Investigation into the Protective Effects of Hypaconitine and Glycyrrhetinic Acid Against Chronic Heart Failure of the Rats

Liqin Wang  
The First Affiliated Hospital of Nanchang University

Haiming Deng  
The First Affiliated Hospital of Nanchang University

Tengyu Wang  
The First Affiliated Hospital of Nanchang University

Yun Qiao  
Jiangxi University of Chinese Medicine

Jianbing Zhu  
The First Affiliated Hospital of Nanchang University

Mingfeng Xiong (✉ ncxfm1213@126.com)  
The First Affiliated Hospital of Nanchang University

Research Article

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Abstract

Background

The present study aimed to determine the protective effects of hypaconitine (HA) and glycyrrhetinic acid (GA) against chronic heart failure (CHF) in the rats and to explore the underlying molecular mechanisms.

Methods

The CHF rat model was established by transverse-aortic constriction (TAC) operation. The total cholesterol (TCHO) and triglyceride (TG) levels were determined by ELISA assay. The protein expression of fibroblast growth factor 2 (FGF2), vascular endothelial growth factor A (VEGFA) and endothelial nitric oxide synthase (eNOS) in the rat ventricular tissues was determined by immunohistochemistry. The serum metabolites were determined by LC-MS/MS assay.

Results

HA + GA treatment significantly reduced the plasma levels of TCHO and TG in the CHF rats. The expression of FGF2 and VEGFA protein was up-regulated and the expression of eNOS protein was down-regulated in the ventricular tissues of CHF rats, which was significantly restored after HA + GA treatment. HA + GA treatment down-regulated serum isonicotinic acid, phosphatidylcholine, cardiolipin, estrogen glucuronide, and glycocholic acid, up-regulated serum sphingosine and deoxycholic acid in the CHF rats.

Conclusion

In conclusion, HA +GA showed protective effects on CHF in the rats, and the HA + GA may exert protective effects by reducing lipid levels, up-regulating the expression of FGF2 and VEGFA proteins, attenuating eNOS protein expression, and modulating metabolic pathways. However, the molecular mechanisms underlying HA + GA-mediated effects still require further examination.

Background

Chronic heart failure (CHF) is a rapidly growing public health problem that occurs secondary to a common chronic phase of cardiac impairment of multiple etiologies [1]. Atherosclerosis is an important risk factor for chronic heart failure and its possible mechanisms include imbalance of lipid metabolism and poor adaptive immune response, leading to chronic inflammation of the arterial vessel wall [2]. Angiotensin-converting enzyme inhibitors, β-adrenergic blockers and diuretics are the main treatments for CHF, while long-term use of these chemical drugs may cause adverse effects such as hypotension, fluid depletion and electrolyte depletion [3]. Traditional Chinese medicines (TCMs), such as Salvia miltiorrhiza and Ginseng, can promote myocardial contractility, dilate blood vessels, diuresis and inhibit ventricular remodelling [4–6]. Some formulae have been clinically applied in the treatment of heart failure. The advantage of multi-channel and multi-target of TCM can attenuate adverse effect caused by the genetic defect of western medicine treatment target.

Hypaconitine (HA) is one of the main aconitum alkaloids in traditional Chinese medicines prepared with herbs from the genus Aconitum. HA has various biological activities including its effects on cardiovascular system. Yi et al., showed that HA could inhibit calmodulin expression and connexin43 phosphorylation in rat cardiac muscle in vivo [7]. Xie et al., showed that HA could induce QT prolongation via inhibiting KCNH2 potassium channels in conscious
dogs [8]. Bai et al., showed that HA could inhibit the apoptosis of endothelial cells via targeting histone deacetylase-high mobility group box 1 pathway [9]. In addition, glycyrrhetinic acid (GA) is one of the main glycyrrhizinic acid in traditional Chinese medicines prepared with herbs from the Glycyrrhiza uralensis Fisch. GA could exert various biological activities. GA could mitigate prophylaxis induced by mesaconitine in the rats, indicating that pretreatment with GA might reduce the toxicity of mesaconitine in the metabolic level [10]; GA has also been found to be a typical active component of Shaoyao-Gancao Decoction for pain relief [11, 12]. In our previous study, we found that HA and GA, the active components of Fucus and Glycyrrhiza, exerted protective effects against CHF through apoptotic signaling pathways [13]. However, the underlying protective effects of HA and GA in CHF remain unclear.

In this study, we performed ELISA to examine the effects of HA + GA treatment on plasma TCHO and TG levels as well as immunohistochemistry to investigate expression levels of FGF, VEGF and eNOS protein in CHF rats after HA + GA treatment. In addition, LC-MS/MS analysis used to determine the metabolic differences between the HA+GA group and the Model group and to find potential biomarkers and their possible metabolic pathways associated with CHF.

### Materials And Methods

#### Animals

Male specific-pathogen free rats (weight 160-170 g) were purchased from Shanghai Slack Laboratory Animal Co., Ltd., (Shanghai, China). The rats were allowed free access to food and water and were kept on a 12-h light/dark cycle at room temperature (20 ± 2 °C) with constant humidity (50 ± 2%). All animal experimental procedures were approved by the Animal Care and Use Committee of Zhejiang Chinese Medical University and complied with laboratory animal management and use regulations (SYXK (Zhejiang)2013-184). The study is reported in accordance with ARRIVE guidelines (https://arriveguidelines.org).

#### Establishment of CHF model in the rats and treatments

The CHF model was established by transverse-aortic constriction (TAC) method based on previous methods [14]. Briefly, the rats were anesthetized with 2.0% isoflurane (Shenzhen Huabao Medical Supplies Industry & Trade Development Co., Ltd., Shenzhen, China), and then were endotracheally intubated with positive pressure ventilation with a tidal volume of 250 mL at a velocity of 120 breaths per minute by a Small Animal Ventilator (Hallowell, Inc., USA). After opening the chest wall via an upper sternotomy, the aorta was carefully dissected out. A self-regulating “L” needle with the external diameter of approximately 0.9 mm was placed on the aorta, on which a 3-0 prolene ligation was placed between the right and left common carotid arteries. The needle was removed cautiously, and the aortic constriction was then created. After the procedure, the wound was closed, and the animal was allowed to recover from anesthesia. Penicillin (REYOUNG Co. Ltd., Shandong, China) was injected intramuscular once a day for 3 days. The TAC-operated rats were assigned as the model group. The rats in Sham group underwent same procedures with the Model group but without TAC operation [15, 16].

During the first 4 weeks after surgery, rats were allowed free access to food and water. The rats were then randomly divided into three groups: Sham group (n=6), Model group (n=10), HA + GA group (n=10). According to our previous work [17], the dosage of 2.07 mg/kg HA (Baoji Chenguang Biotechnology Co. Ltd., Xi’an, China) + 25 mg/kg GA (Baoji Chenguang Biotechnology Co. Ltd.) were orally administered to rats of HA + GA group once a day for 1 week, and an equal volume of normal saline was given to the rats from the Sham and Model groups. At one week after drug treatments, the animals were sacrificed and the relevant tissues were collected for further assays.
Collection of tissue samples

After sacrificing the animals, the blood was collected from the abdominal aortic, and plasma was obtained from supernatant of centrifugation of the blood. The heart tissues were then dissected out and weighed. The left ventricular tissues of the heart were cut into two parts: one part was fixed in 4% paraformaldehyde (PFA) and embedded in paraffin for histological analysis, and the other part was preserved in −80 °C.

Enzyme-linked immunosorbent assay (ELISA) for detecting TCHO and TG levels

The TCHO and TG levels in the plasma samples were detected by using ELISA kits for TCHO and TG according to the manufacturer’s instructions.

Immunohistochemistry

Heart ventricular tissues were fixed in 4% PFA and embedded in paraffin. Then, sections (3 µm) were sequentially permeabilized with 1% Triton X-100 in phosphate buffered saline (PBS) for 30 min at room temperature, boiled in 100 mM sodium citrate (pH 6.0) three times (6 min each) with 5 min intervals for antigen retrieval, washed in 3% hydrogen peroxide for 30 min to remove endogenous peroxidase and blocked for 1 hour in 5% bovine serum albumin at room temperature. Sections were then incubated with anti-FGF (1:200, # Sc-365, Santa Cruz Biotechnology, Dallas, USA), anti-VEGF (1:200), and anti-eNOS antibody (1:200) at 37 °C for 60 min at 37 °C. After being washed PBS, the sections were incubated with horseradish peroxidase-conjugated secondary antibodies for 40 min at 37 °C. The tissues were then washed by PBS and incubated in diaminobenzidine substrate (#1901275A, DAKO Co., Ltd., Denmark, Sweden). The sections were finally counterstained with hematoxylin and xylene before being rewashed in ethanol. Images were collected by DAB light microscope (Leica Microsystems Ltd., Wetzlar, Germany). Integrated optical density (IOD) was obtained to represent the relative amount of positive staining, and 5 fields (magnification 200x) were randomly chosen to analyze.

Statistical analysis

All experimental data were presented as the mean ± the standard deviation (SD); one-way analysis of variance (ANOVA) followed by Tukey’s multiple comparison test was performed using SPSS 19.0 statistical software. P < 0.05 was considered statistically significant.

Results

Analysis of lipid profile in the rat plasma

The effects of HA+GA on TG and TCHO levels in CHF rats were determined by ELISA assay. As shown in Table 1, the TCHO and TG levels were significantly lower in the HG + GA group than that in the Model group. In addition, TG and TCHO levels were significantly lower in the Sham group than that in the Model group (Table 1). The difference in TCHO and TG levels between the HA+GA group and the Sham group was not statistically significant (Table 1).
Table 1
TCHO and TG levels in Sham, Model and HA+GA group

| Group    | TCHO (mg/dl) | TG (mmol/L) |
|----------|--------------|-------------|
| Sham     | 1.44±0.13    | 0.31±0.11   |
| Model    | 2.06±0.29##  | 0.69±0.26## |
| HA+GA    | 1.68±0.16**  | 0.39±0.07*  |

HA = hypaconitine; GA = glycyrrhetinic acid. Data were presented as mean ± SD (n = 6-10). ##P< 0.01 versus Sham group; ∗P < 0.05 and **P< 0.01 versus Model group.

Immunostaining analysis of FGF2, VEGFA and eNOS in the heart tissues of the rats

Immunohistochemistry was used to detect the effect of HA+GA on the expression of FGF2, VEGFA and eNOS proteins in heart tissues of rats with CHF. As shown in Figure 1 and 2, the protein expression levels of FGF2 and VEGFA were significantly higher and the protein expression level of eNOS was significantly lower in the HA + GA group than that in the Model group. In addition, the protein expression levels of FGF2 and VEGFA were significantly higher and the protein expression level of eNOS was significantly lower in sham group than that in the model group. There was no statistically significant difference in FGF2, VEGFA and eNOS between the Sham and HA+GA group. The above results indicated that the protective effect of HA+GA in rats with CHF was related to the regulation of FGF2, VEGFA and eNOS protein expression.

Untargeted metabolomics analysis

The serum metabolites of the rats with different treatments were analyzed by using LC-MS/MS technique. Based on the LC-MS/MS analysis, we detected 9237 metabolite signatures of 9920 metabolites in positive and negative mode (Figure 3). Figure 3A and 3B illustrated total ion flow chromatogram (TIC) in positive and negative mode, respectively, and the response intensity and retention time of peaks of each sample largely overlapped. Using unsupervised principal component analysis, QC samples were used to assess the quality of the experiment. as shown in the figure 3C and 3D, the distribution points of QC samples were clustered, indicating good reproducibility of the instrument.

To better distinguish the differences between groups, we performed the supervised PLS-DA, which enables the prediction of sample categories while reduces the dimensionality. Figure 4A and 4B demonstrated the PLS-DA results for the comparison between the Model group and HA+GA group. In the results, each point represents a sample, and the clustered sample points of the HA+GA group are remarkably distinct from that of the model group, indicating that there is a significant difference between the Model and HA+GA group. The replacement test of the PLS-DA also showed that the PLS-DA was not overfitted in our datasets (Figure 4C and 4D).

Furthermore, hierarchical cluster analysis of the differential metabolites was performed to examine the metabolite subgroups, and the results were presented as a heatmap. Figure 5 demonstrated the heatmap of the hierarchical cluster analysis in positive ion mode and negative ion mode between the HA+GA group and the model group, and highly expressed metabolites were found in both the Model group and HA+GA group. Based on the PLS-DA analysis,
a total of 303 differential metabolites in the positive ion mode and a total of 170 differential metabolites in the negative ion mode between Model and HA + GA group were identified.

Furthermore, the associated metabolic pathways of the differential metabolites between Model and HA + GA group were also explored. As shown in the Table 2, isonicotinic acid, phosphatidylcholine, cardiolipin, estrogen glucuronide, and glycocholic acid were significantly up-regulated, while sphingosine and deoxycholic acid were significantly down-regulated in the HA+GA group compared with the Model group. The associated metabolic pathways included sphingolipid metabolism, sphingolipid signaling pathway, and glycerophospholipid metabolism (Table 2).
Table 2
Differential metabolites and their metabolic pathways between the Model and HA+GA group

| MS2kegg | RT    | M/Z      | Formula     | Regulated | Identification       | Pathway                                           |
|---------|-------|----------|-------------|-----------|----------------------|--------------------------------------------------|
| C07446  | 1.07  | 124.0387 | C6H5NO2     | up        | Isonicotinic acid    | Drug metabolism - other enzymes                  |
| C00319  | 3.20  | 300.2893 | C18H37NO2   | down      | sphingosine          | Sphingolipid metabolism                          |
| C00319  | 3.20  | 300.2893 | C18H37NO2   | down      | sphingosine          | Sphingolipid signaling pathway                   |
| C00319  | 3.20  | 300.2893 | C18H37NO2   | down      | sphingosine          | Metabolic pathways                               |
| C00157  | 4.96  | 580.3945 | C10H18N08PR2| up        | Phosphatidylcholine  | Glycerophospholipid metabolism                   |
| C00157  | 4.96  | 580.3945 | C10H18N08PR2| up        | Phosphatidylcholine  | Arachidonic acid metabolism                     |
| C00157  | 4.96  | 580.3945 | C10H18N08PR2| up        | Phosphatidylcholine  | Linoleic acid metabolism                         |
| C00157  | 4.96  | 580.3945 | C10H18N08PR2| up        | Phosphatidylcholine  | alpha-Linolenic acid metabolism                  |
| C00157  | 4.96  | 580.3945 | C10H18N08PR2| up        | Phosphatidylcholine  | Metabolic pathways                               |
| C00157  | 4.96  | 580.3945 | C10H18N08PR2| up        | Phosphatidylcholine  | Retrograde endocannabinoid signaling             |
| C00157  | 4.96  | 580.3945 | C10H18N08PR2| up        | Phosphatidylcholine  | Choline metabolism in cancer                     |
| C04483  | 3.33  | 391.2854 | C24H40O4    | down      | Deoxycholic acid     | Bile secretion                                   |
| C11133  | 2.35  | 445.1907 | C24H30O8    | up        | Estrone glucuronide  | Steroid hormone biosynthesis                     |
| C01921  | 2.83  | 464.3029 | C26H43NO6   | up        | Glycocholic acid     | Primary bile acid biosynthesis                   |
| C01921  | 2.83  | 464.3029 | C26H43NO6   | up        | Glycocholic acid     | Metabolic pathways                               |
| C01921  | 2.83  | 464.3029 | C26H43NO6   | up        | Glycocholic acid     | Bile secretion                                   |
| C05980  | 5.13  | 483.2734 | C13H18O17P2R2| up       | Cardiolipin          | Glycerophospholipid metabolism                   |

Discussion

We aimed to investigate the protective effects of the active ingredients of Aconite and Glycyrrhiza glabra on rats with CHF. Firstly, we performed ELISA to examine the effects of HA + GA treatment on plasma TCHO and TG levels as well as immunohistochemistry to investigate expression levels of FGF, VEGF and eNOS protein in CHF rats after
HA + GA treatment. In addition, LC-MS/MS analysis used to determine the metabolic differences between the HA+GA group and the Model group and to find potential biomarkers and their possible metabolic pathways associated with CHF.

The biochemical tests revealed that TCHO and TG levels in Model were higher than in the HA + GA group, while the difference between the HA + GA and Sham groups was not statistically significant, suggesting that the protective effect of HA + GA on chronic heart failure was associated with a reduction in lipid levels. In hypercholesterolemic patients, plasma FGF2 levels were significantly lower, which was associated with the inhibitory effect of plasma low-density lipoprotein (LDL) [18]. In atherosclerotic lesions, VEGFA expression expressed in smooth muscle cells was significantly increased [19]. It is well known that eNOS has atherosclerotic effects through the synthesis of NO, which has been shown to promote atherosclerosis by oxidizing LDL. The immunohistochemistry results showed that the HA+GA group achieved a protective effect against CHF by up-regulating the expression of FGF2 and VEGFA proteins and attenuating eNOS protein expression.

Sphingolipids are a complex class of lipids consisting of a backbone sphinx base that can be phosphorylated, acylated, glycosylated, bridged to various head groups via phosphodiester bonds, or otherwise modified, resulting in hundreds of sphingolipid subspecies [10]. Sphingolipids have been found to be closely associated with cardiovascular disease; and sphingomyelin levels are significantly increased in atherosclerotic lesions [11], and Poss AM et al., showed that its serum levels have been suggested as a biomarker for cardiovascular disease [12]. Sphingosine is a biologically active signaling molecule among sphingolipid metabolites, and we observed that sphingosine levels were significantly downregulated in the HA + GA group compared to that in the Model group, and we speculated that HA + GA may achieve protective effects in rats with CHF by modulating sphingolipid metabolic pathways and sphingolipid signaling pathways.

Phosphatidylcholine is a common glycerophospholipid that is widely found in animal and plant tissues and egg yolk. The choline component of dietary phosphatidylcholine is metabolized by human intestinal microorganisms to produce trimethylamine-N-oxide (TMAO), which increased the risk of cardiovascular disease [20]. Paapstel K et al. found a negative correlation between serum phosphatidylcholine levels and vascular injury in patients with atherosclerosis [21]. We found that phosphatidylcholine levels were significantly upregulated in the HA+GA group compared to that in the Model group, suggesting that HA + GA achieves vascular protection in CHF rats by promoting the synthesis of phosphatidylcholine. Cardiolipin is a phospholipid specific to mitochondrial membranes and plays an important role in the composition and function of mitochondrial membranes. Impaired energy metabolism is a feature of heart failure, which is associated with iron deficiency and impaired mitochondrial function in patients with heart failure [14]. The important function of cardiolipin for mitochondria lies in its combined action on protein complexes and super complexes [22]. Even for green plants, cardiolipin plays an important role in the mitochondrial development and function [17]. We found that cardiolipin levels were significantly up-regulated in the HA+GA group, indicating that HA+GA achieved a protective effect against CHF in rats by modulating the glycerophospholipid pathway. In addition to regulating sphingolipid metabolic pathway and glycerophospholipid metabolic pathway, it is also related to regulate sphingolipid signaling pathway, apoptosis, arachidonic acid metabolism, linoleic acid metabolism, α-linolenic acid metabolism, and bile secretion pathway.

**Conclusions**

In conclusion, HA +GA showed protective effects on CHF in the rats, and the HA + GA may exert protective effects by reducing lipid levels, up-regulating the expression of FGF2 and VEGFA proteins, attenuating eNOS protein expression,
and modulating metabolic pathways. However, the molecular mechanisms underlying HA + GA-mediated effects still require further examination.

**Abbreviations**

ANOVA, one-way analysis of variance; CHF, chronic heart failure; ELISA, Enzyme-linked immunosorbent assay; eNOS, endothelial nitric oxide synthase; HA, hypaconitine; FGF2, fibroblast growth factor 2; IOD, integrated optical density; LDL, low-density lipoprotein; GA, glycyrrhetinic acid; PFA, paraformaldehyde; SD, standard deviation; TAC, transverse-aortic constriction; TCHO, total cholesterol; TCMs, Traditional Chinese medicines; TG, triglyceride; TIC, total ion flow chromatogram; VEGFA, vascular endothelial growth factor A.

**Declarations**

**Ethics approval and consent to participate**

All animal experimental procedures were approved by the Animal Care and Use Committee of Zhejiang Chinese Medical University and complied with laboratory animal management and use regulations (SYXK (Zhejiang)2013-184). All methods were performed in accordance with the relevant guidelines and regulations.

**Consent for publication**

Not applicable.

**Availability of data and materials**

All the data generated and analyzed in this study are mentioned in this manuscript.

**Competing interests**

The authors declare that there is no conflict of interests regarding the publication of this paper.

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**Authors’ contributions**
Li Qin Wang and Haiming Deng wrote the main manuscript text, Tengyu Wang and Yun Qiao prepared the figures and tables, Mingfeng Xiong amended the manuscript, all authors reviewed the manuscript.

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**Figures**

**Figure 1**

Immunostaining analysis of FGF2, VEGFA and eNOS in the heart tissues of the rats. (A-C) Immunohistochemical staining micrographs of FGF2 (A), VEGFA (B) and eNOS (C) in the rat heart tissues from Sham group (magnification = 100x). (D-F) Immunohistochemical staining micrographs of FGF2 (D), VEGFA (E) and eNOS (F) in the rat heart tissues from Model group (magnification = 100x). (G-I) Immunohistochemical staining micrographs of FGF2 (D), VEGFA (E) and eNOS (F) in the rat heart tissues from HA +G A group (magnification = 100x).
Figure 2

Quantitative analysis of FGF2, VEGFA and eNOS protein expression levels in the rat heart tissues. (A) Quantitative image analyses of FGF2 protein were performed based on the IOD of positive immunostaining (brown) in the heart tissues from Sham, Model and HG + GA group. (B) Quantitative image analyses of VEGFA protein were performed based on the IOD of positive immunostaining (brown) in the heart tissues from Sham, Model and HG + GA group. (C) Quantitative image analyses of eNOS protein were performed based on the IOD of positive immunostaining (brown) in the heart tissues from Sham, Model and HG + GA group. Data are presented as mean ± SD (n = 6-10). Significant differences between treatment groups were shown as *P < 0.05 and **P < 0.01.
Figure 3

LC/MS-MS analysis of serum metabolites of rats. (A and B) Total Ion Chromatography plots in positive (A) and negative (B) ion modes. (C-D) PCA plots in positive (C) and negative (D) ion modes.

Figure 4

PLS-DA analysis of serum metabolites from Model and HG + GA group. (A and B) PLS-DA plots of HA+GA group and Model group in positive (A) and negative (B) ion modes. (C and D) The corresponding substitution test plots in the positive (C) and negative (D) ion models.

Figure 5

Heatmap plots of the differential serum metabolites between Model and HG + GA group (A and B) Heatmap plots of differential serum metabolites between HA+GA group and Model group in positive (A) ion mode and negative (B) ion mode, respectively.