% sodium pentobarbital was injected intra-peritoneally at a dose of 30 mg/kg. After anesthesia, blood samples were collected, placed at room temperature for 30 minutes, and centrifuged (4500 rpm, 4°C) for 15 minutes to obtain serum samples. Store at -80°C until further processing.

Non-targeted metabolomics sample preparation.

Rat serum samples were thawed at 4°C and mixed by vortexing. Take 100 µL of serum into a 1.5 mL EP tube, add 400 µL of pre-cooled acetonitrile, 20 µL of methanol-dissolved 0.06 mg/mL L-2-chloro-phenylalanine internal standard solution, vortex for 1 min, 4°C environments. Under static for 15 min, the serum protein was fully removed. Then centrifuge at 15000 r·min⁻¹ for 15 min. Put 400 µL of the supernatant in a 1.5 mL EP tube, dry it with nitrogen, and add 100 µL of the initial mobile phase (water:acetonitrile (50:50, v/v)) to redissolve, vortex for 1 min, 15000 rpm was centrifuged for 10 min, and the supernatant was taken for LC-MS analysis. 5 µL of each serum sample was taken, vortexed and mixed to prepare a quality control (QC) sample. Before injection, 6 QC samples were run to stabilize the instrument, and after every 6 samples were injected, QC was run once samples to monitor instrument stability.

Pseudo-targeted metabolomics sample preparation.

Sample preparation for pseudo-targeted metabolomics. Rat serum samples were thawed at 4°C and mixed by vortexing. Take a certain amount of serum into a 1.5 mL EP tube, add overnight pre-cooled acetonitrile in a ratio of 1:4 (serum sample: acetonitrile), and add 0.06 mg/mL of methanol dissolved in methanol in a ratio of 5:1 (serum sample: internal standard solution). The internal standard solution of L-2-chloro-phenylalanine was vortexed for 1 min and left at 4°C for 15 min to fully remove serum proteins. Then centrifuge at 15000 rpm for 15 min. Place the supernatant in a 1.5 mL EP tube, dry it with nitrogen, add the initial mobile phase (water:acetonitrile (50:50, v/v)) to redissolve, and finally dilute the sample to different concentrations: 0.001, 0.004, 0.008, 0.016, 0.032, 0.064, 0.128, 0.25, 0.5, 1, 1.5, 2, 2.5, 4, 6, 8, 10, 16, 20, vortex for 1 min, centrifuge at 15000 rpm for 10 min, take The supernatant was used for LC-MS pseudo-targeted metabolomic analysis.
2.7 Chromatographic mass spectrometry conditions

Ultra-high-performance liquid chromatography analysis was performed on an Ultimate 3000 UPLC system (Thermo Fisher, CA, USA). Chromatographic conditions: C18 column (2.1mm×100mm, 2.7 µm, AMT, USA) was selected for chromatographic separation, and the mobile phases of gradient elution were 0.05% formic acid aqueous solution (A) and 0.05% formic acid acetonitrile solution (B), linear-gradient program as follows: 0 min, 2% B; 3 min, 40% B; 9 min, 98% B; 18 min, 98% B; 18.1 min, 2% B; and 3.0 min equilibration. The flow rate was 0.3 mL·min⁻¹.

The mass spectrometer was a quadrupole-electrostatic field orbitrap ultra-high-resolution mass spectrometer (UPLC-Q-Orbitrap-MS) from Thermo Scientific. Detection in positive and negative ion mode. Detection conditions were: ion source HESI; capillary voltage 3500 V (positive ion mode), capillary voltage 3000 V (negative ion mode); capillary temperature: 300°C; source temperature: 350°C; sheath gas: 45 arb; auxiliary gas 10 arb; mass spectrometry Acquisition range: 80-1200 m/z; resolution 70000; S-Lens RF Level 55.

2.8 Data processing

After metabolomic analysis of serum samples, the original metabolomic spectrum information (ie.,the metabolomics raw map information was converted in “. Raw” format into”.mzXML” format metabolomics data to complete the data preprocessing process of peak identification, peak filtering, peak correction, peak alignment, peak screening and retention time correction, RT), a metabolomic dimension data array including peak intensities. The above data matrix was analyzed by PCA and PLS-DA using SIMCA-P software (version 13.0; Umetrics, Umea, Sweden). According to the metabolic profiles of the two groups of animals of different ages, 100 random permutation experiments were performed, and R2 and Q2 tests were performed. The accuracy of the mathematical model.

2.9 Biomarker identification

The metabolomics dimensional data array obtained after data preprocessing was imported into SIMCA-P for pattern recognition. In the case of good model accuracy, the VIP > The variables of 1 were further analyzed by MPP (Mass Profiler Professional, Agilent, USA) software, and compared between groups. Screening P< 0.05, Fold Change(FC) ≥ 1.5 was considered statistically significant. Endogenous metabolites were identified and confirmed by MS/MS, HMDB database (http://www.hmdb.ca/), and KEGG (https://www.kegg.jp/). Metaboanalyst 5.0 software (http://www.metaboanalyst.ca) was used for metabolite heat map analysis and pathway enrichment analysis.

The m/z values of the variables obtained above were imported into HMDB (HTTP://www.hmdb.ca) and KEGG (HTTP://www.genome.jp/kegg/) databases and the maximum database search error was set to 30 ppm. Inc.) software to validate the identification results, construct mass spectra by deconvolution, and also combine the information of fragmented fragments of secondary mass spectra for the identification of biomarkers as potential biomarkers. Lysophosphatidylcholine (20:0) (Lysophosphatidylcholine (20:0), LysoPC (20:0)) was used as an example to characterize the marker identification process. The exact molecular mass of the molecular ion m/z 552.4030 was imported into the HMDB database, and the search deviation was set at 30 ppm, and three molecular weight-matched metabolites, (2-Acetyloxy-3-octadecanoyloxy)ethyl phosphate, LysoPC (20:0/0:0), Aliskiren, and the spectra module revealed that LysoPC (20:0), the secondary mass spectrum of LysoPC(20:0) was found to overlap with the second mass spectrum of this experiment, which was further verified by Mass Frontier and found to be the m/z value corresponding to the [M + H]⁺ peak of LysoPC(20:0).

2.10 Standard curve establishment

The samples were diluted proportionally, and the concentration of the mixed reference substance was successively injected from low to high, 10 µL each time, and a linear relationship was established between the reference substance dilution ratio (X) and the peak area (Y).

2.11 Precision and Accuracy

The precision of the method was determined by analyzing all samples (n = 6 at each dilution ratio). Precision is measured in parallel on the same sample under the same conditions, and the degree of closeness between the measured values is evaluated. Between-day precision was calculated similarly for each concentration point of the 18 replicate three-day validations.

The stability of 7 metabolites in rat serum was examined under different conditions by repeating 6 times at 3 different dilution ratios. Short-term stability was assessed by placing the samples at room temperature (25°C) for 12 h. Freeze-thaw stability of the samples was assessed by repeating the freeze-thaw cycle 3 times at -20°C. The sample stability was calculated as (area ratio of tested samples/area ratio of fresh samples) × 100%.

2.12 Statistical Analysis

Data were analyzed with SPSS 25.0 statistical software. Data are presented as mean ± SD. Two groups of data were compared using an independent samples t-test, significant differences in the above tests are shown in the figure by *P< 0.05, **P< 0.01 (compared to the normal group), *P< 0.05, **P< 0.01 (compared to the model group).
3 Results

3.1 Effects of SZD on blood pressure, body weight, and Lee's index in rats

SZD intervention was given to the SZD-treat, and 0.9% saline was given to the normal group and the model group at the same time (Fig. 1). At 14 weeks, compared with the normal group, the systolic blood pressure (SBP), diastolic blood pressure (DBP) and mean arterial pressure (MAP) of the model group were significantly higher than those of the normal group. After the intervention, the blood pressure of the SZD-treat group was significantly decreased (Fig. 2A1, A2, A3), compared with the model group; the indicators reflect the degree of obesity (visceral obesity) in rats. As shown in Fig. 2B and C, the body weight and Lee's index of the rats in the model group were significantly higher than those in the normal group (P < 0.05). Then, body weight and Lee's index decreased significantly after the intervention of SZD. The above results showed that the obese prehypertensive rat model was successfully established at 8 weeks, and SZD had the exact effect of lowering blood pressure and improving obesity.

3.2 Effects of SZD on biochemical indexes in rat serum

At 14 weeks, the contents of TC, TG, and LDL in the model group and the SZD-treat group were significantly higher than those in the normal group. There was no significant difference in the HDL levels among the groups, and the hs-CRP content was significantly higher than that in the normal group (P < 0.01), combined with the blood pressure and Lee's results in this section, proving that the obese prehypertensive rat model was successful at 8 weeks of age. After 10 weeks of intervention with different doses of SZD, it was found that TC, TG, LDL, and hs-CRP were significantly lower in the high-dose SZD intervention group, which was significantly different from the model group and close to the normal level, so the high-dose group had better antihypertensive and lipid-lowering effects than the other two groups. The effect was poor in lowering HDL, as shown in Fig. 3.

3.3 Non-targeted metabolomic analysis

3.3.1 Metabolic profiling and multivariate statistical analysis

Comprehensive phenotypic and serum biochemical results analysis revealed that the effect of high-dose treatment in the SZD-treat group was better than that in the medium and low-dose groups, so the high-dose SZD intervention group was selected for the metabolomic analysis. To systematically screen the potential intervention targets of SZD on the obese prehypertensive rat model, UPLC-Q-Orbitrap-MS was used to analyze the normal group, model group, and SZD-treat group in positive and negative ion mode. In the total ion current map of serum samples in the decoction intervention group (Fig. 4A), it was found that there were significant differences in the total ion current map of different groups, indicating that there were significant differences in metabolic profiles between different groups. In the unsupervised PCA mode, the QC samples were found to be clustered, which suggested good instrument stability and reliable data quality (Supplementary Figure). To evaluate the metabolic differences between the normal group, the model group, and the SZD-treat group, supervised PLS-DA analysis was applied (Fig. 4B1, B2), positive ions: R2 = 0.984, Q2 = 0.916; negative ion: R2 = 0.982, Q2 = 0.931. In addition, 200 permutation tests were performed to verify the PLS-DA model and avoid overfitting (Fig. 3C1, C2), the intercept of positive ion R2 = 0.737 Q2 = -0.28 and negative ion R2 = 0.72 Q2 = -0.358 were generated in the model, indicating that the established PLS-DA model has adaptability and predictability. Multivariate analysis showed that the normal group, model group, and the SZD-treat soup aggregation within the group is better, and could clear separation between different groups, the normal group and model group significantly separated model, indicating that the model is successful, SZD-treat group gradually approached the normal group, suggested that the SZD-treat group regulate metabolism network model rats after the intervention.

Metabolites were carefully screened before being identified as potential biomarkers. The scatter plot with VIP > 1 was obtained in the PLS-DA model (Figure. 4D1, D2). The larger the VIP value, the greater the separation contribution in this mode, and the more important metabolites that could reflect the SZD intervention model. Compared with the normal group and the model group or the SZD-treat group compared with the model group, the serum endogenous metabolites with VIP value greater than 1.0, P value less than 0.05, and FC >= 1.5 were screened out. KEGG database for chemical information of potential biomarkers.

3.3.2 Identification of potential endogenous biomarkers and analysis of metabolic pathways

Based on information from MS/MS results and database matching results, we have identified endogenous biomarkers under the conditions of VIP > 1, P < 0.05, and FC >= 1.5. Compared with the normal group, 35 differential biomarkers were identified in the model group (Supplementary Table), and 31 differential biomarkers were identified in the SZD-treat group compared to the model group, (Supplementary Table). Metabolic pathways were further analyzed by MetPA, and it was found that there were 7 metabolic pathways related to the metabolism of the obese prehypertensive rat model (Fig. 5A), among which the pathways with influence values over 0.01 were glycerophospholipid metabolism, steroid hormone biosynthesis, phenylalanine metabolism, glycerol metabolism, ether lipid metabolism, and sphingolipid metabolism. After the
intervention model of SZD, 8 metabolic pathways were found (Fig. 5B), and the pathways with influence values greater than 0.01 were sphingolipid metabolism, linoleic acid metabolism, unsaturated fatty acid biosynthesis, glycerol phospholipid metabolism, arachidonic acid metabolism, tryptophan metabolism, primary bile acid biosynthesis, and steroid biosynthesis. The pathways of the model and SZD group have certain changes, the reason why the changes in metabolic pathways are that SZD causes part of metabolic (especially the sphingolipid metabolism, linoleic acid metabolism, and arachidonic acid metabolism) transformation in obese prehypertensive rats, which promotes the recovery of abnormal metabolic state. Compared to the two groups of differential metabolites, the SZD could adjust 7 generation biomarkers, including Estradiol, Sphingosine, TXB2, LysoPC(20:2), LysoPE(22:0), LysoPC(22:5), and LysoPC(20:0), as shown in Fig. 6 and the fragment ion information of the seven metabolites is shown in Table 1

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<th>No.</th>
<th>tR/min</th>
<th>[M + H]+</th>
<th>Error (ppm)</th>
<th>Formula</th>
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<td>C18H24O2</td>
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</tr>
<tr>
<td>2</td>
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<td>264.2675</td>
<td>10</td>
<td>C18H27NO2</td>
<td>264.268, 265.272, 121.101, 97.101, 91.054, 107.085, 109.101</td>
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<tr>
<td>3</td>
<td>8.15</td>
<td>331.2399</td>
<td>8</td>
<td>C20H34O6</td>
<td>67.054, 79.054, 83.049, 95.085, 97.065, 107.086, 119.085, 121.101, 137.096</td>
<td>TXB2</td>
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<tr>
<td>4</td>
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<td>552.4030</td>
<td>4</td>
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</table>

3.4 Analysis of Pseudo-Targeted Metabolomics Results

3.4.1 Standard Curve, Precision, and Accuracy

Pseudo-targeted metabolomics combines the advantages of non-targeted and targeted analysis. In this study, non-targeted metabolomics was used to screen out specific biomarkers of SZD in obese prehypertensive rats. Therefore, we adopted a strategy to mix three sets of different samples, dilute or concentrate them according to different concentration gradients, so that the metabolites with different abundances were included, and inject the samples in order from low to high concentration. The metabolite linear relationship was established by the sample dilution ratio (X) and peak area (Y). As shown in Fig. 7 and Table 2, the results showed that the correlation coefficient was > 0.99, indicating that the method is highly linear.

The precision was measured under this method, and the stability results were shown in Table 3. The intra- and inter-day precisions ranged from 0.87–11.01%, which indicated that the method has good precision. The short-term stability of samples with different dilution ratios is 1.32–8.17%, and the freeze-thaw stability of samples with dilution ratios is 1.24–8.65%. The results showed that the freeze-thaw cycles were repeated 3 times within 24 h at room temperature or -20 °C back. All validation experiments demonstrated that the proposed method for targeted metabolomics by utilizing UPLC-Q-Orbitrap-MS was accurate and reliable for the simultaneous analysis of 7 metabolites in rat serum samples.

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<th>Compounds</th>
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<th>R²</th>
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<td>y = 8E + 06x + 7E + 07</td>
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<td>Sphingosine</td>
<td>y = 6E + 06x - 537048</td>
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</tr>
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<td>TXB2</td>
<td>y = 2E + 08x + 5E + 08</td>
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</tr>
<tr>
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<td>y = 5E + 07x + 9E + 06</td>
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</tr>
<tr>
<td>LysoPE(22:0)</td>
<td>y = 4E + 07x - 1E + 07</td>
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</tr>
<tr>
<td>LysoPC(22:5)</td>
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<td>LysoPC(20:0)</td>
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### Table 3
Precision and stability of seven metabolites

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<th>Compounds</th>
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<th>Precision intra-day (%)</th>
<th>Precision inter-day (%)</th>
<th>Precision short-term (%)</th>
<th>Precision freeze-thaw (%)</th>
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#### 3.4.2 Effects of SZD on the contents of biomarkers

The peak areas of endogenous metabolites in different groups were brought into their respective standard curves, and the relative contents of the 7 metabolites in serum were obtained as shown in Fig. 8. Compared with the normal group, the serum contents of TXB2, LysoPC, and LysoPE in the model group were significantly higher than those in the normal group, while the contents of estradiol and sphingosine were lower in the model group. After intervention by SZD, the contents of TXB2, LysoPC, and LysoPE in serum decreased, among which LysoPC (20:0) decreased significantly, which was lower than that of the normal group after the intervention, while the content of estradiol and sphingosine increased after the intervention.

#### 3.5 Metabolic pathway analysis of SZD intervention

Figure 9 Metabolic network analysis of SZD in the intervention of obese Pre-hypertension. The arrow indicates up-regulated or down-regulated. Yellow: glyceride metabolism; pink: glycerophospholipid metabolism; blue: arachidonic acid metabolism; grey: sphingolipid metabolism; red circle: linoleic acid metabolism.

#### 4 Discussion

Obesity leads to the increasing prevalence of pre-hypertension and metabolic syndrome (Leong, Jayasinghe et al., 2020), which seriously endangers people's cardiovascular health, and its prevention and treatment are diverse and complex. SZD is a classical prescription in TCM, in which the active ingredients of ST have good antihypertensive effects, and is often used to treat hypertension. However, the overall mechanism of action and efficacy benefits of SZD in obese pre-hypertension have not been thoroughly investigated. In the present study, non-targeted metabolomics was used to analyze differential biomarkers in serum between normal and model groups and before and after SZD intervention, and a total of 35 metabolites were identified as biomarkers in obese prehypertensive rats and 31 biomarkers related to the intervention.
of SZD, of which 7 were affected by the intervention of SZD. 7 signature metabolites of SZD were validated by pseudo-targeted metabolomics for the treatment of obese Pre-hypertension, namely estradiol, sphingosine, TXB2, LysoPC (20:2), LysoPE (22:0), LysoPC (22:5), and LysoPC (20:0). From the above results, it was found that the modulatory effect of SZD resulted in blood pressure, body weight, and obesity index of rats close to the normal group, and improved serum levels of TG, TC, HDL, LDL, and Hs-CRP as well as increased serum levels of estradiol and sphingosine, and increased levels of LysoPC (20:2), LysoPE (22:0), LysoPC (22:5) and LysoPC (20:0) decreased, involving pathways such as phospholipid, arachidonic acid, linoleic acid, and hormone metabolism. In conclusion, the above results suggested that SZD could improve obesity and reduce blood pressure mainly by regulating lipid metabolism.

The metabolic syndrome caused by obesity is partly due to an excess of free fatty acids, caused by improper lipolysis, which is mainly derived from triglycerides stored in adipose tissue. During lipolysis, the free fatty acids associated with obesity are released through the action of cyclic adenosine acids, and the increase in free fatty acids contributes to an increase in smooth muscle tone, peripheral resistance, and blood pressure (Kachur et al., 2018). TGs produced from diglycerides under the action of diglyceride acyltransferase, and diglycerides are produced through monoaoylglycerols under the action of acyl-coenzyme A. In addition, TC and TG are the main carriers of lipid production and transport in the body, which are ultimately expressed as lipoproteins, such as LDL, HDL, etc. (Fan, Xu et al., 2021), where LDL contributes to the occurrence of cardiovascular disease, and HDL is cholesterol that improves atherosclerosis (Luo, Yang et al., 2020). The content of TC, TG, and LDL in serum decreased after SZD intervention, and the body weight and Lee index of rats also decreased significantly, indicating that SZD reduced the content of free fatty acids in serum by regulating the lipid transport pathway to improve the degree of obesity (abdominal obesity) in rats. In this study, the disturbances in phospholipid metabolism observed by SZD intervention such as glycerophospholipid metabolism and sphingolipid metabolism.

Sphingolipids are important components of cell membranes, and sphingolipid metabolism plays an important role in cardiovascular disease (Borodzicz, Czarzasta et al., 2015, Seah, Chew et al., 2020). In sphingolipid metabolism sphingosine (SP), a metabolite of sphingolipids is phosphorylated by sphingosine kinase to form sphingosine 1-phosphate (S1P), which is an important vaso-protective regulator of angiogenesis, changes in permeability and changes in tone. (Kurek, Piotrowska et al., 2013). It was found that S1P can regulate endothelial function by activating nitric oxide synthase to induce nitric oxide concentration, leading to vasodilatation (Catalupo, Gargiulo et al., 2017, Catalupo, Zhang et al., 2015). S1P has multiple receptors, among which S1P1R1 is mainly distributed in vascular endothelial cells and plays a role in vascular regulation through receptors 1–3 (S1P1R1-3) (Lee, Thangada et al., 1999). In this study, the relevant sphingolipid metabolites, including S1P and SP, were screened by non-targeted metabolomics, and the changes in sphingolipid content were analyzed by pseudo-targeted metabolomics. The content of sphingosine in the model group was significantly lower than that in the normal group, while the content of SP increased significantly after SZD intervention, which was significantly different from that of the model group. This indicates that sphingosine content can be used as a specific index in obese hypertensive rats and as an index for the evaluation of the efficacy of SZD. SZD can regulate sphingolipid metabolism by increasing the content of SP, protecting the vascular endothelium, and regulating blood pressure.

As a major component of glycerophospholipids in biological cell membranes, glycerophospholipid metabolism is closely related to fat production and elevates blood pressure and its metabolite phosphatidylycholine (PC) acts not only as an intermediate in phospholipid metabolism but also as a cellular messenger, promoting cell proliferation, growth, differentiation, and other important biological behaviors. PC acts as a precursor compound for linoleic acid metabolism and arachidonic acid and is hydrolyzed by phospholipase A2 to lysophosphatidylycholine (LysoPC) (Brown, Chambers et al., 2003). Studies have shown that LysoPC is the main active component of oxidized LDL, which promotes vascular inflammation and atherosclerosis, and thus LysoPC is considered a pro-inflammatory lipid metabolite capable of altering the function of many cell types, such as endothelial cells, where it is biologically active (Matsumoto, Kobayashi et al., 2007), is closely related to the occurrence and development of cardiovascular disease. In this study, we screened 15 metabolites of SZD related to glycerophospholipid metabolism. The pseudo-targeted metabolomics study revealed that SZD could regulate the serum levels of LysoPC (20:2), LysoPE (22:0), and LysoPC (22:5) in the model group of rats, with the levels of LysoPC (20:0), were significantly higher than those in the normal group, while the levels of metabolites in serum decreased significantly after SZD intervention, with the most pronounced decrease in LysoPC (20:2). Therefore, SZD exerts its therapeutic effect mainly through the regulation of glycerophospholipid metabolites, and LysoPC and LysoPE are the main regulatory targets of SZD.

In this study, both the metabolism of arachidonic acid and linoleic acid were disturbed. PC produces linoleic acid, which further facilitates the metabolism of arachidonic acid. When cell membrane phospholipase A2 is activated, it catalyzes the hydrolysis of phospholipids to release arachidonic acid, generating prostaglandins (PGs) and thromboxanes (TXs) in the presence of cyclooxygenase, which plays an important role in blood pressure regulation and promoting vasoconstriction to raise blood pressure (Sun, Shelat et al., 2010). Arachidonic acid is first generated by cyclooxygenase, and the unstable PG2 is catalyzed by peroxidase to PGH2, which is not only an intermediate in the synthesis of prostaglandins but also a precursor of thromboxane. TXA2 is further generated by thromboxane synthease, and TXA2 is rapidly converted to TXB2. In the present study, prostaglandin H2 and TXB2 were screened as endogenous biomarkers of SZD intervention. The levels of TXB2 were significantly higher in the model group than in the normal group, and the serum levels of TXB2 decreased significantly after SZD intervention, reminding us that TXB2 is a potential therapeutic target and that lowering TXB2 levels has an important role in lowering blood pressure. Linoleic acid produces PC and also promotes the metabolism of arachidonic acid. Studies have shown that linoleic acid can act as a substrate for vasoactive prostaglandins to lower blood pressure (Iacono & Dougherty, 1993) and promote the relaxation of vascular smooth muscle cells (Pompuestosio, Alva et al., 1998, Tsukamoto & Sugawara, 2018), thereby lowering blood pressure. It was found that linoleic acid was associated with the development of obesity while lowering
blood pressure, leading to increased adipogenesis through the activation of different types of PPAR (Ailhaud, Massiera et al., 2006; Massiera, Saint-Marc et al., 2003). Linoleic acid metabolism is a metabolic pathway with a greater impact related to SZD intervention and linoleic acid is a biomarker of SZD. It showed that SZD lowers blood pressure to improve obesity through the regulation of the linoleic acid metabolic pathway in the treatment of obese prehypertensive rats.

Estrogens are an important class of fat-soluble steroid hormones. All steroid hormones in the human body are produced through the conversion of cholesterol. Estrogens are mainly composed of estradiol, estrone, and estriol, of which estradiol has the strongest physiological effect (Murphy & Steenbergen, 2014). A study by Taguchi et al. found that estradiol reduces cardiovascular injury caused by hypertension, and the mechanism may be that estradiol promotes phosphorylation of p38 mitogen-activated protein kinase and endothelial nitric oxide synthase, which promotes vasodilation and lowers blood pressure (Taguchi, Morishige et al., 2012). Shoemaker et al. revealed that estradiol promotes the production of angiotensin-converting enzyme 2 (ACE2), which inhibits the production of angiotensin II, resulting in an antihypertensive effect (Wang, Shoemaker et al., 2015). In the present study, we suggested that estradiol levels in the model group were significantly lower than those in the normal group and were significantly higher after SZD intervention. According to our study, estradiol metabolism is an important factor in the mechanism of SZD intervention in obese Pre-hypertension. Based on the present findings, we have identified potential targets of biological importance. Most importantly, SZD was found to reverse abnormal levels of metabolites related to sphospholipid, arachidonic acid, linoleic acid, and hormone metabolism.

Conclusion

This study revealed that the metabolic phenotype of SZD-interventional obese prehypertensive rats by establishing a non-targeted and pseudo-targeted metabolomics analysis method, and the effect of SZD on improving obesity and lowering blood pressure was evaluated by combining blood pressure, body weight, Lee's index, blood lipids, and Hs-CRP total of 31 intervention-related biomarkers were screened by multivariate statistical analysis, and SZD was verified to modulate the content changes of 7 of these metabolites by pseudo-targeted metabolomics, which can be used not only as biomarkers for obese Pre-hypertension but also as endogenous efficacy evaluation indexes of the model for assessing the effect of SZD on obese Pre-hypertension. The intervention pathways include linoleic acid metabolism, glycerophospholipid metabolism, sphingolipid metabolism, arachidonic acid metabolism, and estrogen metabolism. This study is the first to analyze the efficacy of SZD in the treatment of obese Pre-hypertension rats using a non-targeted combined pseudo-targeted metabolomics approach, which increases our understanding of the pathogenesis of obese Pre-hypertension, and enriches the pharmacological effects of SZD. The non-targeted combined pseudo-targeted metabolomics approach is promising to be a useful tool for exploring the mechanism of action of TCM in treating diseases, as well as providing a systematic and comprehensive evaluation strategy for elucidating the potential mechanism of TCM.

Abbreviations

SZD: San-Zi-Yang-Qin Decoction; TCM: Traditional Chinese Medicine; ST: sinapine thiocyanate; TG: Triglyceride; TC: total cholesterol; LDL: Low-density lipoprotein cholesterol; HDL: High-density lipoprotein cholesterol; Hs-CRP: Hypersensitivity C-reactive protein; SBP: systolic blood pressure; DBP: diastolic blood pressure; TXB2: Thromboxane B2; LysoPC: Lysophosphatidylcholine; LysoPE: Lysophosphatidylethanolamine; UPLC-Q-Orbitrap-MS: Ultra-high performance liquid chromatography-quadrupole electrostatic field orbital trap tandem mass spectrometry; WKY: Wistar-Kyoto Rat; PCA: Principal Component Analysis; QC: Quality Control; PLS-DA: Partial Least Squares Discriminant Analysis; VIP: variable importance in the projection; FC: Fold Change.

Declarations

Acknowledgments

The author would like to thank all the participating members and experimental animals.

Authors’ Contributions

SL and MD contributed to the study design, study conduct, contributed to data analysis, and drafting of the manuscript. SL contributed to the data collection and data interpretation. YL, HJ, and DQ revised the manuscript.

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Availability of data and materials

The data used to support the findings of this study are available from the corresponding author request.
Ethics approval and consent to participate

All procedures were performed in accordance with the guidelines of the Shandong University of Traditional Chinese Medicine ethics committee.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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**Figures**
Figure 1

The schematic of the whole study. When the model was established (at 14 weeks), the model group and SZD-treat group were continued to give high-fat chow and 6% saline for maintaining the obese prehypertension rat model, while SZD was given to the treatment group with the same volume of saline After administration for 10 weeks, serum was collected for biochemical and non-targeted metabolomics analysis. 7 metabolites were screened by multivariate statistical analysis and examined methodologically by pseudo-targeted metabolomics, and finally, relative content was determined.
Figure 2

Effects of SZD on blood pressure (A: systolic blood pressure, diastolic blood pressure, and mean arterial pressure), body weight (B), and Lee's index (C) before and after the intervention. 

*P < 0.05,* P < 0.01, compared with the normal group

#P < 0.05, compared to the model group
Figure 3

Changes of TC(A), TG(B), HDL(C), LDL(D), and Hs-CRP(E) before and after SZD intervention. Control: the normal control group; Model: the model group; H: high dose of SZD group; M: the middle dose of SZD group; L: the low dose of SZD group. *P < 0.05, **P < 0.01, compared with the normal control group. #P < 0.05, ##P < 0.01 compared to the model group.
Figure 4

Metabolomics analysis of serum samples. A: Total ion chromatograms of the three groups; B: The PLS-DA score maps of the three groups under the positive(B1) and negative ion(B2) model respectively; C: 200 this permutation test under positive(C1) and negative ions(C2); D: Scatter plot of VIP>1 under positive(D1) and negative ions(D2); the X-axis is the variable number, and the Y-axis is the VIP value.
Figure 5

Figure 6

MS² mass spectrometry cleavage map of LysoPC (20:0). A: mass spectrometry; B: fragmentation pathways of LysoPC(20:0)
Figure 7

Standard curves of seven metabolites
Figure 8

Changes in the contents of 7 metabolites after SZD intervention. * $P < 0.05$, ** $P < 0.01$, compared with the normal group. # $P < 0.05$, ## $P < 0.01$ compared to the model group.
Figure 9

Metabolic network analysis of SZD in the intervention of obese Pre-hypertension. The arrow indicates up-regulated or down-regulated. Yellow: glyceride metabolism; pink: glycerophospholipid metabolism; blue: arachidonic acid metabolism; grey: sphingolipid metabolism; red circle: linoleic acid metabolism.

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