Analysis of Phytochemical Constituents of Zuogui Wan in Rat Serum and its Effects on Early Embryonic Development of Mice

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Abstract

Objective: Zuogui Wan (ZGW) has been used as a typical prescription for tonifying kidney essence in traditional Chinese medicine. The objective of this study is to elucidate the phytochemical constituents of ZGW-treated rat serum (ZGWRS) using ultra-performance liquid chromatography-electrospray ionization/quadrupole-time-of-flight high-definition mass spectrometry (UPLC-ESI-Q-TOF-MS). Methods: ZGW was administered to rats, and the phytochemical constituents in rat serum were determined using UPLC-ESI-Q-TOF-MS. MetaboLynx analysis in negative ion mode was adopted to characterize the chemical constituents of ZGWRS. Orthogonal partial least squares discriminant analysis was applied for the discovery of constituents of ZGW that entered the serum of rats. The fertilized eggs collected from the same experiment were randomly divided into four groups, including the normal, 40 mmol/L glucose, 40 mmol/L glucose 5% control rat serum, and 40 mmol/L glucose 5% ZGWRS groups. They were cultivated at 37℃ and 5% CO2 incubator. The blastocyst rate and two-cell rate were used to evaluate the effects of ZGWRS on embryonic development. Results: Thirteen constituents were identified in the ZGWRS, among which sweroside, loganin, morroniside, loganic acid, and 8-epiloganic acid were from Fructus Corni (Shan Zhu Yu). 5-Hydroxymethyl-2-furfural-glucuronide, 2-H-5-hydroxymethyl-2-furfural-glucuronide, 3-hydroxy-2,6,6-trimethyl-1-cyclohexene-1-carboxylic acid, and β-D-ribofuranuronic acid methyl ester triacetate were from Radix Rehmanniae Preparata (Shu Di Huang). Coumaric acid was from Fructus Lycii (Gou Qi Zi). Kaempferol-3-beta-O-glucuronide and cuscutamine were from Semen Cuscutae (Tu Si Zi). The embryonic development was significantly inhibited using 40 mmol/L glucose. Compared with the normal group, the blastocyst rate of the glucose group was decreased. The blastocyst rate of the 40 mmol/L glucose 5% ZGWRS group was significantly higher than that of the glucose group, indicating that ZGWRS negates the effect of glucose on mouse embryonic development. Conclusion: The results verified that a rapid and robust UPLC-ESI-Q-TOF-MS-based platform had been successful for identifying multiple constituents of ZGW. ZGWRS is rich in active constituents of iridoid glycosides. The results of this study also showed that ZGW could negate the effect of glucose on mouse embryonic development.

Keywords: Orthogonal partial least squares discriminant analysis, ultra-performance liquid chromatograph-electrospray ionization/quadrupole-time-of-flight high-definition mass spectrometry, Zuogui Wan

INTRODUCTION

The formula of Zuogui Wan (ZGW) originates from a traditional Chinese medicine (TCM) book titled “Jingyue Quanshu.” It has been used as a classic TCM prescription for tonifying the kidneys, such as for the treatment of diabetic nephropathy. The prescription consists of several medicines, such as Radix Rehmanniae Preparata (Shu Di Huang), Semen Cuscutae (Tu Si Zi), Fructus Corni (Shan Zhu Yu), Fructus Lycii (Gou Qi Zi), Rhizoma Dioscoreae (Shan Yao), and Radix Cyathulae (Chuan Niu Xi). As a valuable TCM prescription, ZGW has also been used to treat osteoporosis and type 2 diabetic nephropathy. Ju et al. reported that ZGW could prevent and treat osteoporosis.[1] Besides, ZGW can significantly increase bone Gla-protein (BGP) and reduce...
calcitonin content in an osteoporosis rat model without ovaries. ZGW can promote the two-cell rate and blastocyst rate in mice and can reduce the damage caused by alcohol.[2] When the drug was administered in the embryo stage of the intrauterine growth retardation (IUGR) mice model, ZGW improved the immunity of IUGR mice by enhancing the activity of hexokinase and glutamate dehydrogenase in the liver tissue and creatine kinase in skeletal muscle.[3-5]

The composition of this TCM compound is remarkably complicated, especially after entering the body. Its therapeutic effect could be attributed to the synergistic effects of its multiple components. High-performance liquid chromatograph-mass spectrometry can be used to analyze the composition of ZGW.[6] Methods of serum pharmacochemistry have been introduced on the analysis of the therapeutic substances of TCM compounds.[7,8] Previous studies showed that the active components in the ZGW-treated rat serum (ZGWRS) can significantly influence the secretion of osteocalcin and BGP by osteoblasts in vitro, and improve proliferation and the differentiation of osteoblasts, most likely being related to estrogen-induced ERK/SMAD signaling pathways.[9-11] In addition, ZGWRS can inhibit the apoptosis of thyocytes induced by corticosterone, possibly by regulating the ratio of Bcl-2 to Bax.[12] The characteristics of complicated chemical compositions and multiple targets of ZGW can exert a systematic influence on rats.

As a method used in metabolomics, the statistical approach of principal component analysis (PCA) has been used to analyze the serum pharmacochemistry of TCM. The orthogonal partial least squared discriminant analysis (OPLS-DA), a type of supervised classification, has been recently developed, which has a better performance on processing two datasets to discriminate the constituents in biological samples.[13]

The present work aimed to identify the phytochemical constituents of ZGWR using ultra-performance liquid chromatograph-electrospray ionization/quadrupole-time-of-flight high-definition mass spectrometry (UPLC-ESI-Q-TOF-MS). The combination of UPLC-MS with OPLS-DA is supposed to find differential metabolites in ZGWRS and analyze the effects of ZGW on mouse blastocysts cultured in high glucose conditions.

**Methods**

**Chemicals and drugs**

HPLC acetonitrile was purchased from Merck (Darmstadt, Germany). HPLC formic acid was purchased from Fisher (Fisher, USA). Analytical methanol was purchased from Tianjin Fu Ya Fine Chemical Co., Ltd. (Tianjin, China). Leucine enkephalin was purchased from Sigma-Aldrich (MO, USA). Distilled water was purchased from Watson’s Food and Beverage Co. (Guangzhou, China). Radix Rehmanniae Preparata (Shu Di Huang), Semen Cuscutae (Tu Si Zi), Fructus Corni (Shan Zhu Yu), Fructus Lycii (Gou Qi Zi), Rhizoma Dioscoreae (Shan Yao), Radix Cyathulae (Chuan Niu Xi), and other herbs were all purchased from Beijing Tongrentang Pharmacy Chain Co., Ltd. (Shanxi, China). Prof. Qianjin Feng authenticated all herbs at Shanxi University of Traditional Chinese Medicine.

**Preparation of Zuogui Wan**

ZGW was prepared with all herbs in a constant proportion, according to “Jingyuan Quanshu.” The herbs were immersed in 810 mL of water at 60°C, decocted for 1.5 h, and filtered. The same decoction and filtration process were repeated for a second time. The filtrates were combined and concentrated to 1 g/mL crude drug.[14]

**Serum preparation**

SPF female rats, weighing 200 ± 20 g, were bought from National Institutes for Food and Drug Institute, China (License number: SCXK-(JING) 2009-0017), and randomly divided into two groups. The rats were housed in a Good Laboratory Practice animal room in China Institute for Radiation Protection (24°C ± 2°C, 60% ± 5% relative humidity). All rats were free to access water and standard food and set in a light-dark cycle of 12 h/12 h for 1 week before being subjected to experiments.

One group of rats was fed a normal diet, referred to as the control group. The other groups of rats were fed ZGW for 7 days, referred to as the drug groups. The rats were executed with diethyl ether. Blood was collected directly from their hearts and incubated at 4°C for 30 min, followed by centrifuging at 4,000 rpm for 15 min at 4°C. The serum was then collected and stored at −75°C before use. Serum from the control group is denoted as control rat serum (CRS) and that from the drug group is denoted as ZGWRS.

**Serum sample preparation for liquid chromatograph/mass spectrometry analysis**

OASIS HLB solid-phase extraction C18 columns (Waters Corporation, USA) were activated with 3 mL methanol, followed by 3 mL of water, before use. A volume of 2 mL rat serum was added to 40 µL phosphoric acid, ultrasonicated for 1 min, and vortexed for 30 s. The sample mixture was applied to preactivated OASIS HLB solid-phase extraction C18 columns and allowed to pass through. The column was then washed using 2 mL of water and eluted using 4 mL of methanol. The sample eluent was dried under N2 at 37°C. The residue was dissolved in 100 µL of 50% methanol, ultrasonicated for 1 min, vortexed for 30 s, and centrifuged at 13,000 rpm for 10 min at 4°C. An aliquot (5 µL) was injected for UPLC-MS analysis.

**Ultra-performance liquid chromatograph-electrospray ionization/quadrupole-time-of-flight high-definition mass spectrometry analysis**

UPLC/ESI-Q-TOF-MS analysis was performed at the National TCM Key Lab of Serum Pharmacochemistry, Heilongjiang University of Chinese Medicine. The UPLC/ESI-Q-TOF-MS system consists of a Waters Acquity™ ultra-performance LC system (Waters Corporation, Milford, USA) controlled by
MassLynx (V4.1), and a Waters Synapt™ High Definition MS (HDMS/MS) System (Waters Corporation, Milford, USA) equipped with an electrospray ionization source, operated in either positive or negative mode. An ACQUITY UPLC HSS T3 column (1.8 µm, 100 mm × 2.1 mm, Waters Corp, Milford, USA) was used for separation. The mobile phase was a gradient elution system of A (HCOOH: CH₃CN = 0.1:100, v/v) and B (HCOOH: H₂O = 0.1:100, v/v), and the elution was programmed as follows: 4%–10%A for 0–5 min, 10%–32%A for 5–12 min, 32%–60%A for 12–14 min, and 60%–100%A for 14–18 min. The flow rate was 0.4 mL/min, and the column temperature was 40°C.

The ESI-Q-TOF-MS was operated in negative electrospray ionization mode. The optimal conditions for MS detection were as follows: ESI mode, capillary voltage of 2.6 kV, sampling cone voltage of 30.0 V, and extraction cone voltage of 3.5 V. The source temperature was set to 110°C; desolvation gas temperature was 350°C, and desolvation gas flow was 600 L/h. The full-scan MS data were produced in the mass range of 100–1000 Da. All data were acquired using an independent reference spray via the LockSpray interference. Leucine enkephalin was used as the lock mass in negative ionization mode (m/z = 554.2615) to ensure the accuracy and reproducibility of the method.

**Statistical data analysis**

The accurate masses and compositions were calculated using MassLynx V4.1 software (Waters Corporation, USA). The parameters for the data analysis were set as follows: retention time from 0.1 to 18.0 min, mass ranging from 100 to 1000 Da, mass tolerance of 0.05 Da, noise elimination level at 6, and peak intensity threshold at 100, and isotopic data were excluded from the analysis. Peaks were identified using the retention time (t<sub>R</sub>) and mass transition (m/z) to determine their intensities. All MS raw data were analyzed using the MarkerLynx and EZinfo 2.0 software (Waters Corporation, USA), to identify the potential discriminated variables. PCA and OPLS-DA were conducted to obtain three-dimensional data with peak number (RT−m/z pair), sample name, and intensity.

**Effects of Zuogui Wan-treated rat serum on early embryonic development of mice**

ICR mice (6–7 weeks) were housed at 20°C–27°C, with free access to water and standard mouse chow, and set in a light-dark cycle of 12 h/12 h for 1 week, before subjected to experiments. Female mice were intraperitoneally injected with pregnant mare’s serum gonadotropin 7.5 IU and human chorionic gonadotropin 7.5 IU after 48 h for the superovulation and to mate with sexually matured male mice. The vaginal suppository was checked after 12 h, and fertilized eggs were collected as described in the literature. The fertilized eggs collected from the same experiment were randomly divided into four groups, including the normal, 40 mmol/L glucose, 40 mmol/L glucose 5% CRS, and 40 mmol/L glucose 5% ZGWRS groups. All groups were cultivated at 37°C and 5% CO₂ in a saturated humidity CO₂ incubator. The blastocyst and two-cell rates were calculated to evaluate the effects of ZGWRS on embryonic development.

**Ethics approval and consent to participate**

All animal procedures were approved and conducted according to the guidelines of the Laboratory Animal Care Committee of Shanxi University of Chinese Medicine, China (license number: 2019 LL138).

**Results**

**Characterization of the chemical constituents of control rat serum and Zuogui Wan-treated rat serum using ultra-performance liquid chromatograph-mass spectrometry**

Figure 1 shows the based peak intensity chromatograms of CRS and ZGWRS in ESI mode under the optimal conditions. The two chromatograms are very similar, indicating that both retention time and precise molecular mass are required to identify the peaks.

Due to the interferences from endogenous components and similarity of the BPIs of CRS and ZGWRS, multivariate analysis, OPLS-DA, was performed to discriminate the origin of each constituent.

**Multivariate statistical analysis and chemical constituents of Zuogui Wan-treated rat serum**

The score plots of the constituents in the OPLS-DA model revealed that the constituents were separated into two groups with Group 1 for ZGWRS and Group 2 for CRS [Figure 2a], indicating that the constituents in serum had changed after the rats were dosed with ZGW.

An S-plot was graphed to determine the components contributing the most to the differences between ZGWRS and CRS [Figure 2b]. Each point of the S-plot represents an ion with t<sub>R</sub>−m/z. The X-axis represents the variable contribution, in which the farther the ion t<sub>R</sub>−m/z pair point is from zero, the more the ion contributes to the difference between ZGWRS and CRS. Y-axis represents variable confidence, in which the farther the ion t<sub>R</sub>−m/z pair point is from zero, the higher is the confidence level of the ions contributing to the difference between ZGWRS and CRS. Therefore, the ion points at the two ends of the “S” represent characteristic markers with the most expression of the two serum groups.

The trend plot of ion t<sub>R</sub>−m/z pair of t<sub>n</sub> = 8.31 min, m/z = 183.0978 ion in Figure 2c clearly demonstrates that the ion found in the ZGWRS is absent in CRS. The OPLS-DA of the datasets of ZGWRS and CRS identified or tentatively assigned 27 ions in negative mode to ZGWRS, by comparing the samples with the reference compounds or matching them with the empirical molecular formulae.

To confirm the herbs from which the identified components originated from, and to help the assignment of each component, water extracts of each constituent herb were also analyzed under the same conditions. Among them, 13
prototype components and metabolites were identified. The ion at $t_R = 8.31$ min with an $m/z$ of 183.0978 was identified as 3-hydroxy-2,6,6-trimethyl-1-cyclohexene-1-carboxylic acid, a metabolite of *Radix Rehmanniae Preparata* (Shu Di Huang) [Figures 3 and 4]. The extracted ion chromatograms (EICs) of ZGWRS, CRS, and ZGW samples in negative mode at $m/z = 183.0978$ are shown in Figure 3a-c. From the extracted EICs, it can be observed that 3-hydroxy-2,6,6-trimethyl-1-cyclohexene-1-carboxylic acid was extracted at a retention time of 8.31 min. All identified components are listed in Table 1.

**Zuogui Wan-treated rat serum on early embryo development in mice**

Table 2 lists the blastocyst rate and the two-cell rate of all four groups. The embryonic development was significantly inhibited by 40 mmol/L glucose. Compared with the...
normal group, the blastocyst rate of the glucose group was decreased. The blastocyst rate of the ZGWRS group was significantly higher than that of the glucose group, indicating that ZGWRS promoted the development of mouse embryos.

**Discussion**

ZGW can be used for the treatment of gestational diabetes and has a protective effect on embryonic development. Among all the herbs in ZGW, *Radix Rehmanniae Preparata* (Shu Di Huang), *Rhizoma Dioscoreae* (Shan Yao), *Fructus Lycii* (Gou Qi), and *Semen Cuscutae* (Tu Si Zi) are commonly used for the treatment of gestation diabetes. *Radix Cyathulae* (Chuan Niu Xi) and *Deerhorn glue* (Lu Jiao Jiao) are often used to prevent miscarriage. *Fructus Corni* (Shan Zhu Yu) and *Glue of Tortoise Plastron* (Gui Ban Jiao) are two commonly used gynecological drugs. The results of UPLC-ESI-Q-TOF-MS in this study showed that the constituents of ZGW that can enter blood were mainly iridoids.[16-20]

The active constituents in ZGWRS were mainly iridoid glycosides, which can promote the blastocyst rate and development of mice embryos cultured using high glucose. Morroniside, loganin, sweroside, loganic acid, and 8-epiloganic acid are the bioactive herbal ingredients from *Fructus Corni* (Shan Zhu Yu), a major herb in ZGW.

It has been reported that morroniside and loganin can improve the morphological changes of rat mesangial cells and regulate their growth by reducing oxidative stress, which provides a molecular mechanism for the use of morroniside and loganin in the early stages of diabetic nephropathy.[21] Besides, loganin can significantly inhibit the expression of fibronectin and interleukin-6, which are harmful to the mesangial cells in the kidney.[22]

Sweroside can attenuate and inhibit apoptosis and has a direct

| Treatment | Number of embryos | Number of two-cell embryos | Number of blastocysts | The two-cell rate (%) | The blastocysts rate (%) |
|-----------|-------------------|----------------------------|-----------------------|-----------------------|-------------------------|
| Normal group | 39                | 21                         | 12                    | 54                    | 57                      |
| 40 mmol/L glucose group | 31                | 22                         | 9                     | 71                    | 41                      |
| 40 mmol/L glucose 5% CRS group | 30               | 19                         | 8                     | 63                    | 42                      |
| 40 mmol/L glucose 5% ZGWRS group | 48               | 26                         | 14                    | 54                    | 54                      |

The blastocysts rate%: Number of blastocysts/number of two-cell embryos) × 100%. The two-cell rate%: Number of two-cell embryos/number of embryos) × 100%. ZGW: Zuogui Wan, CRS: control rat serum, ZGWRS: ZGW-treated rat serum
osteogenic effect on the proliferation and differentiation of human MG-63 cells and rat osteoblasts in vitro.[23] *Fructus Corni* (Shan Zhu Yu) has been safely used for the treatment of osteoporosis in postmenopausal women or elderly men in Asia with a long history.

Loganic acid, also an active iridoid in *Cornus officinalis* (Shan Zhu Yu), is a polar compound. An hour after 0.7% loganic acid extract in vehiculum containing 0.15% sodium hyaluronate was administered directly into the conjunctival sac, the intraocular pressure of the animal model (New Zealand rabbit) was reduced by 15%, indicating its potential application in ocular hypertension therapy.[24]

Coumaric acid, a hydroxyl derivative of cinnamic acid, is a bioactive herbal ingredient from *Fructus Lycii* (Gou Qi Zi). It can reduce the peroxidation of low-density lipoprotein and has several biological functions, such as anti-mutagenesis, anti-genotoxicity, antimicrobial, and antioxidant activity. Coumaric acid can also inhibit cellular melanogenesis, and it plays a role in immune regulation. In addition, coumaric acid can capture peroxide substances and reduce the incidence of vascular atherosclerosis.[25]

Kaempferol-3-glucuronide, a bioactive herbal ingredient from *Semen Cuscutae* (Tu Si Zi), can be efficiently absorbed by the human body, even at low oral doses. Kaempferol-3-glucuronide is the major metabolite found in plasma and urine.[26] Studies have shown that dietary kaempferol can reduce the risk of chronic diseases, especially cancers. Kaempferol may augment antioxidation in the body against free radicals, preventing the development of cancer.[27]

5-Hydroxymethyl-2-furfural (5-HMF) glucuronide and dihydro-5-hydroxymethyl-2-furfural glucuronide are bioactive herbal ingredients from *Radix Rehmanniae Preparata* (Shu Di Huang). They can be hydrolyzed to 5-HMF in vivo; therefore, they have almost the same biological effects, including antioxidant effects, inhibiting the sickling of red blood cells, and reducing hypoxic injury.[28,29]

The growth and development of early embryos were inhibited when they were exposed to a high concentration of glucose. In the case of ZGWRs, the blastocyst rate was significantly increased, showing that ZGWRs can protect embryonic development under a high glucose environment. This study on ZGW, a kidney-tonifying prescription, provides an experimental basis for the theory of TCM that “the kidney being the origin of the congenital constitution” and that “the kidney governs growth, development, and reproduction.”[30]
CONCLUSION
Overall, the evaluation of the chemical constituents of ZGWRS was conducted using UPLC-ESI-Q-TOF-MS for the first time. The introduction of multivariate statistical analysis revealed the constituents of ZGW that can enter the blood. The present study further supports the treatment of diabetic nephropathy and related kidney diseases with ZGW. ZGWRS was also analyzed using ESI in positive mode. No new substances or metabolites were detected, possibly because their structures were difficult to identify.

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Conflicts of interest
There are no conflicts of interest.

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