Postprint of Plasma Metabolomics of Guifu Dihuang Wan Intervention in Yang-Deficiency Constitution

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Abstract

Objective To evaluate the clinical efficacy of Guifu Dihuang Wan intervention for Yang-deficiency constitution and explore its therapeutic mechanism. Methods Sixty-two subjects with Yang-deficiency constitution were enrolled from healthy and sub-healthy populations to exclude the influence of disease on constitution, and randomly divided into two groups (n=31 each). The control group received lifestyle guidance, while the experimental group received Guifu Dihuang Wan intervention for 1 month in addition to lifestyle guidance. The Yang-deficiency constitution scores of subjects were evaluated; plasma metabolomic analysis was performed using nuclear magnetic resonance (NMR) technology before and after intervention, and multivariate statistical analysis methods were employed to identify endogenous differential metabolites in plasma before and after intervention. Results After 1 month of intervention, the Yang-deficiency constitution transformation score in the experimental group was significantly lower than that in the control group ($P<0.05$). Metabolomic results showed that lifestyle intervention could increase the levels of lactate, valine, proline, 3-hydroxybutyrate, and arginine in blood of Yang-deficiency constitution individuals. Guifu Dihuang Wan intervention based on lifestyle guidance, in addition to increasing the levels of lactate, valine, proline, 3-hydroxybutyrate, and arginine in blood of Yang-deficiency constitution individuals, could also increase the levels of alanine, glutamine, -glucose, isoleucine, betaine, and propylene glycol. Conclusion The mechanism by which Guifu Dihuang Wan improves Yang-deficiency constitution may be related to its ability to increase the levels of alanine, glutamine, -glucose, isoleucine, betaine, and propylene glycol, ameliorate energy metabolism disorder, and increase body energy production.

Full Text

Preamble

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Plasma Metabonomics of Guifu Dihuang Wan in the Treatment of Yang Deficiency Constitution

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Abstract

Objective: To evaluate the clinical efficacy of Guifu Dihuang Wan (GFDHW) in intervening yang deficiency constitution and explore its therapeutic mechanism. **Methods:** Sixty-two healthy and sub-healthy individuals with yang deficiency constitution were enrolled to exclude disease influence on constitution. Participants were randomly divided into two groups (n=31 each). The control group received lifestyle guidance alone, while the experimental group received GFDHW intervention for one month in addition to lifestyle guidance. Yang deficiency constitution scores were evaluated. Plasma metabolomics before and after intervention was analyzed using nuclear magnetic resonance (NMR) spectroscopy, and multivariate statistical analysis was employed to identify endogenous differential metabolites. **Results:** After one month of intervention, the yang deficiency constitution conversion score decreased significantly in the experimental group compared with the control group ($P<0.05$). Metabolomics results showed that lifestyle intervention increased plasma levels of lactate, valine, proline, 3-hydroxybutyrate, and arginine. GFDHW intervention on the basis of lifestyle guidance not only elevated these metabolites but also increased alanine, glutamine, -glucose, isoleucine, betaine, and propylene glycol levels. **Conclusion:** The mechanism by which GFDHW improves yang deficiency constitution may be related to its ability to increase the levels of alanine, glutamine, -glucose, isoleucine, betaine, and propylene glycol, thereby ameliorating energy metabolism disorders and enhancing energy production.

Keywords: Guifu Dihuang Wan; yang deficiency constitution; metabonomics

Introduction

Traditional Chinese Medicine (TCM) constitution refers to the comprehensive, relatively stable inherent characteristics formed during human life processes based on innate endowment and acquired factors, encompassing morphological structure, physiological function, and psychological state [1]. Constitution determines disease susceptibility and represents a crucial factor influencing disease nature, location, stage, and progression [2-3]. Yang deficiency constitution is a fundamental TCM constitution type characterized by insufficient yang qi and failure to warm the body, manifesting primarily as aversion to cold, cold extremities, preference for hot food and drinks, and listlessness. While constitution possesses relative stability, it is also dynamically modifiable through intervention. Constitution regulation methods include health education, dietary adjustment, exercise, emotional regulation, lifestyle modification, and pharmacological intervention [4]. Chinese herbal formulas, with their holistic and bidirectional regulatory characteristics, serve as important means for constitution intervention. Guifu Dihuang Wan, derived from the Shenqi Wan formula in *Jin Gui Yao Lue* (Synopsis of the Golden Chamber), consists of eight medicinal herbs: cinnamon, processed aconite, prepared Rehmannia root, processed Cornus officinalis, Moutan bark, Chinese yam, Poria, and Alisma. The formula warms and tonifies kidney yang and is commonly used to regulate yang deficiency constitution [5], though its pharmacological mechanism remains incompletely elucidated.

Current research on TCM constitution mechanisms has primarily focused on genetic polymorphisms and differential gene expression [6-8]. However, TCM constitution arises from the combined effects of innate genetic factors and acquired environmental influences, and genetic differences alone cannot fully explain its molecular mechanisms. Metabonomics represents another important field in systems biology following genomics and proteomics. It employs modern analytical techniques with high throughput, efficiency, and sensitivity to conduct dynamic tracking analysis of small-molecule metabolites in metabolic cycles (generally low-molecular-weight compounds with relative molecular mass <1000). Through multivariate statistical analysis, metabonomics can sensitively and instantaneously reflect functional state changes in organisms caused by internal and external factors such as genes and environment, providing objective information for clinical practice [9-11]. Constitution phenomena reflect the dynamic changes of yin-yang, qi-blood, and body fluids within the body. Individuals with the same constitution phenomenon should share common material bases, which should be reflected at various levels including genes, proteins, cells, tissues, organs, and the whole organism. Metabolites reside at the terminal end of life regulation, and compared with other "omics"approaches, metabonomics is closer to phenotypes and reflects the integrated results of genotype-environment interactions [10]. Therefore, metabonomics research provides more comprehensive insights into TCM constitution phenomena.

This study enrolled healthy and sub-healthy individuals with yang deficiency constitution to exclude disease influence. Participants were randomly divided

into two groups: a control group receiving lifestyle guidance and an experimental group receiving GFDHW intervention in addition to lifestyle guidance. Yang deficiency constitution scores were evaluated, and plasma metabolomics before and after intervention was analyzed using NMR spectroscopy. The therapeutic mechanism of GFDHW intervention for yang deficiency constitution was explored from a metabonomics perspective to provide scientific evidence for TCM-based constitution intervention.

Methods

1.1 Study Subjects

Study participants were recruited from university students at a medical university in Guangzhou. Using SPSS 22.0 software to generate random numbers and following inclusion/exclusion criteria, 62 individuals with yang deficiency constitution were randomly divided into two groups (31 each) based on informed consent. The clinical protocol was approved by the Ethics Committee of Nanfang Hospital, Southern Medical University (Approval No.: [2012] 伦审字 (035) 号).

1.2 Criteria for Yang Deficiency Constitution

According to the *Classification and Determination of TCM Constitutions* published by the China Association of Chinese Medicine [12], participants answered all questions in the questionnaire, with each item scored on a 5-level scale (original score range: 1-5 points). Raw and conversion scores were calculated to determine constitution type. Raw score = sum of all item scores. Conversion score = [(raw score - number of items) / (number of items × 4)] × 100.

Determination criteria: Conversion score 40 points indicates "yes" ; 30-39 points indicates "tendency" ; <30 points indicates "no."

1.3 Inclusion Criteria

Individuals meeting yang deficiency constitution criteria; normal routine physical examination or no definite disease diagnosis; age 18-25 years; voluntary signed informed consent.

1.4 Exclusion Criteria

Individuals with mixed constitutions; severe unhealthy lifestyle; recent infection (within 4 weeks); psychiatric disorders; pregnant or lactating women; allergy to trial medication; participation in other drug clinical trials; researchers' determination of unsuitability; family genetic diseases.

1.5 Intervention Protocol

Control group: Received individualized lifestyle guidance including dietary adjustment, exercise, psychological intervention, and daily living regulation. Specific measures: (1) **Diet:** Consume foods with yang-warming and qi-tonifying properties (grains: glutinous rice, polished rice; meats: hairtail, silver carp, brown trout, loach, eel, mutton, pork stomach, dog meat, sea cucumber; vegetables: chili pepper, onion, leek, ginger, scallion, coriander, wood ear, mustard greens; fruits: longan, lychee, chestnut, peach, apricot, papaya, olive, jujube, cherry, pomegranate). Avoid cold-natured foods that damage yang qi, various cold drinks, raw and cold fruits. Limit green tea consumption and avoid bitter tea. (2) **Exercise:** "Movement generates yang" —engage in outdoor physical activities, preferably in spring/summer or sunny, warm weather. Exercise intensity should be moderate to avoid excessive sweating that damages yang qi. Recommended activities: walking, stair climbing, stretching exercises, badminton, hiking, rope skipping, focusing on upward and jumping movements to assist yang qi elevation. (3) **Psychology:** Identify stress sources, build confidence, strengthen willpower cultivation, maintain optimism. Develop 1-2 sustainable hobbies to bring life pleasure and improve emotional states, which in turn effectively relieve mental stress. Seek social support from multiple perspectives through effective communication with family or participation in cultural, entertainment, and sports activities with colleagues and friends. (4) **Daily living:** Living environment should feature warm, gentle colors. Avoid prolonged work and life in dark, damp, cold environments. Pay attention to waist, back, and lower limb warmth.

Experimental group: Received GFDHW concentrated pills (produced by Henan Wanxi Pharmaceutical Co., Ltd., composed of prepared Rehmannia root, processed aconite, cinnamon, processed Cornus officinalis, Chinese yam, Alisma, Moutan bark, and Poria) in addition to lifestyle guidance. Oral administration: 8 pills per dose, 3 times daily for one month.

Safety evaluation indicators: After one month of intervention, blood routine, liver and kidney function, blood lipids, urine routine, electrocardiogram, and chest X-ray were examined in both groups.

1.6 Collection of Metabolomics Serum Samples

Ten participants from each group were randomly selected for metabolomics analysis. Subjects avoided strenuous and dangerous sports and blood donation for 3 days before sample collection and consumed a light diet the day before. Blood was drawn in the morning after overnight fasting, collected in heparin sodium-pretreated centrifuge tubes, mixed, centrifuged at 4°C and 1000×g for 10 minutes. Plasma supernatant was aspirated and stored at -80°C for ^{1}H-NMR detection.

1.7 NMR Sample Preparation

Plasma samples were thawed at room temperature and centrifuged (14,000×g, 10 min, 4°C). Then 300 L supernatant was transferred to 5 mm NMR test tubes, mixed with 100 L of 0.2 mol/L phosphate buffer (Na HPO , pH 7.4) and 100 L deuterated water, and vortexed.

1.8 NMR Data Acquisition and Analysis

NMR experiments were performed on a BRUKER Avance 500 MHz superconducting NMR spectrometer at 298K. The Carr-Purcell-Meiboom-Gill (CPMG) T filter pulse sequence was used with echo time 2n =100 ms, spectral width 10 kHz, 3 s delay time, 64k data points, and 128 scans. Free induction decay signals were converted to one-dimensional NMR spectra via 32K Fourier transformation with 0.3 Hz exponential window function applied before transformation. MestReNova 5.3.1 software (MestreLab Research SL, Spain) was used for phase and baseline correction, with chemical shift calibrated to alanine at 1.48 ppm. All ^{1}H NMR spectra were automatically integrated over 0.5-9.0 ppm with 0.005 ppm intervals. The 4.7-5.2 ppm region was set to zero to eliminate residual water peak effects. To eliminate concentration-based analytical errors, segmented integral values were normalized before principal component analysis.

Processed data were exported to Excel format for analysis.

1.9 Statistical Analysis

Clinical data were analyzed using SPSS 22.0 software. All statistical inferences employed two-sided tests with significance level =0.05, and 95% confidence intervals were used for parameter estimation. Two independent sample t-tests compared measurement data between groups. For metabolomics analysis, Excel data were imported into Simca-P 12.0.1.0 software (Umetrics AB, Sweden) for multivariate statistical analysis. Principal Component Analysis (PCA) and Orthogonal Partial Least Squares Discriminant Analysis (OPLS-DA) were performed. VIP values (variable importance in projection) were extracted from OPLS-DA models. Chemical shift values with VIP>1 were selected for further statistical analysis of normalized integrals (significance level a=0.05). Chemical shift values with P<0.05 were identified as differential metabolites.

Results

2.1 General Characteristics

The control group comprised 31 participants (5 males, 26 females) with mean age 19.77±1.31 years and BMI 19.28±1.76. The experimental group comprised 31 participants (7 males, 24 females) with mean age 20.26±1.83 years and BMI 18.85±1.63. No significant differences in age, gender ratio, or BMI existed between groups (P>0.05), indicating comparable baseline characteristics.

2.2 Comparison of Yang Deficiency Constitution Scores

Before intervention, no significant difference in yang deficiency constitution conversion scores existed between groups. After one month of intervention, the experimental group showed significantly decreased conversion scores compared with the control group (Table 1 , $P<0.05$), indicating that GFDHW significantly improved yang deficiency constitution beyond lifestyle intervention alone.

Table 1 Comparison of yang deficiency constitution scores between control and experimental groups

Group	Before treatment	After treatment
Control group (n=31)	47.12±12.42	51.96±16.36
Treatment group (n=31)	47.35±12.12	39.17±18.10

$P<0.05$ vs before treatment.

2.3 Safety Indicators

Before intervention, both groups showed normal blood routine, urine routine, liver and kidney function, blood lipids, electrocardiogram, and chest X-ray. After one month, all indicators remained normal in the experimental group with no significant differences from the control group ($P>0.05$). No adverse drug reactions occurred in the experimental group during follow-up.

2.4.1 PCA Pattern Recognition (Control Group)

Figure 1 [Figure 1: see original paper] shows the PCA score plot. PCA model parameters were $R^2X=0.968$ and $Q^2X=0.879$, indicating the model explained 96.8% of original data with cumulative contribution rate of 87.9%. Samples were primarily distributed within the T^2 ellipse (95% confidence interval) in the scatter plot, demonstrating good model fit. Samples before and after intervention in the control group separated significantly along the t1 axis, suggesting lifestyle intervention altered plasma metabolites in yang deficiency constitution.

The loading plot (Figure 2 [Figure 2: see original paper]) showed that while some components clustered together, certain metabolites changed significantly and deviated from the cluster, suggesting these metabolites contributed to differences between pre- and post-intervention samples. We further extracted normalized integral values of variables with VIP>1 to identify differential metabolites.

2.4.2 Differential Metabolite Identification (Control Group)

Combining VIP values and statistical analysis of normalized integrals, nine statistically significant chemical shift values were identified and annotated using the Human Metabolome Database (http://www.hmdb.ca) and literature review.

As shown in Table 2 , differential metabolites contributing significantly to classification before and after lifestyle intervention included valine, lactate, proline, choline, arginine, myo-inositol, leucine, creatine, and 3-hydroxybutyrate, all of which increased after intervention.

Table 2 Differential metabolites in the control group before and after intervention

Shift	Potential metabolites
Valine	
Lactate	
Proline	
Choline	
Arginine	
Myo-inositol	
Leucine	
Creatine	
3-Hydroxybutyrate	

2.5.1 PCA Pattern Recognition (Experimental Group)

PCA was performed for pattern recognition. The model calculated four principal components with parameters R^2X=0.896 and Q^2X=0.646, explaining 89.6% of original data with cumulative contribution rate of 64.6%. The score plot (Figure 3 [Figure 3: see original paper]) showed samples primarily distributed within the T^2 ellipse (95% confidence interval), indicating good model fit. Samples before and after intervention separated along the t1 axis, suggesting GFDHW intervention significantly affected plasma metabolite profiles in yang deficiency constitution. The loading plot (Figure 4 [Figure 4: see original paper]) revealed that while some components clustered, certain metabolites changed significantly and deviated from the cluster. We further extracted normalized integral values of variables with VIP>1 for statistical analysis.

2.5.2 Differential Metabolite Identification (Experimental Group)

Combining VIP values and statistical analysis, eleven statistically significant chemical shift values were identified and annotated using the Human Metabolome Database and literature review. As shown in Table 3 , differential metabolites contributing significantly to classification after GFDHW intervention included valine, isoleucine, lactate, alanine, proline, glutamine, arginine, betaine, -glucose, propanediol, and 3-hydroxybutyrate, all of which increased after intervention.

Table 3 Differential metabolites in the experimental group before and after intervention

Shift	Potential metabolites
Valine	
Isoleucine	
Lactate	
Alanine	
Proline	
Glutamine	
Arginine	
Betaine	
-glucose	
Propanediol	
3-Hydroxybutyrate	

2.6 Changes in Related Metabolite Levels

Lifestyle guidance increased plasma levels of valine, lactate, proline, choline, arginine, myo-inositol, leucine, creatine, and 3-hydroxybutyrate in yang deficiency constitution. GFDHW intervention on this basis further elevated alanine, glutamine, -glucose, isoleucine, betaine, and propylene glycol levels in addition to the aforementioned metabolites.

Discussion

This study employed 500 MHz superconducting NMR spectroscopy combined with pattern recognition analysis to examine plasma metabolites before and after yang deficiency constitution intervention. Results showed that lifestyle intervention partially improved metabolic disorders in yang deficiency constitution by increasing plasma lactate, valine, proline, 3-hydroxybutyrate, and arginine levels. GFDHW intervention further elevated these metabolites and additionally increased alanine, glutamine, -glucose, isoleucine, betaine, and propylene glycol levels, suggesting that GFDHW' s improvement of yang deficiency constitution may be associated with enhanced energy production through increased levels of these metabolites. These differential metabolites primarily involve multiple metabolic pathways including gluconeogenesis, glycolysis, fatty acid metabolism, amino acid metabolism, tricarboxylic acid cycle, inositol metabolism, and neurotransmitter transmission.

Approximately 50-70% of human energy requirements derive from glucose. Major glucose metabolic pathways include anaerobic glycolysis, aerobic oxidation, pentose phosphate pathway, glucuronic acid pathway, and polyol pathway. Glucose oxidation provides essential energy for human life activities. Previous studies reported that blood glucose levels were generally low in family members with kidney-yang deficiency [13] and that individuals with yang deficiency constitution had lower fasting blood glucose levels [14]. Our study found that lifestyle intervention had no significant effect on -glucose content, whereas GFDHW

intervention increased -glucose levels in yang deficiency constitution, suggesting that GFDHW may improve symptoms such as aversion to cold and cold extremities by increasing -glucose content and enhancing energy production.

Glutamine is the most important nitrogen source in the human body. As a neutral amino acid under physiological pH, it contains -amino and amide groups that serve as carriers for nitrogen transfer between tissues, completing approximately 30-50% of amino acid transport in circulation. The amide group also serves as a precursor for purine and pyrimidine nucleotide synthesis in cells. Glutamine is a precursor for reduced glutathione, an extremely important antioxidant, and can enhance antioxidant capacity and reduce oxygen free radical damage by maintaining and increasing reduced glutathione reserves in tissues and cells [15-16]. Glutamine also regulates protein metabolism by promoting intracellular protein synthesis and reducing skeletal muscle protein breakdown, playing important roles in immune function modulation and acid-base balance maintenance [17-19]. Studies have shown that peripheral blood leukocytes in yang deficiency constitution exhibit altered expression of immune-related genes, characterized by decreased immune surveillance function and upregulated inflammatory cytokine expression [7]. While lifestyle intervention had no significant effect on glutamine content, GFDHW intervention increased plasma glutamine levels in yang deficiency constitution, which may be related to enhanced immune function. Research has demonstrated that GFDHW can significantly increase IL-2, INF- , TNF- , IL-4, IL-6, and IL-10 levels and restore Th1/Th2 cell balance in yang deficiency constitution [20].

Alanine is a non-essential amino acid that can be synthesized from pyruvate in vivo. It is abundant in muscle tissue and serves as an important amino acid released by muscle and a key energy substrate. Alanine regulates blood glucose through participation in the "glucose-alanine cycle" (glucose released by the liver enters muscle as blood glucose, pyruvate generated through glycolysis can be converted to alanine via transamination with branched-chain amino acids, re-enters the liver, and forms glucose through gluconeogenesis) [21]. Hepatic gluconeogenesis produces 25-40% of glucose from alanine [22-23]. Serum alanine concentration fluctuates with blood glucose levels, decreasing during hypoglycemia and ketosis [24]. We observed that lifestyle intervention had no significant effect on alanine content, whereas GFDHW intervention increased plasma alanine levels in yang deficiency constitution, suggesting that GFDHW may enhance energy production by increasing alanine levels and consequently glucose content.

In summary, yang deficiency constitution is a constitution type characterized by slowed metabolism and reduced energy production, involving alterations in glucose and lipid metabolism, amino acid metabolism, tricarboxylic acid cycle, and neurotransmitter pathways. GFDHW may improve yang deficiency constitution by increasing alanine, glutamine, -glucose, isoleucine, betaine, and propylene glycol levels, thereby modulating multiple metabolic pathways including gluconeogenesis, glycolysis, fatty acid metabolism, amino acid metabolism,

tricarboxylic acid cycle, inositol metabolism, and neurotransmitter transmission.

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Note: Figure translations are in progress. See original paper for figures.