Journal of Ethnopharmacology 135 (2011) 270–277

Contents lists available at ScienceDirect

Journal of Ethnopharmacology

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

Comparison of toxic reaction of *Tripterygium wilfordii* multiglycoside in normal and adjuvant arthritic rats

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ARTICLE INFO

Article history:
Received 17 November 2010
Received in revised form 2 March 2011
Accepted 3 March 2011
Available online 9 March 2011

Keywords:
Tripterygium wilfordii multiglycoside
Toxicity
Adjuvant arthritis
Rat

ABSTRACT

Aim of the study: Tripterygium wilfordii multiglycoside (GTW), an authorized Chinese patent drug, is used for treatment of rheumatoid arthritis and other immune disease. This study was to determine whether GTW induced different toxic reactions in adjuvant arthritis rats (AA rats) compared to those in normal rats.

Materials and methods: To prepare arthritic rat model, male Sprague–Dawley (SD) rats were immunized by injecting complete Freund's Adjuvant into right hind footpad. And then, GTW was given to rats intragastrically at dosage of 7 or 105 mg kg^{-1} day^{-1} from day 15 to day 28 after immunization. Routine clinical parameters and histopathologic changes of liver, kidney and testis were examined. Metabolic profiling in serum of groups was analyzed by LC–MS. A principal component analysis (PCA) and partial-least-squares discriminate analysis (PLS-DA) were carried out combined with mass spectrometry (MS) data set. All the quantitative data were performed by two-way ANOVA analysis following Student's *t*-test.

Results and conclusions: Treatment with GTW at both doses could diminish the right and left hind paws swelling. There was slight lipoid degeneration in hepatic tissue of normal rats treated by high dose of GTW, but there were not distinctly pathological changes in hepatic tissue of AA rats treated by GTW. Compared normal rats administered with GTW, no statistically significant difference in the serum alanine aminotransferase (ALT), creatinine (CRE), and blood urea nitrogen (BUN) levels were observed. However, the serum aspartate aminotransferase (AST) level was significant decreased in AA rats under exposure GTW compared with normal rats in the same conditions ($p < 0.05$), which indicated that GTW could offer a different liver toxic reaction in normal and AA rats. The metabolic analysis showed that a clear separation of PCA and PLS-DA score spot in normal rats, but not separation was seen in AA rats perturbed with low dosage GTW. The result indicated low dosage GTW might arouse a general toxic in normal rats but not in AA rats. The biomarker analysis showed that the level of lysophosphatidylcholines (LPCs) was down-regulated, but the level of ursodeoxycholic acid (UDCA) and chenodexycholic acid (CDCA) was up-regulated in AA rats compared with normal rats under exposure GTW. According to pathway analysis of metabolic markers, we conceived that LPC, UDCA and CDCA were the critical intermediates of choline and fatty acid metabolism. And the lipid metabolism was a correlative outcome of GTW induced toxicity in the liver in physiological condition animals. Taken together, GTW could induce different toxic reactions between normal and AA rats, and the lipid metabolism might be part of the mechanism for the hepatic lipidosis or the other liver injury.

Abbreviations: ALT, alanine aminotransferase; AST, aspartate aminotransferase; AA, adjuvant arthritis rat model; BUN, blood urea nitrogen; CFA, complete Freund's adjuvant; CRE, creatinine; CDCA, chenodexycholic acid; GTW, *Tripterygium wilfordii* multiglycoside; LC/MS, high pressure liquid chromatography combined mass spectrometry; LPC, lysophosphatidyl choline; MS, mass spectrometry; PCA, principal component analysis; PLS-DA, partial-least-squares discriminate analysis; RA, rheumatoid arthritis; UDCA, ursodexycholic acid; VIP, variable importance parameters.

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0378-8741/$ – see front matter.
doi:10.1016/j.jep.2011.03.007

1. Introduction

Tripterygium wilfordii multiglycoside (GTW), an authorized Chinese patent drug by State Food and Drug Administration, is the ingredient extracted from the medical plant *Tripterygium wilfordii* hook F. (*Celastraceae*). GTW showed anti-inflammatory and immunosuppressive activities in human clinical trials for rheumatoid arthritis (RA), systemic lupus erythematosus, nephritis, asthma, and ankylosing spondylitis (Tao et al., 2001). In 2000, the U.S. Food and Drug Administration had approved a clinical trial to evaluate the effects of a chloroform/methanol extract of the herb *Tripterygium wilfordii* in the treatment of RA (Pyatt et al., 2000). Based on many clinical and experimental researches (Goldbach-Mansky et al., 2009; Tao et al., 2002), GTW is regarded as a promising drug for the treatment of RA. Despite its potential clinical utility, many side effects, such as gastrointestinal upset, diarrhea, vomit, bellyache, etc. have been reported (Canter et al., 2006; Sun et al., 2001). More seriously, Triptolide, an essential active component of GTW, could induce hepatic injury and increasing of the serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST) if it was taken for a long time or at an over dose (Jones et al., 2010). Furthermore, the antifertility activity of *Tripterygium wilfordii* also suggested the reproductive toxicity (Huynh et al., 2000). Taken into account these drawbacks, it is critical to understand the toxicological property associated with the long-term use of GTW.

However, the research on GTW-induced toxicity was scarce, and the data on toxicity of GTW were mostly from the experimental animals under normal physiological conditions (Chen et al., 2008). Recently, results in a study showed that triptolide could cause a significant difference in the toxicity between different sexes because of the alteration of activity of microsomal enzymes in liver at different sex rats (Liu et al., 2010a,b). Another study exhibited that thyroid metabolism in buffalo changed remarkably in different physiological states (Ambrosio et al., 2009). Thus it is reasonable to hypothesize that GTW might induce different toxic reactions in individuals under healthy state and non-healthy state which could be recovered by application of GTW. In order to address the different profiles in the toxicities of GTW, the normal and adjuvant arthritis rats were used in this study to evaluate the toxicity of GTW in different status by examining blood biochemistry parameters and histological changes of liver, kidney and testis. And then, we adopted LC/MS approach combined with pattern recognition techniques to investigate the differences of metabolic profiling between normal and adjuvant arthritis rats exposed to GTW.

2. Materials and methods

2.1. Drug and reagents

Oral GTW (10 mg/tablet, Lot No. Z32021007) was purchased from Jiang Su Mei Tong Pharmaceutical Co. Ltd (GMP certificated), China. All aqueous solution was prepared using purified distilled water from a Milli-Q system (Milli-Q Adventage, Millipore MA). HPLC grade acetonitrile and formic acid was purchased from Merck (USA). Formic acid, extra pure grade (98–100%) was purchased from Fluka (WI, USA). Leucine–enkephalin, complete Freund's adjuvant (CFA) and other materials were purchased from Sigma–Aldrich (USA).

2.2. Animal handling procedure

Male Sprague–Dawley (SD) rats (160 ± 20 g) were purchased from Institute of Experimental Animals in Chinese Academy of Medical Science (the rodent license No.: SYXK 11-00-0039). They were housed under standard laboratory conditions. Food and tap water were provided ad libitum. Half of rats were randomly chosen for adjuvant arthritis (AA) model. The induction and assessment of AA were performed as our previously described (He et al., 2006). Briefly, SD rats were injected with 0.1 ml CFA in right hind footpad. 7 days after the immunization, the degree of arthritis was examined every 2 days. 14 days after the immunization, the rats were grouped based on the assessment of arthritis index ($n = 10$). With the addition of normal rats, the groups are: (1) normal rats with vehicle treatment as normal control, (2) normal rats with 7 mg/kg GTW treatment (clinical equivalent dosage), (3) normal rats with 105 mg/kg GTW treatment (15 times of clinical equivalent dosage), (4) AA rats with vehicle treatment as model control, (5) AA rats with 7 mg/kg GTW treatment and (6) AA rats with 105 mg/kg GTW treatment. The experimental procedures were reviewed and approved by the Animal Care and Use Committee in China Academy of Chinese Medical Sciences before animal experiments were carried out.

2.3. GTW administration and sample collection and preparation

GTW was orally administered in a volume of 1 mL/100 g body weight from 15 days after immunization. All of the treatment groups were given GTW or equivalent dose vehicle solution (saline). All animals were sacrificed after 14 days treatment by GTW, and then blood samples from each rat were collected and centrifuged at $3000 \times g$ for 15 min at room temperature to obtain serum. A portion of serum was used for routine clinical parameters analysis including alanine aminotransferase (ALT), aspartate aminotransferase (AST), creatinine (CRE), and blood urea nitrogen (BUN). The other portion was used for LC/MS analysis. Liver, kidney and testis tissues were fixed in 10% formalin for histological examination.

2.4. Chromatography

Chromatography was performed on an ultra performance liquid chromatography system (Waters, USA). A 10 μL aliquot of diluted rat plasma was injected onto a 10 cm × 2.1 mm, 1.7 μL BEH C18 column (Waters, USA). The column was maintained at 35 °C. Mobile phases A and B consisted of 0.1% aqueous formic acid and acetonitrile, respectively. The elution followed a linear gradient of 2–100% B in 35 min at a flow rate of 0.3 mL/min. The standard sample ran six ties continuously for the study of the stability of the method. All the samples were kept at 4 °C during the analysis and analyzed once at a random order.

2.5. Mass spectrometry

A Waters Q-TOF micro-MS system (Waters MS Technologies, Manchester, UK) operated in positive ionization modes was used in this study. Tune page was used to regulate the sample cone voltage. Data was collected in full scan mode from 100 to 1000 m/z from 0 to 20 min. Samples were analyzed randomly for unbiased measurement with tune mixture solution ($m/z = 118.09$, 622.05, or 922.02). Argon was employed as the internal standards for quality control. Target MS analysis was used for the identification of the potential biomarkers.

2.6. Metabolomical data analysis

The raw data were analyzed by MarkerLynx (Waters, UK) for peak deconvolution and alignment. The parameters were set as: the mass tolerance was set at 0.5 amu, peak width was set at 5%, baseline noise elimination was level 5, and the mass window was set at 0.5 amu. The data was combined into a single matrix by aligning peaks with the same mass/retention time pair together from each data file in the dataset, along with their associated normalized intensities. Based on SIMCA-P 12.0 (Version 12.0, UMETRICS AB,

Table 1
The effect of GTW on body weight and hepatosomatic index (mean ± S.D.), $n = 10$.

Group	Body weight (g)				Liver/body (%)
	Day 1	Day 15	Day 21	Day 27	
Normal rat + vehicle	184.3 ± 23.6	314.0 ± 15.0	352.5 ± 17.3	378.6 ± 19.5	3.3 ± 0.2
Normal rat + GTW low	193.1 ± 6.8	315.0 ± 15.6	349.5 ± 19.6	371.1 ± 22.9	3.3 ± 0.3
Normal rat + GTW high	185.7 ± 11.6	315.5 ± 13.2	350.4 ± 19.4	375.8 ± 21.9	3.3 ± 0.3
AA rat + vehicle	197.2 ± 5.7	308.5 ± 12.3	349.1 ± 17.9	382.2 ± 24.8	3.0 ± 0.3*
AA rat + GTW low	196.8 ± 8.9	306.8 ± 21.0	339.0 ± 26.2	366.9 ± 30.9	3.0 ± 0.2
AA rat + GTW high	195.5 ± 8.7	311.0 ± 15.9	337.6 ± 21.3	364.5 ± 23.4	3.1 ± 0.3

* $p < 0.05$, compared to normal control group.

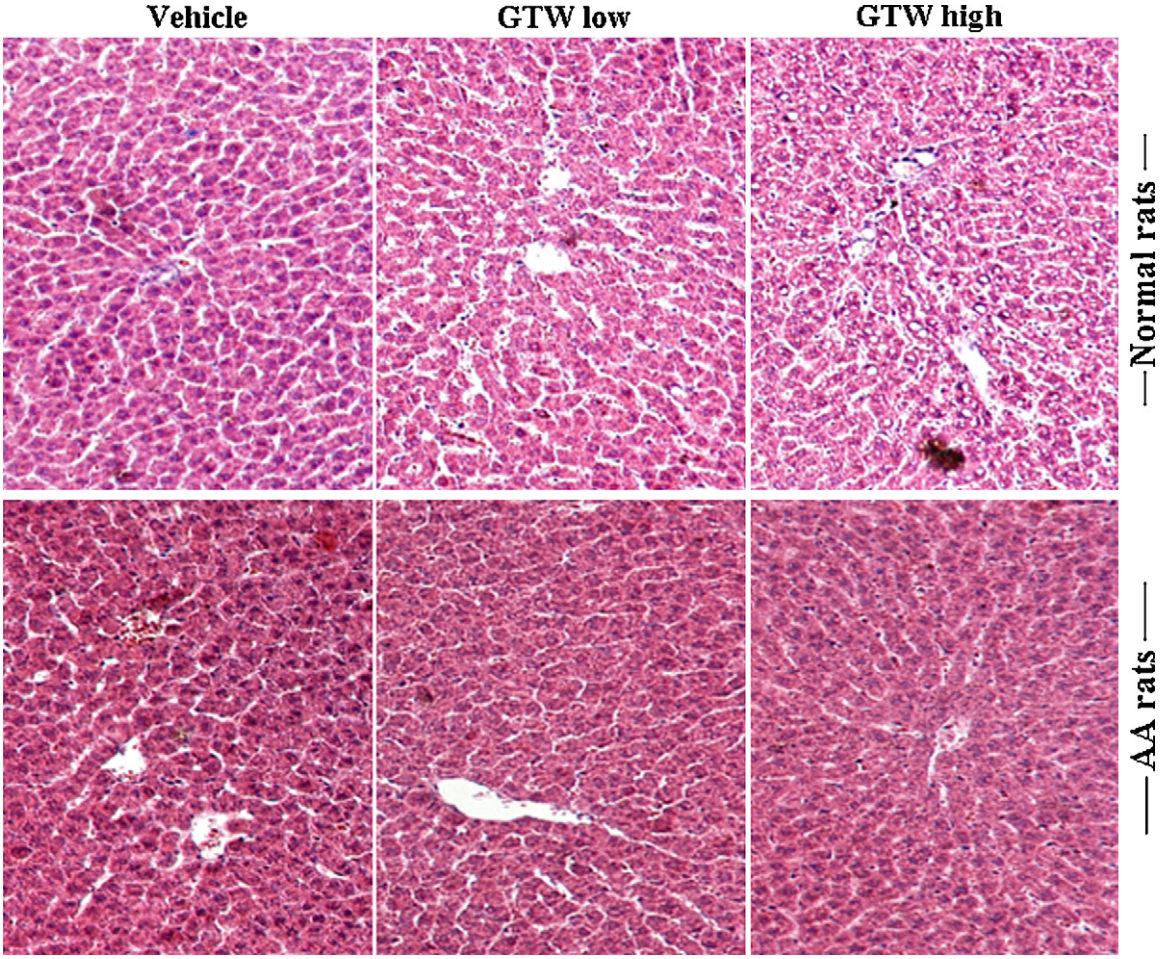

Fig. 1. Paraffin section and H & E staining of liver tissue at the end of the study (100×). A slight lipoid degeneration was detected in hepatic tissue of normal rats treated by high dose of *Tripterygium wilfordii* multiglycoside (GTW), but there were not any pathological changes in hepatic tissue of adjuvant arthritis (AA) rats treated by two doses of GTW.

Box 7960, SE 90719, Umea, Sweden), principal component analysis (PCA) was initially used to visualize general clustering. For further identifying the differentially expressed metabolites accountable for the separation between the groups and dosed, a more sophisticated partial-least-squares discriminate analysis (PLS-DA) was carried out on the combined MS data set. Parameters of PLS-DA such as $R^2\gamma$ and Q^2 were used in the study for the evaluation of the models indicating the goodness of fit and ability of prediction.

2.7. Statistical analysis

On the basis of the threshold of p values and fold change values from the nonparametric, Wilcoxon–Mann–Whitney test was implemented in the SIMCA-P statistical toolbox. Response permutation test was used to assess a model to avoid over-fitting due to chance correlation. All the statistical analyses of quantitative indexes were performed using the SPSS11.5 software package. All results were expressed as mean ± S.D. Significance of the differences between the various groups was evaluated by two-way ANOVA following Student's t-test. The results were considered statistically significant when $p < 0.05$.

3. Results

3.1. General observations

Arthritis was induced reproducibly in control rats given CFA, all the onset and distribution of arthritis were similar to the patterns previously described in our lab (He et al., 2006). Treatment with GTW could diminish the right and left hind paws swelling from day

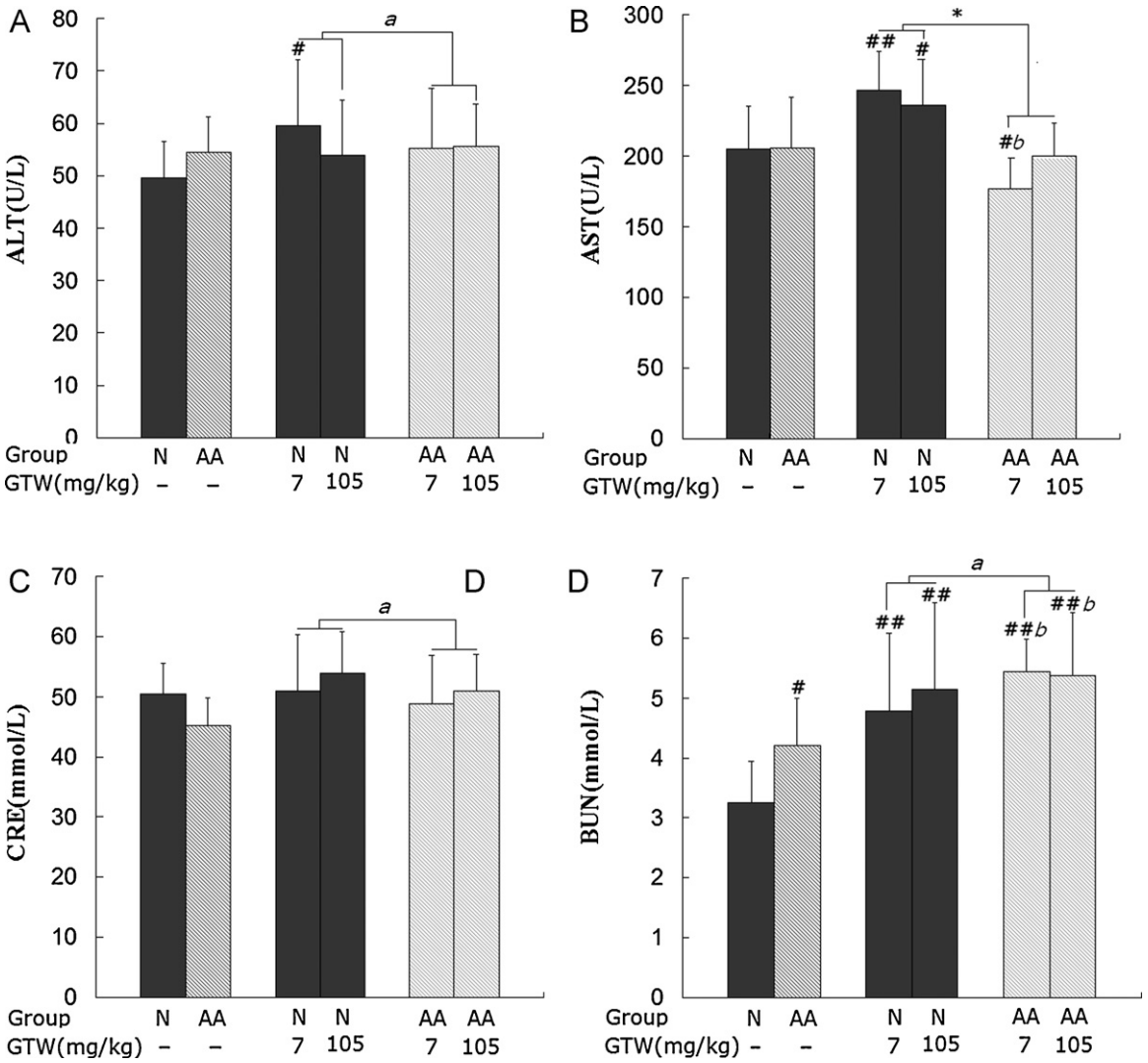

Fig. 2. (A) Serum alanine aminotransferase (ALT) level in different groups. (B) Serum aspartate aminotransferase (AST) level in different groups. (C) Serum creatinine (CRE) level in different groups. (D) Serum blood urea nitrogen (BUN) level in different groups. All of the samples were collected from the rats at the end of the study. $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ vs. the normal control rats and $^{a}p > 0.05$, $^{b}p < 0.05$ vs. the model control rats (one-way ANOVA, followed by Student's *t*-test). $^{*}p < 0.05$ vs. normal rats treated by GTW (two-way ANOVA, followed by Student's *t*-test).

23 to 27 after immunization (data not shown). All animals tolerated the experimental procedures well with no deaths up to the study termination at day 28. Some of rats in normal group under exposure GTW showed dysphoria, piloerection, and sometimes thin feces. No significant differences of body weight were observed among groups in different time points, except that organ index of liver was significantly decreased in AA model group ($p < 0.05$) (Table 1).

After all the rats sacrificed, histopathology of liver, kidney and testis was examined. No significant difference was detected in renal and testicle tissue under two doses of GTW treatments both in normal rats and AA rats (data not shown). Correspondingly, there was slight lipoid degeneration in hepatic tissue of normal rats treated by high dose of GTW, but there were not distinctly pathological changes in hepatic tissue of AA rats treated by two doses of GTW (Fig. 1).

3.2. Biochemical parameter measurements in serum

In this study, serum ALT and AST levels were used to assess liver function. As shown in Fig. 2A, an increasing ALT level was observed in the normal rats following treatment with 7 mg/kg GTW ($p < 0.05$, vs. normal control). However, ALT level was unchanged in the AA rats with or without GTW treatment. Fig. 2B shows that the serum AST level increased significantly in the normal rats treated by GTW at both dosages ($p < 0.05$–0.01, vs. normal control), while a decreased AST level was observed in AA rats treated by 7 mg/kg GTW ($p < 0.05$, vs. normal control or model control). To assess the kidney function, serum CRE and BUN levels were selected. As shown in Fig. 2C, the serum CRE level was similar in different groups. Fig. 2D shows that BUN concentration increased obviously not only in model control group but also in respective groups with GTW treatment at both dosages ($p < 0.05$–0.01, vs. normal control). In addition, compared to model control, the serum BUN level increased continuously in AA rats treated by GTW at both dosages ($p < 0.05$, vs. model control). The results of multivariate analysis showed that except for the serum AST level, no statistically significant difference in the serum ALT, CRE and BUN levels were detected between normal group and AA group under exposure GTW.

3.3. Visual examination of MS spectra and identification of the differential metabolites

In order to understand AA, the abnormal condition of the body, serum metabolic profiling of normal rats and AA rats was performed

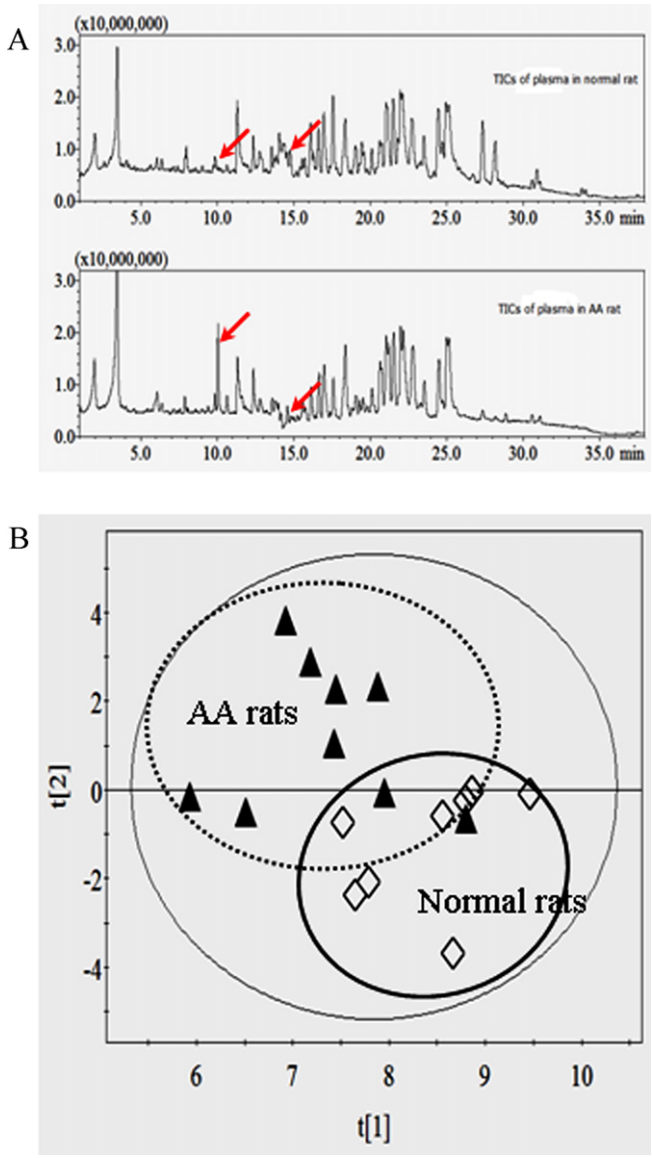

Fig. 3. (A) Typical ion flow charts (TICs) of serum metabolites between normal and AA rats the end of the study. Arrows show the different peaks between normal rats with AA rats. (B) Principal component analysis (PCA) scores (component 1 vs. component 2) of serum metabolites derived from normal and AA rats. A clear separation of the score spot was observed between two groups.

and a typical LC-TOF TIC chromatography was detected. Fig. 3A shows that some of the metabolic changes in AA rats could be found directly in the TIC chromatograms (parts of peaks marked with arrows). Fig. 3B reveals a trend of the metabolic changes between the normal rats and the AA rats using the unsupervised analysis of PCA.

Based on above experimental results, we examined the metabolic changes of serum in normal rats and AA rats under GTW exposure. In low dose GTW treatment group, a clear separation of score spot was shown in normal rats, but not separation was seen in AA rats (Fig. 4A), suggests that GTW could offer a different toxic reactions in normal and adjuvant induced arthritic rats with low dosages. By contrast, there was a clear separation of the score spot from all the rats treated with high dose of GTW respectively (Fig. 4B). Furthermore, the separation between low dose and high dose of GTW could be seen both in normal and AA rats (Fig. 5), which indicated GTW possessed obvious dosage dependence.

Using LC/MS analytical protocols coupled with a software-based peak deconvolution procedure, a total of 14 individual metabolites were detected in at least 85% of the serum samples. Compound identification was performed with the commercially available authentic standards. Based on this approach, these compounds were lysophosphatidylcholines (LPC C14:0, C16:0, C16:1, C18:0, C18:1, C18:2, C20:4), ursodexycholic acid (UDCA), chenodexycholic acid (CDCA), and 5 amino acid fragment. Among these metabolites, 4 biomarkers were remarkably changed. As shown in Fig. 6A, the variable importance parameter (VIP) value was decreased remarkably in model control group and AA rats with 7 mg/kg GTW treatment group ($p < 0.05$, vs. normal control). However, the VIP value of LPC C16:0 was increased largely in normal rats treated by 105 mg/kg GTW ($p < 0.05$, vs. normal control). Fig. 6B shows that LPC C18:1 was increased significantly in model control rats, normal rats treated by both dose of GTW, and AA rats treated by 105 mg/kg GTW group ($p < 0.05$–0.01, vs. normal control). In addition, compared to model control, a statistically significant increase of LPC C18:1 was observed in AA rats treated by 105 mg/kg GTW ($p < 0.05$). As shown in Fig. 6C and D, the VIP value of UDCA and CDCA were all decreased in normal rats treated by GTW at both dosages and in AA rats treated by GTW at 105 mg/kg dosage ($p < 0.05$–0.01, vs. normal control). Compared to model control, the VIP value both in UDCA and CDCA were increased obviously in AA rats treated by 7 mg/kg GTW ($p < 0.05$–0.01). The results of multivariate analysis showed that the level of LPC C16:0 and LPC C18:1 was down-regulated, while the level of UDCA and CDCA was up-regulated in AA rats compared with normal rats under exposure GTW ($p < 0.05$, Fig. 6).

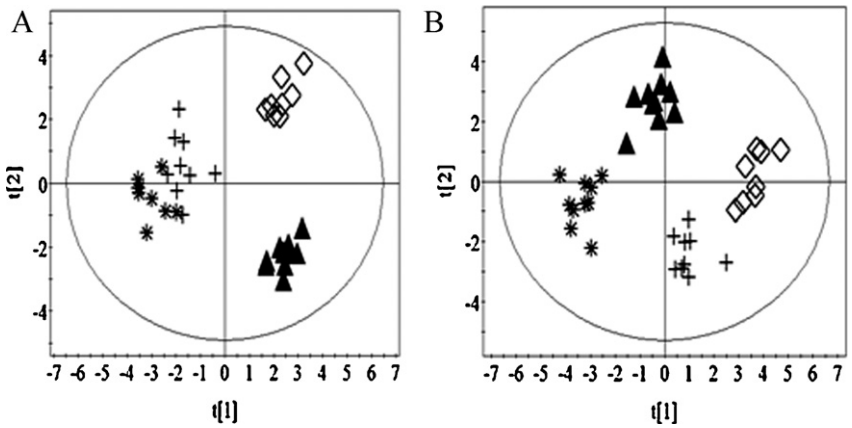

Fig. 4. (A) OSC-PLS-DA scores of serum metabolites impacted by different groups in low dosage of GTW (7 mg/kg). (B) OSC-PLS-DA scores of serum metabolites impacted by different groups in high dosage of GTW (105 mg/kg). ◊ Normal rat treated by vehicle, ▲ normal rats treated by GTW, + AA rats treated by vehicle and * AA rats treated by GTW. Under exposure low dosage of GTW, a clear separation of score spot can be observed in normal rats, but unclear separation in AA rats. In high dosage of GTW treatment, a clear separation of score spot can be observed both in AA rats and normal rats.

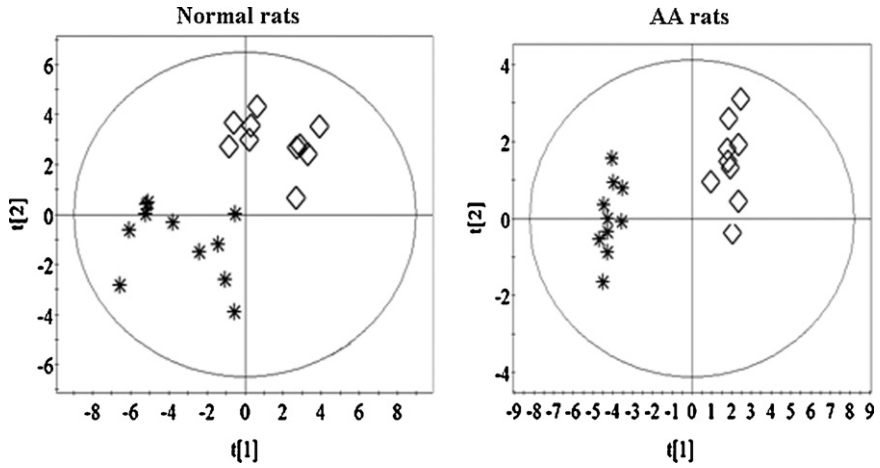

Fig. 5. (A) OSC-PLS-DA scores impacted by different doses of GTW in normal rats. (B) OSC-PLS-DA scores impacted by different doses of GTW in AA rats. ◊ Low dose of GTW (7 mg/kg) and * high dose of GTW (105 mg/kg). A clear separation between low dose and high dose GTW could be detected both in AA and normal rats.

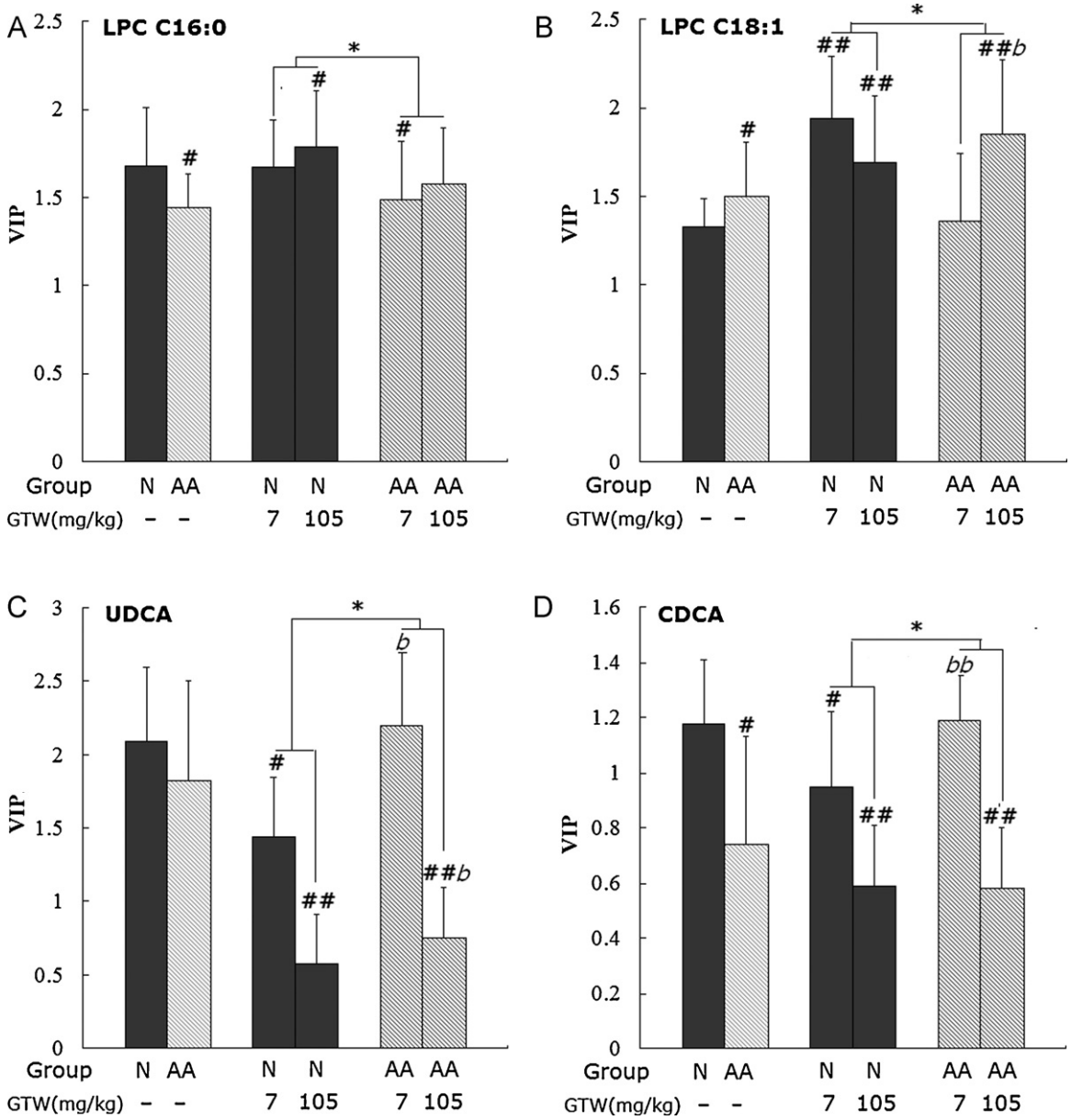

Fig. 6. (A) The value of variable important parameter (VIP) of lysophosphatidyl choline (LPC) C16:0 in different groups. (B) The value of VIP of LPC C18:1 in different groups. (C) The value of VIP of ursodexycholic acid (UDCA) in different groups. (D) The value of VIP of chenodexycholic acid (CDCA) in different groups. $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ vs. the normal control group and $^{b}p < 0.05$ vs. the model control rats. (one-way ANOVA, followed by Student's *t*-test). $^{*}p < 0.05$, $^{**}p < 0.01$ vs. the normal rats treated by GTW (two-way ANOVA, followed by Student's *t*-test).

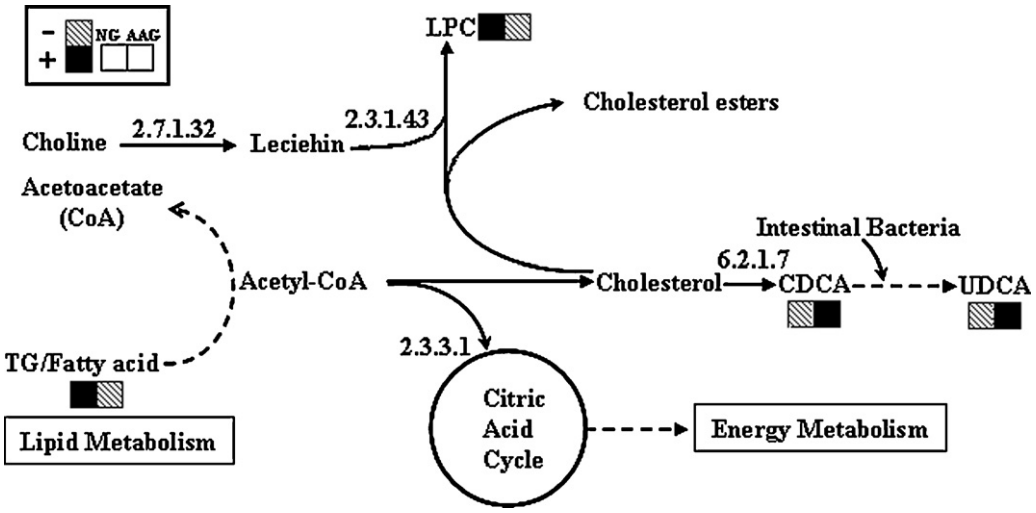

Fig. 7. Perturbed metabolic regulatory network in response to GTW exposure. The black square denotes an elevated plasma level of metabolites in AA rats (■), the gray with twill square means a reduced level of metabolites in this group as compared to that in the normal group (▨). The solid line represents the direct reaction; the dashed line indicates the omission of several intermediate processes.

4. Discussion

Recent studies have revealed some several serious side effects of herbs including *Tripterygium wilfordii* (Liu et al., 2010a,b). Some further studies on the toxicities of the extracts of *Tripterygium wilfordii* have been reported, but most of them were tested on normal animals (Chen et al., 2007; Ni et al., 2008; Zheng et al., 2008). However *Tripterygium wilfordii* was widely used for the treatment of autoimmune diseases, such as RA in clinic. And then, only testing the toxicity of GTW in normal animals is not proper to show the reality in its toxicity. In this study, we reported that oral administration of GTW to normal rats lead to the higher level of ALT, AST, and BUN in serum, which indicated liver and kidney function abnormalities. And some hepatic histopathology changes could be detected when high dose of GTW (15 times of clinical application dosage) was used to treat normal rats. However, a novel phenomenon was detected that GTW offered a very slightly influence to biochemical parameters of serum and histopathological changes in AA rats.

To our knowledge, toxicities induced by GTW are unpredictable and poorly understood, presumably not only due to the multiple targeting sites involved in its in vivo toxic reactions but also the complexity of multiple chemical ingredients, such as diterpenoids, triterpenoids, sesquiterpenoids, dulcitol, glycosides and so on. Hence, the conventional studies on GTW toxicity have faced many difficulties. Fortunately, metabolic profiling strategy enables us to identify the varying metabolites in the complex regulatory network by monitoring many endogenous low-molecular-weight metabolites using liquid chromatography/mass spectrometry (LC/MS) in combination with multivariate statistical techniques and pattern recognition techniques, e.g. PCA and PLS-DA (Nicholson, 2006). Metabolic profiling methods offer the opportunity to identify biomarkers or patterns of biomarker changes related to drug toxicity in blood or urine samples. Based upon the toxicity, the patterns of metabolic biomarkers are expected to be different in organs, especially the organ that is targeted by a particular drug compound (Nicholson et al., 2007). Recently, LC/MS approach has begun to be used for metabonomic analysis to provide crucial information on TCM toxicity (Li et al., 2008). To be emphasized, more and more herbs related potential biomarkers have been identified in recent researches (Van Wietmarschen et al., 2009; Yin et al., 2008). Limited by background noise, analytical techniques and metabolic data library, only 3 kinds of metabolites (LPC, UDCA, and CDCA) were confirmed in this study. As well known, LPC should be constituted less than 2% of the total phospholipids in the membranes of tissues. Since it has cytolytic and membrane perturbing properties, the level of this must be strictly controlled. Researchers have demonstrated the fact that LPC increases under reactive oxygen species (ROS) and inflammatory conditions, such as patients suffering from RA, lung infection, atherosclerosis, diabetes, and kinds of liver injury etc. (Matsumoto et al., 2007; Schober et al., 2009). CDCA, the primary bile acids, which is produced by the liver, can be transformed into UDCA by the intestinal bacteria. UDCA helps cholesterol regulation by reducing the rate at which the intestine absorbs cholesterol molecules while breaking up micelles containing cholesterol. Our initial results indicated that the metabolic profiles were different remarkably between normal physiological states and arthritic pathological states when perturbed by GTW in the same conditions. In brief, the reduced level of LPC followed with raised levels of UDCA and CDCA in AA rats were observed in metabolites of serum compared to those in the normal rats in response to GTW exposure. According to documented data and biochemical databases (e.g. KEGG and METLIN), we demonstrated that LPC, UDCA and CDCA were critical intermediates of choline and fatty acid metabolism. As shown in Fig. 7, in response to GTW exposure, the raised level of LPC was observed in serum in the rats with physiological state. This compound was the critical end product of choline and leciehin metabolism. With the raised LPC, the level of choline might be upregulated yet. Choline, product of lipid catabolism mediated by phosphopase, plays an important role in protecting the liver from lipidosis. On the other hand, the reduced UDCA and CDCA could directly mediate cholesterol metabolism, and indirectly reduce acetyl-CoA consumption. We thus conceived that the alteration of TG or fatty acid metabolism was a correlative outcome of GTW induced toxicity in the liver in physiological condition animals. Further more, it offered a conceivable mechanism consisted with the hepatic lipidosis observed in normal rats treated by GTW in this study. Taken together the biochemical and histopathological data, we demonstrated a conclusion that GTW may induce lower toxic reactions in AA rats compared to normal rats by regulation of lipid metabolism.

In summary, the occurrence of different toxic reactions induced by GTW in normal or AA rats was highly associated with lipid metabolites. These findings demonstrated that the herbal safety evaluation could be tested not only in healthy subjects but also in the state which could be regarded as the therapeutic indication for the herbal product. Further studies should be directed toward

exploring the detailed pathway of lipid metabolism in different functional status of animal.

Acknowledgements

This research is supported in part by the projects from Ministry of Sciences and Technology 2009ZX09502-019, National Science Foundation of China, No. 30825047 and 30902000, and by E-institutes of Shanghai Municipal Education Commission No. E03008

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