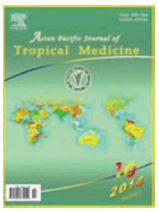
Contents lists available at ScienceDirect

Asian Pacific Journal of Tropical Medicine

journal homepage:www.elsevier.com/locate/apjtm



Document heading doi: 10.1016/S1995-7645(14)60142-5

^{1}H nuclear magnetic resonance-based metabolomics reveals sex-specific metabolic changes of gastrodin intervention in rats

Xin Li[1], Yuan-Wei Jia[1,2], Jun-Song Wang[3*], Ming-Hua Yang[1], Kelvin D. G. Wang[1], Ling-Yi Kong[1*]

[1]*State Key Laboratory of Natural Medicines, Department of Natural Medicinal Chemistry, China Pharmaceutical University, Nanjing210009, China*
[2]*Anhui provincial Center for Drug Clinical Evaluation, Yijishan Hospital, Wannan Medical College, Wuhu241001, China*
[3]*Center for Molecular Metabolism, School of Environmental and Biological Engineering, Nanjing University of Science and Technology, Nanjing210094, China*

ARTICLE INFO

Article history:
Received 10 April 2014
Received in revised form 15 May 2014
Accepted 15 July 2014
Available online 20 October 2014

Keywords:
Gastrodin
Metabolomics
^{1}H NMR spectroscopy
Sex differences
Health protection

ABSTRACT

Objective: To explore ^{1}H nuclear magnetic resonance-based metabolomics on sex-specific metabolic changes of gastrodin intervention in rats. **Methods:** In this research, ^{1}H NMR-based metabolomics was used for the first time to investigate metabolic changes following chronic intervention with gastrodin in rats. **Results:** 24 endogenous metabolites were identified. Body weight, daily diet and the total volume of urine in in each day of each rat were measured synchronously. Modifications in 12 metabolites were observed following gastrodin intervention, indicating gastrodin-induced alterations in carbohydrate and energy metabolism. Interestingly, these metabolic changes were not totally identical in female and male rats. Some metabolic changes arising from gastrodin intervention showed sexual dimorphism including LDL/VLDL and lactate which were on the decrease in the female but on the increase in the male, together with arginine/ornithine, creatine, and glycerol which were on the increase in the female but on the decrease in the male. While the decrease in pyruvate, succinate and glutamate was only shown in the male and the increase in valine, α-ketoglutarate, glycine and glucose was only in the female. **Conclusions:** This research shows the sex-specific metabolic response to GAS intervention, weather GAS is a healthy dietary supplement for the male merits further investigation.

1. Introduction

Traditional Chinese medicine "Tianma" (Gastrodia elata Bl.), first recorded in "Sheng Nong herbal classic" in eastern Han dynasty, has been used as one of the most important medicinal plants in oriental countries. Besides medicinal application, "Tianma" is commonly used as raw material in medicated diet. Gastrodin (GAS p-hydroxymethylphenyl- β -D-glucopyranoside) (Figure 1) the main bioactive component of "Tianma", has been reported with comprehensive pharmacological functions, including anticonvulsant and neuroprotective effects, and hypoxia tolerance[1-3]. As a dietary supplement, GAS has been also reported to prevent neurasthenia and to invigorate brain[4,5].

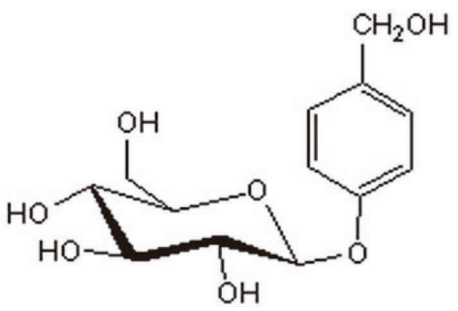


Figure 1. Structure of GAS.

*Corresponding author: Ling-Yi Kong, professor, China Pharmaceutical University, Nanjing210009, Jiangsu, China.
Tel.: +86-25-8327-1405
E-mail: cpu_lykong@126.com
Jun-Song Wang, professor, Nanjing University of Science and Technology, Nanjing210094, Jiangsu, China.
Tel.: +86-025-8327-1402
E-mail: wang.junsong@gmail.com
Foundation project: This research was funded by the National Science Foundation of China (81173526) and the Fundamental Research Funds for the Central Universities (No.30920130112014).

However, the role of GAS played in healthy conditions remains unclear and calls for a systematic research. In recent years, the new "omics" sciences such as metabolomics, proteomics, and transcriptomics have become strong tools to solve and fully understand the impact of body exposure to stimuli (*eg*., nutrients, noxious agents, stressors, genetic modification, pathophysiological or environmental conditions) on the network of metabolites, proteins and genes found within cells, tissues, or organisms[6-9]. Nowadays, with the development of analysis instrument, several analytical methods are available for metabolomics research, such as nuclear magnetic resonance (NMR) and gas chromatography/mass spectrometry (GC/MS), and liquid chromatography/mass spectrometry (LC/MS). NMR-based metabolomics has emerged as a preferred platform with minimal specimen preparation and disturbance prior to the quantitative spectral acquisition step, providing a comprehensive metabolic profile of proton-containing, low-molecular-weight metabolites[10].

In this study, a metabolomics strategy has been applied to investigate the effect of oral administration of GAS on the plasma profiles in normal rats. Considering possible sex-dependent mode of action[11-18], we have evaluated the metabolite changes following GAS intervention in the female and the male for better understanding the mechanisms of health benefits of GAS.

2. Materials and methods

2.1. Chemical

Gastrodin (purity>98%) was purchased from Zelang Biotechnology Co. Ltd. (Nanjing, China). Trimethylsilyl-2,2,3,3-tetradeuteropropionic acid (TSP) was purchased from Sigma (St. Louis, MO., USA). Deuterium oxide (D_2O, 99.9%) was purchased from Sea Sky Biotechnology Co. Ltd. (Beijing, China). Saline was purchased from Jiangsu Medicine Company (Nanjing, China).

2.2. Animal handling and study protocol

24 Sprague-Dawley rats (8-9 weeks old, half male and half female, Certificate No. SCXIL-Su 2009-0001) were purchased from Comparative Medicine Center of Yangzhou University (Yangzhou, China), and were housed individually in metabolism cages. The animals were allowed to acclimate for 7 days with free access to food and water, and under room humidity of (50±5)%, temperature of (23±1) ℃ and a light-dark cycle 12 h and 12 h. The study was approved by the Animal Ethics Committee of China Pharmaceutical University and it was in compliance with the National Institute of Health guidelines for the Care and Use of Laboratory.

2.3. Experimental animals

After a week of acclimation, the metabolomics investigation was conducted to probe gender-specific metabolism differences in rats. The female (n=12) and the male (n=12) rats were all randomly divided into two groups: the administration group rats received a thrice-daily oral dose of GAS (100 mg/kg, n=6 animals/group/sex) for 14 days, and the control groups consisting of gender-matched rats (n=6 animals/group/sex) received saline vehicle (0.9%) for 14 days. GAS dosage was selected based on the reference[19]. The daily diet (rodent chow and water) and the total volume of urine in each day for each rat were measured. These data were statistically analyzed using Microsoft Excel (Microsoft office 2003). Student's t test was used and data were expressed as mean± SD, and P<0.05. After dosing with GAS, 0.5 mL 12 h-fasting blood sample was taken from the ocular veins of each rat in heparinized tubes and immediately centrifuged at 4 500 g for 10 min at 4 ℃ on day 1, 4, 7, 10, and 14. Plasma was separated and stored at −80 ℃ until analysis by NMR spectroscopy.

2.4. ^1H NMR spectroscopy

The plasma samples were thawed at room temperature, and 300 μL plasma was mixed with 150 μL trimethylsilyl-2,2,3,3-tetradeuteropropionic acid (TSP, 1 mg/mL) dissolved in D_2O and 150 μL phosphate buffer solution (0.2 mol/L Na_2HPO_4 and 0.2 mol/L NaH_2PO_4 in D_2O, pH 7.4), then the mixture was centrifuged at 12 000 rpm for 10 min at 4 ℃. The 550 μL supernatants from the mixture were transferred into 5 mm NMR tubes. D_2O and phosphate buffer were added for signal locking and maintaining normal plasma osmolality[20].

All ^1H-NMR measurements were carried out on a Bruker AV 500 MHz spectrometer at 25 ℃. Each spectrum was recorded by using the Carr-Purcell-Meiboom-Gill (D [−90°-(τ-180°-τ)$_n$-FID]) sequence. The sequence suppresses broad signals arising from macromolecules (*i.e*. proteins) and makes micro-molecular metabolite signals prominent. The spin-echo delay (2nτ) was 64 ms. A total 128 transients were acquired into 32 000 data points using a spectral width of 7 500 Hz and an acquisition time of 1.95 s. RD is a relaxing decay of 2 s. The FIDs were weighted by an exponential function with a 0.5 Hz line-broadening factor prior to Fourier transformation (FT). Phase and baseline were manually corrected by using TOPSPIN software (version 3.0, Bruker Biospin, Germany).

2.5. Data reduction and chemometric analysis of the ^{1}H-NMR spectra

The ^{1}H-NMR spectra were automatically reduced to ASCII files using MestRe-C 2.3 (www.mestrec.com). Then all ASCII files were input into R software package (http://cran.r-project.org/)[10], which is a freely available open-source software package and processed with an in-house developed R-script to further reduce phase and baseline distortions.

A peak alignment script was built in R software to reduce phase and baseline distortion prior to statistical analysis. For input, reduced NMR spectra were binned into integrated segments of equal width of 0.015 ppm to minimize the effects of pH and ionic concentration differences. Each bin of the aligned spectra over the range of δ H 0.0-4.5 was normalized by integral normalization and vector length normalization[21] as our initial analysis indicated that the region δ H 6.0-10.0 had no significant metabolite signals contributing to GAS intervention, and the region δ H 4.6-5.3 was removed to suppress residual water signals.

The mean-centered and Pareto-scaled NMR data were analyzed by a supervised method orthogonal partial least squares discriminant analysis (OPLS-DA), to eliminate the contribution of systematic variation arising from features in the data that were unrelated to the class. OPLS-DA was used to explore a model discriminating between control and post-GAS intervention and the model was tested on female and male rats individually. All OPLS-DA models were cross-validated using a seven-fold method by default, the validity of the models against over-fitting was assessed by the parameter R^2Y, and the predictive ability was described by Q^2[22]. This multivariate analysis was used to assess the integration areas of metabolites by using R software (http://cran.r-project.org/), which is a freely available, open-source software package. These data were first tested for the normality of the distribution, and then parametric (Student's t test) or nonparametric (Mann-Whitney test) tests were performed to detect statistically significant metabolites that increased or decreased between groups.

3. Results

3.1. Statistical results of diet, body weight, and urine volume

The statistical results of diet, body weight and urine volume of female and male rat samples are shown in Figure 2. The mean values of water intake (Figure 2c, 2d) and urine volume (Figure 2e, 2f) compared the control and GAS groups over the time in each sex group. Female rats consumed more water than males after GAS intervention, and produced more urine, fluctuated consistently with the water intake over the time (P<0.05). The mean body weight (Figure 2b) comparison between pre-GAS and post-GAS after 14 days (n=12 animals/sex group) represented an overall change of body status arising from GAS intervention. Male and female rats consumed the same amount of food during dosage of GAS as shown on Figure 2a. However, as illustrated in Figure 2b, the mean value of male body weight was higher in post-GAS ingestion rats than pre-GAS rats but the female body weight maintained a same level in pre- and post-GAS rats.

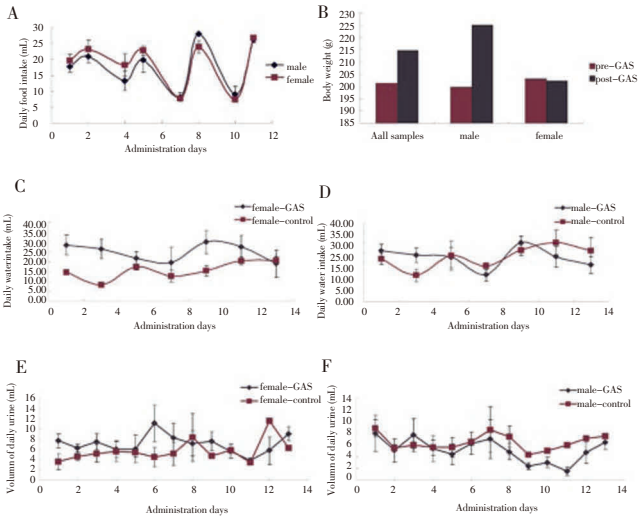


Figure 2. Statistical results of food intake, body weight, water intake and urine volume.

P<0.05 (n=12 animal/sex group).Food intake trends over GAS intervention periods (A), mean body weight comparison from pre-GAS and post-GAS after 14 days in all samples and in individual sex group (B), water intake variation between control and post-GAS intervention of the female and the male rats individually (C, D), urine volume variation between control and post-GAS intervention of the female and the male rats individually (E, F).

3.2. ^{1}H NMR spectral analysis of blood plasma

Figure 3 shows the average normalized 500-MHz ^{1}H NMR spectra of plasma samples from both female and male control rats (Figure 3a, 3c), and both female and male post-GAS rats (Figure 3b, 3d). The assignment of metabolites (Figure 3, Table 1) was made by searching public metabolomics databases, such as HMDB (http://www.hmdb.ca), METLIN (http://metlin.scripps.edu) and KEGG (http://www.kegg.jp) and by comparison with reference[23]. Direct inspection of the ^{1}H NMR spectra of plasma samples could hardly tell the metabolic changes related to GAS intervention, which might suggest the mild action of GAS. To understand and explain

the health benefits and the underlying mechanism of GAS, chemometric analysis was performed to help interpret and detect latent variables associated with GAS intervention.

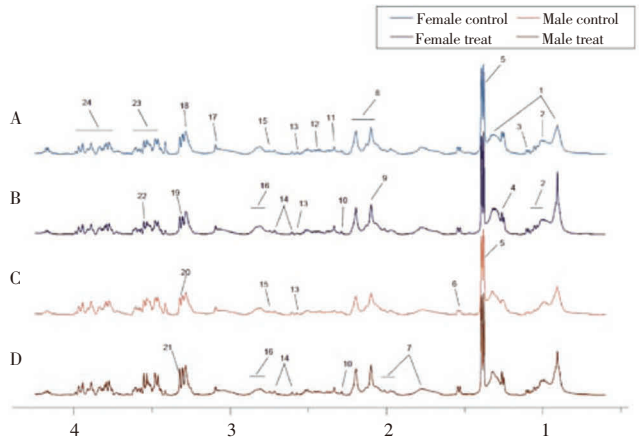


Figure 3. Average normalized 500-MHz high-resolution ^{1}H CPMG NMR spectra of plasma from control female rats.
(A), post-GAS female rats (B), control male rats (C), post-GAS male rats (D). Identified metabolites: 1. LDL/VLDL, 2. leucine/isoleucine, 3. valine, 4. β-hydroxybutyrate, 5. lactate, 6.alanine, 7. arginine/ornithine, 8.glycoprotein, 9. glutamate/glutamine, 10. acetoacetate, 11. pyruvate, 12. succinate, 13. α-ketoglutarate, 14. citrate, 15. dimethylamine, 16. polyunsaturated fatty acid (PUFA), 17. creatine, 18. choline/phosphocholine, 19. glycerophosphocholine (GPC), 20. trimethylamine oxide (TMAO), 21. betaine, 22. glycine, 23. glycerol,. glucose.

Table 1
Assignment of 24 metabolites.

Number	Metabolites	δ ^{1}H region
1	LDL/VLDL	0.85-0.95(m), 1.25-1.32(m)
2	Leucine/Isoleucine	0.97(t), 1.00(t)
3	Valine	1.07(d), 1.12(d)
4	β-hydroxybutyrate	1.27(d)
5	Lactate	1.38(d)
6	Alanine	1.54(d)
7	Arginine/Ornithine	1.67-1.71(m), 1.80-1.83(m), 1.95-2.00(m)
8	Glycoprotein	2.10(br s), 2.20(br s)
9	Glutamate/Glutamine	2.08-2.10(m)
10	Acetoacetate	2.27(s)
11	Pyruvate	2.38(s)
12	Succinate	2.45(s)
13	α-ketoglutarate	2.55(s)
14	Citrate	2.61(AB), 2.72(AB)
15	Dimethylamine	2.74(s)
16	PUFA	2.80-2.85(m)
17	Creatine	3.01(s), 3.10(s)
18	Choline/Phosphocholine	3.26-3.31(s)
19	GPC	3.31-3.33(s)
20	TMAO	3.30(s)
21	Betaine	3.34(s)
22	Glycine	3.60(s)
23	Glycerol	3.56(m), 3.79(m)
24	Glucose	3.75-4.00(m)

3.3. Chemometric analysis of the ^{1}H NMR data set

Initial OPLS-DA of all samples indicated the clustering samples were dominated by intersubject variation including innate gender-specific distinction[24] which obscured all separation owing to GAS intervention. However, when data sets were calculated for respective sex groups, a difference between the control and post-GAS ingestion plasma profiles was evident. In the OPLS-DA score plots (Figure 4a, 4d), each point manifests a sample and each clustering represents a corresponding metabolic pattern in different groups. Separation between the control and post-GAS groups of the female was more obvious than that of the male, with R^2Y at 0.912 and Q^2 at 0.734. The S-plots (Figure 4b, 4e), considering both the covariance p (1) and correlation p (corr) loading profiles resulting from the OPLS-DA model, illustrate the variable influence in the model (P<0.05), thus filtering interesting metabolites in the projection. These data were first tested for the normality of the distribution, and then parametric (Student's t test) or nonparametric (Mann-Whitney test) tests were performed to detect statistically significant metabolites that increased or decreased between groups. The significant metabolites increased in GAS groups were in the lower-left quadrant and the decreased ones were in the upper-right quadrant. The loading plots (Figure 4c, 4f) colored according to the correlation coefficients (|r|) of each variable to class separation (red means a large value; blue a lesser value) provide additional information on the physicochemical variations between groups. Negative regions in the loading plot corresponded to metabolites that increased in plasma of post-GAS rats; conversely, positive regions corresponded to metabolites that decreased in plasma of post-GAS rats. From the OPLS-DA S-plots and color-coded loading plots, compared with the control, post-GAS female samples indicated a decrease in low-density-lipoprotein (LDL) / very-low-density-lipoprotein (VLDL), β-hydroxybutyrate, lactate, glycoprotein, choline and an increase in valine, arginine/ornithine, α-ketoglutarate, creatine, glycine, glycerol and glucose; and post-GAS male samples showed a decrease in β-hydroxybutyrate, arginine/ornithine, glycoprotein, glutamate, pyruvate, succinate, creatine, choline, glycerol and an increase in LDL/VLDL, lactate and PUFA. Metabolic changes were not totally identical in female and male rats. Some metabolic changes arising from gastrodin intervention in female and male rats were opposite which included LDL/VLDL, lactate, arginine/ornithine, creatine, and glycerol-LDL/VLDL and lactate were on the decrease in the female but on the increase in the male, together with arginine/ornithine, creatine, and glycerol which were on the increase in the

female but on the decrease in the male, while the decrease in pyruvate, succinate and glutamate was only shown in the male and the increase in valine, α-ketoglutarate, glycine and glucose was only shown in the female (Figure 4 & 5).

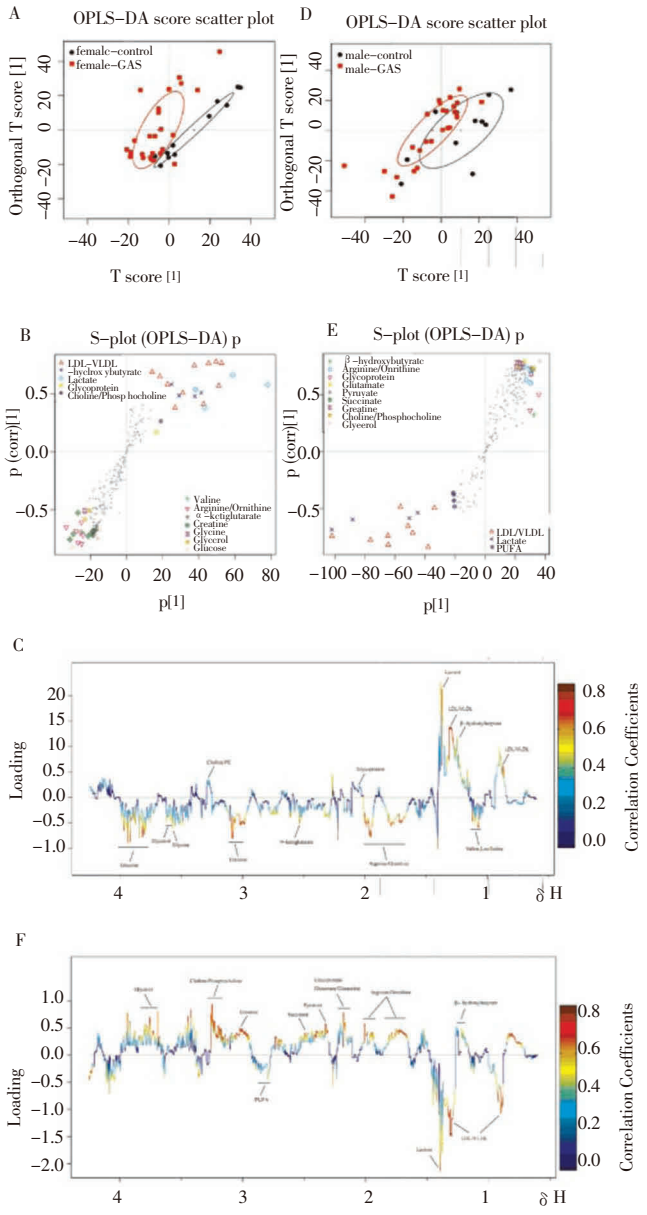


Figure 4. OPLS-DA analysis of 1H NMR plasma data.
Control (blank filled circle) and post-GAS groups (red filled square) calculated for individual sex (ABC for female, DEF for male), score plot of female rats (A), s-plot of female rats (B), color-coded loading plot of female rats (C), score plot of male rats (D), s-plot of male rats (E), color-coded loading plot of male rats (F).

3.4. Metabolic profile changes relating to GAS intervention

It can be seen in Figure 4a and Figure 4d that the metabolic state of post-GAS groups of both female and male rats moved away from control groups to a certain extent, but the degree of group shift was observed to be mild, which indicates GAS is relatively safe for normal rats without eliciting severe metabolic perturbation. The subtle changes in metabolic profiles relating to GAS interventions were highlighted by OPLS-DA s-plots and loading plots. It is the first study that metabolomics approach was applied to determine the health benefit of GAS and its sexual specific action on metabolomics profiles (Figure 5).

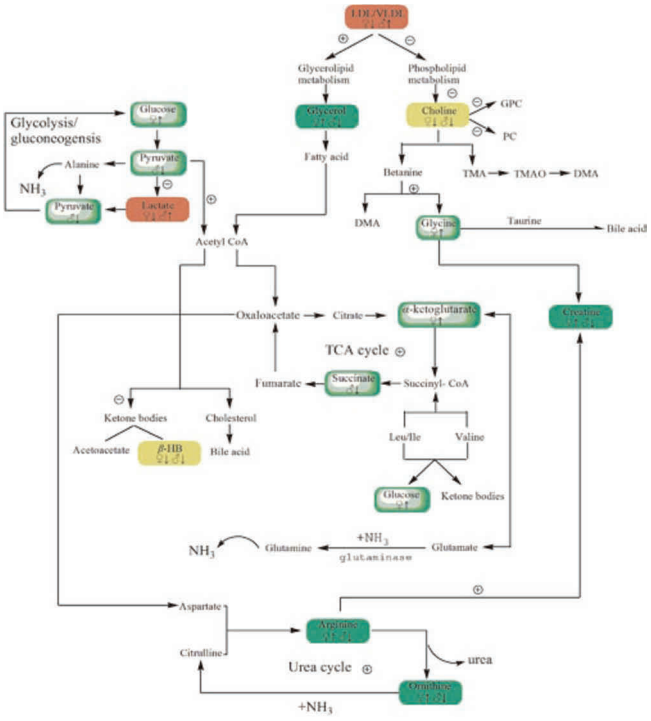


Figure 5. Biochemical pathways illustrating the spectral changes after GAS intervention highlighted by chemometric analysis.
Red rounded rectangle (solid): decrease in female and increase in male after GAS intervention (♀ ↓ and ♂ ↑); Green rounded rectangle (solid): increase in female and decease in male after GAS intervention (♀ ↑ and ♂ ↓); Yellow rounded rectangle (solid): decrease in both female and male after GAS intervention (♀ ↓ and ♂ ↓); Green rounded rectangle (shaded): only increase in female or decease in male after GAS intervention (♀ ↑ or ♂ ↓). The function of GAS might be more beneficial in female than in male rats, processes that may be inhibited in the female by GAS consumption, processes that may be promoted in the female by GAS consumption.

4. Discussion

The alterations in carbohydrate metabolism could be reflected through the changes in the plasma LDL/VLDL, choline and β-hydroxybutyrate levels. LDL and VLDL are associated with cardiovascular disease (CVD)[25].

Accumulation of LDL/VLDL in plasma leads to a high risk of CVD. The decreased level of LDL in female rats after GAS intervention is conducive to preventing the proliferation of smooth muscle cell which is a key step in the pathogenesis of atherosclerosis. GAS was reported to inhibit cell proliferation in vascular smooth muscle cells[26]. Choline can be synthesized from the one degradation pathway of LDL/VLDL and be degraded in body to creatine via betaine and glycine or to methylamines (*eg.*, trimethylamine TMA, trimethylamine oxide TMAO, and dimethylamine DMA) by gut microbiota[27]. GPC, together with phosphocholine, is the major form of choline storage in cytosol[28]. Glycerol is a catabolite through the other degradation pathway of LDL/VLDL via glycerolipid metabolism. The increased level of glycerol and the decreased level of choline and concomitant increase in glycine and creatine substantiate that the degradation of LDL/VLDL is facilitated in female rats and the pathway concerning the degradation of choline to creatine is emphasized in the female. Fatty acid is a catabolism product of LDL/VLDL via glycerolipid metabolism. β-hydroxybutyrate is generated when acetyl-CoA produced from β-oxidation of fatty acid. The decrease in β-hydroxybutyrate of the female resulting from the reduction in acetyl-CoA conversion to ketone body suggests that fatty acid consumption exceeds generation, that is to say, the enhanced-consumption of fatty acid is achieved in the female by means of more acetyl-CoA entering the tricarboxylic acid (TCA) cycle. In addition, the increased female level of glycerol substantiates again an enhanced lipid metabolism, which is consistent with the above viewpoints. However, the above-mentioned results may be confined to the female, the male rats showed the increase in average body weight and LDL/VLDL level, and the glycerol level was on the decrease, these were different compared with the female. Weather GAS is a healthy dietary supplement for the male merits further investigation.

Energy metabolism was observed from the gluconeogenesis precursors (*eg.*, alanine, lactate, and pyruvate) and the TCA cycle intermediates including succinate, α-ketoglutarate and citrate. There also existed the gender variation in energy metabolism. Glucose oxidation is the main way of supplying energy under a well-functioning TCA cycle, known as aerobic metabolism. Glucose catabolism and anabolism are also highly associated with the glucose-alanine and Cori (glucose-lactate) cycles between peripheral tissues and the liver. Both lactate and alanine are formed peripherally by glucose-derived pyruvate which is transported to liver, where it is finally reconverted to glucose. The male rats with an augmented lactate level were converse to the female with a decrease. This result in the male may indicate the activation of glycolysis exceeds the oxidative phosphorylation, *i.e.* extreme exercise, hypoxia, or from muscle activity generally under conditions where energy demand exceeds energy supply. Under normal biochemical circumstances, lactate is shuttled into gluconeogenesis and thus is not maintained at a high level in plasma. The augmented lactate level of the male rats may suggest the inhibition of gluconeogenesis, and the decrease in glycoprotein may suggest an affected protein turnover by GAS. In contrast, the decrease in lactate and slight increase in glucose of the female rats indicate a suppression of anaerobic metabolism. Additionally, subtle enhancement of TCA cycle intermediate α-ketoglutarate in the female agree with that GAS may facilitate mitochondrial aerobic metabolism, which, however, holds not true for the male with the increase in lactate and decrease in succinate which is also a TCA cycle intermediate.

Another marked variation is creatine level. Endogenous creatine is synthesized from amino acids arginine, glycine and methionine in kidney, liver, pancreas, and possibly brain, and transfers from blood plasma by specific sodium and chloride-dependent creatine transporters located in skeletal muscle, kidney, heart, brain and liver[29,30]. Generally, the primary physiological function of creatine is to buffer energy supply in tissues, especially in muscles and the brain[31]. The majority of ATP synthesis occurs during aerobic cellular respiration, beginning with glycolysis and ending with the coupling of adenosine diphosphate (ADP) and phosphate group via oxidative phosphorylation. However, these complex, multi-step metabolic pathways require time and energy. In brain and other tissues where there are sudden and significant energy demands, creatine kinase isoenzyme catalyzes the phosphorylation of creatine to form pools of phosphocreatine, serving as energy reserves. When energy supply is insufficient, creatine kinase isoenzyme catalyzes the transfer of the phosphate group from phosphocreatine to ADP to synthesize ATP. Thus, creatine is the key metabolite in the rapid synthesis of ATP when energy demand increases. Bearing this in mind, the creatine-creatine kinase-phosphocreatine circuit may be thought as a bioenergetic thermostat that quickly replenishes ATP in tissue to maintain stable levels when there are sudden and significant energy demands[31]. The favorable effects of creatine supplementation on energy metabolism increase the plausibility of creatine having

a positive effect on brain cognitive processes and mood states. Converging evidences by Allen attached importance of endogenous creatine for normal brain development and cognitive function[32]. The expression of the majority of creatine kinase isoenzyme in the hippocampus and frontal cortex provided a clue that creatine metabolism participates in higher mental functioning[33]. There are numerous reports showing that GAS may improve learning and facilitate memory consolidation and retrieval[34,35]. Furthermore, we firstly found that creatine might be the endogenous metabolite that was responsible for neuroprotective activity of GAS. Interestingly, in this research, GAS could only facilitate the female synthesis of creatine with the increased arginine, glycine and creatine levels but in male rats which showed the decreased level of arginine and creatine.

In conclusion, this research showed the sex-specific response to GAS intervention for some metabolites which included LDL/VLDL, lactate, arginine/ornithine, creatine, glycerol, pyruvate, succinate, glutamate, valine, α-ketoglutarate, glycine, and glucose. The function of GAS might be more beneficial in female than in male rats. Further studies should be carried out to clarify the mechanism underlying the above phenomena.

Conflict of interest statement

We declare that we have no conflict of interest.

Acknowledgments

This research work was funded by the National Science Foundation of China (81173526), the Program for New Century Excellent Talents in University, State Education Ministry of China (#NCET-11-0738), the Fundamental Research Funds for the Central Universities (No.30920130112014), the Program for Changjiang Scholars and Innovative Research Team in University (PCSIRT-IRT1193) and the Priority Academic Program Development of Jiangsu Higher Education Institutions (PAPD).

References

[1] Hsieh CL, Chiang SY, Cheng KS, Lin YH, Tang NY, Lee CJ, et al. Anticonvulsive and free radical scavenging activities of *Gastrodia elata* Bl. in kainic acid-treated rats. *Am J Chin Med* 2001; **29**(2): 331-341.

[2] Zeng X, Zhang Y, Zhang S, Zheng X. A microdialysis study of effects of gastrodin on neurochemical changes in the ischemic/reperfused rat cerebral hippocampus. *Biol Pharm Bull* 2007; **30**(4): 801-804.

[3] Xu X, Lu Y, Bie X. Protective effects of gastrodin on hypoxia-induced toxicity in primary cultures of rat cortical neurons, *Planta Med* 2007; **73**(7): 650-654.

[4] Wang Q, Chen G, Zeng S. Pharmacokinetics of Gastrodin in rat plasma and CSF after i.n. and i.v.. *Int J Pharm* 2007; **341**(1): 20-25.

[5] Zhang Q, Yang YM, Yu GY. Effects of gastrodin injection on blood pressure and vasoactive substances in treatment of old patients with refractory hypertension: a randomized controlled trial. *Chin J Integr Med* 2008; **6**(7): 695-699.

[6] Nicholson JK, Wilson ID. Understanding "global" systems biology: metabonomics and the continuum of metabolism. *Nat Rev Drug Discov* 2003; **2**(8): 668-676.

[7] Goodacre R, Vaidyanathan S, Dunn WB, Harrigan GG, Kell DB. Metabolomics by numbers: acquiring and understanding global metabolite data. *Trends Biotechnol* 2004; **22**(5): 245-252.

[8] Nicholson JK, Connelly J, Lindon JC, Holmes E. Metabonomics: a platform for studying drug toxicity and gene function. *Nat Rev Drug Discov* 2002; **1**(2): 153-161.

[9] Viant MR, Rosenblum ES, Tjeerdema RS. NMR-based metabolomics: a powerful approach for characterizing the effects of environmental stressors on organism health. *Environ Sci Technol* 2003; **37**(21): 4982-4989.

[10] Song X, Wang J, Wang P, Tian N, Yang M, Kong L. ^{1}H NMR-based metabolomics approach to evaluate the effect of Xue-Fu-Zhu-Yu decoction on hyperlipidemia rats induced by high-fat diet, *J Pharm Biomed Anal* 2013; **78**(79): 202-210.

[11] Allen PJ, D'Anci KE, Kanarek RB, Renshaw PF. Chronic creatine supplementation alters depression-like behavior in rodents in a sex-dependent manner. *Neuropsychopharmacology* 2010; **35**(2): 534.

[12] Hamakawa H, Kato T, Shioiri T, Inubushi T, Kato N. Quantitative proton magnetic resonance spectroscopy of the bilateral frontal lobes in patients with bipolar disorder. *Psychol Med* 1999; **29**(3): 639-644.

[13] Kato T, Takahashi S, Shioiri T, Inubushi T. Brain phosphorous metabolism in depressive disorders detected by phosphorus-31 magnetic resonance spectroscopy. *J Affect Disord* 1992; **26**(4): 223-230.

[14] Kato T, Takahashi S, Shioiri T, Murashita J, Hamakawa H, Inubushi T. Reduction of brain phosphocreatine in bipolar II disorder detected by phosphorus-31 magnetic resonance spectroscopy. *J Affect Disord* 1994; **31**(2): 125-133.

[15] Nery FG, Stanley JA, Chen HH, Hatch JP, Nicoletti MA, Monkul

ES, et al. Normal metabolite levels in the left dorsolateral prefrontal cortex of unmedicated major depressive disorder patients: A single voxel ^{1}H spectroscopy study. P*sychiatry Res: Neuroimaging* 2009; **174**(3): 177-183.

[16]Moore CM, Christensen JD, Lafer B, Fava M, Renshaw PF. Lower levels of nucleoside triphosphate in the basal ganglia of depressed subjects: a phosphorous-31 magnetic resonance spectroscopy study. *Am J Psychiatry* 1997; **154**(1): 116-118.

[17]Volz HP, Hubner G, Rzanny R, Rossger G, Preussler B, Eichhorn M, et al. High-energy phosphates in the frontal lobe correlate with Wisconsin Card Sort Test performance in controls, not in schizophrenics: a ^{31}phosphorus magnetic resonance spectroscopic and neuropsychological investigation. *Schizophr Res* 1998; **31**(1): 37-47.

[18]Volz HP, Rzanny R, Riehemann S, May S, Hegewald H, Preussler B, et al. ^{31}P magnetic resonance spectroscopy in the frontal lobe of major depressed patients. *Eur Arch Psychiatry Clin Neurosci* 1998; **248**(6): 289-295.

[19]Ju XH, Shi Y, Liu N, Guo DM, Cui X. Determination and pharmacokinetics of gastrodin in human plasma by HPLC coupled with photodiode array detector. *J Chromatogr B Analyt Technol Biomed Life Sci* 2010; **878**(22): 1982-1986.

[20]Tian N, Wang J, Wang P, Song X, Yang M, Kong L. NMR-based metabonomic study of Chinese medicine Gegen Qinlian Decoction as an effective treatment for type 2 diabetes in rats. *Metabolomics* 2013; **9**(6): 1228-1242.

[21]Dieterle F, Ross A, Schlotterbeck G, Senn H. Probabilistic quotient normalization as robust method to account for dilution of complex biological mixtures application in ^{1}H NMR metabonomics. *Anal Chem* 2006; **78**(13): 4281-4290.

[22]Bro R, Kjeldahl K, Smilde AK, Kiers H. Cross-validation of component models: A critical look at current methods. *Anal Bioanal Chem* 2008; **390**(5): 1241-1251.

[23]Lindon JC, Nicholson JK, Everett JR. NMR spectroscopy of biofluids. *Ann Rep NMR Spectrosc* 1999; **38**: 1-88.

[24]Kochhar S, Jacobs DM, Ramadan Z, Berruex F, Fuerholz A, Fay LB. Probing gender-specific metabolism differences in humans by nuclear magnetic resonance-based metabonomics. *Anal Biochem* 2006; **352**(2): 274-281.

[25]Campos H, Walsh BW, Judge H, Sacks FM. Effect of estrogen on very low density lipoprotein and low density lipoprotein subclass metabolism in postmenopausal women. *J Clin Endocrinol Metab* 1997; **82**(12): 3955-3963.

[26]Zhu L, Guan H, Cui C, Tian S, Yang D, Wang X, et al. Gastrodin inhibits cell proliferation in vascular smooth muscle cells and attenuates neointima formation *in vivo*. *Int J Mol Med* 2012; **30**(5): 1034.

[27]Dumas ME, Barton RH, Toye A, Cloarec O, Blancher C, Rothwell A, et al. Metabolic profiling reveals a contribution of gut microbiota to fatty liver phenotype in insulin-resistant mice. *Proc Natl Acad Sci USA* 2006; **103**(33): 12511-12516.

[28]Tyler-Burt C, Ribolow H. Glycerolphosphorylcholine(GPC) and serine ethanolamine phosphodiester(SEP): evolutionary mirrored metabolites and their potential metabolic roles. *Comp Biochem Physiol Part B: Comp Biochem* 1994; **108**(1): 11-20.

[29]Andres RH, Ducray AD, Schlattner U, Wallimann T, Widmer HR. Functions and effects of creatine in the central nervous system. *Brain Res Bull* 2008; **76**(4): 329-343.

[30]Snow RJ, Murphy RM. Creatine and the creatine transporter: a review. *Mol Cell Biochem* 2001; **224**(1-2): 169-181.

[31]Wyss M, Schulze A. Health implications of creatine: can oral creatine supplementation protect against neurological and atherosclerotic disease? *Neuroscience* 2002; **112**(2): 243-260.

[32]Allen PJ. Creatine metabolism and psychiatric disorders: Does creatine supplementation have therapeutic value? *Neurosci Biobehav Rev* 2012; **36**(5): 1442-1462.

[33]Kaldis P, Hemmer W, Zanolla E, Holtzman D, Wallimann T. "Hot Spots" of Creatine Kinase Localization in Brain: Cerebellum, Hippocampus and Choroid Plexus. *Dev Neurosci* 1996; **18**(5-6): 542-554.

[34]Hsieh MT, Wu CR, Chen CF. Gastrodin and *p*-hydroxybenzyl alcohol facilitate memory consolidation and retrieval, but not acquisition, on the passive avoidance task in rats. *J Ethnopharmacol* 1997; **56**(1): 45-54.

[35]Wu CR, Hsieh MT, Huang SC, Peng WH, Chang YS, Chen CF. Effects of *Gastrodia elata* and its active constituents on scopolamine-induced amnesia in rats. *Planta Med* 1996; **62**(4): 317-321.