"Dose-Effect-Response" Relationships of *Paeoniae Radix Rubra* on Alpha-Naphthylisothiocyanate-Induced Acute Cholestatic Hepatitis in Rats

WEI Si-si[1, 2], ZHAO Yan-ling[1*], JIANG Feng-juan[1, 2], JIA Lei[1, 2], ZHU Yun[1], WANG Jia-bo[1], SUN Zhi-yong[1, 2], LI Rui-sheng[3], XIAO Xiao-he[1]

1. Integrative Medicine Center of 302 Military Hospital, China Military Institute of Chinese Materia Medica, Beijing 100039, China
2. Chengdu University of Traditional Chinese Medicine, Chengdu 610075, China
3. Animal Center, 302 Military Hospital of China, Beijing 100039, China

Abstract: **Objective** To investigate the hepatoprotective effects of *Paeoniae Radix Rubra* (PRR) at different doses against alpha-naphthylisothiocyanate (ANIT)-induced acute cholestatic hepatitis in rats. **Methods** Rats were ig administrated with vehicle or PRR [(1, 9, 18, 36, 54, 72, and 81 g/(kg·d)] 3 d before and 2 d after ANIT (60 mg/kg) ig administration. The general status of rats, histopathology of liver, serum alanine aminotransaminase, aspartate aminotransaminase, total bilirubin, direct bilirubin, and alkaline phosphatase levels, were observed at respective time points (24 and 48 h) after ANIT administration. Using cluster analysis and correspondence analysis, the "dose-effect-response" relationships of PRR were evaluated. **Results** The results showed that compared with model group, the serum biochemistry index significantly decreased with the increasing of PRR dosage ($P < 0.01$), and the change and necrosis of hepatic cellula, and inflammatory cell infiltration were gradually alleviated. However, the improvement was not obviously found in the low-dose group [1 g/(kg·d)]. The cluster analysis and correspondence analysis results showed that different doses of PRR could significantly ameliorate ANIT-induced acute cholestatic hepatitis of rats in a dose-dependent manner. **Conclusion** The experiments show that administration doses of PRR in clinical use should be added properly in order to gain the expectant therapeutic effect, especially in the treatment of heavy acute cholestasis hepatitis.

Key words: acute cholestatic hepatitis; alpha-naphthylisothiocyanate; cluster analysis; correspondence analysis; "dose-effect-response" relationships; *Paeoniae Radix Rubra*

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Introduction

The striking worldwide prevalence of liver diseases is a serious health problem. But, lack of curative treatment for this disease and high cost of new drugs are some reasons for renewed public interest in other medicines. Traditional Chinese medicine (TCM) has been shown by many modern pharmacological studies to be efficient in the treatment of liver diseases including cholestasis (Zhai and Zhao, 2007), and it has become more imperative to explore hepatoprotective drug with high efficacy, safety, and low cost.

Paeoniae Radix Rubra (PRR), the dried root of *Paeonia lactiflora* Pall. or *P. veitchii* Lynch, is called "Chishao" in Chinese and stipulated in *Chinese Pharmacopoeia* (Pharmacopoeia Committee of P. R. China, 2010). Pharmaceutical studies have demonstrated that PRR containing glucosides and alkaloids could exhibit pharmacological activities such as augmenting blood of coronary artery, antiplatelet agglutination, antithrombogenesis, decreasing blood fat, improving blood hyperviscosity syndrome, depressing blood pressure, and protecting liver (Ruan *et al*, 2003; Ye *et al*, 2001; Sun *et al*, 2009), and be widely used by Chinese medicine practitioners to treat cardiovascular,

* Corresponding author: Zhao YL Address: Integrative Medicine Centre, 302 Military Hospital, China Military Institute of Chinese Materia Medica, Beijing 100039, China Tel/Fax: +86-10-6693 3320 E-mail: zhaoyl2855@126.com
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inflammatory, female reproductive diseases, and liver diseases (Zhu, Fang, and Gong, 1985; Wu, Wu, and Chen, 2010; Zhan *et al*, 2006). Some reports showed that, Wang (1997) explored a high dose of PRR ranged from 120 to 150 g for the therapy of acute severe hepatitis; He and Wang (1997; 1998a; 1998b) treated acute cholestatic hepatitis clinically with high dose (80－120 g/d) of PRR with 89.7% theraputic effect, and also the jaundice-relieving effect was observed; Du (1999), using Kuhuang Chishao Decoction (PRR, 40 g), remedied acute cholestatic hepatitis; Feng (1999) made a feasible use of Huoxue Tuihuang Decoction which contained 30 g PRR to cure the acute cholestatic hepatitis; Xu (2001) made the alanine aminotransaminase (ALT) and total bilirubin (TBIL) of acute cholestatic hepatitis patients recover to normal level after using 30－80 g PRR; Feng (2001) applied a prescription called Chishao Tuihuang Decoction using 60 g PRR and drove TBIL to normal level. In the previous studies, the dose of PRR used in clinic ranged from 30 to 150 g, exceeding the statutory dose (6－12 g/d) of *Chinese Pharmacopoeia*, and showed outstanding therapeutic effect. However, for a number of reasons, the existing optimal therapeutic range of PRR has not proved entirely satisfactory assessment of criteria for high-dose therapy. Because of the increasing use of high-dose therapy for the treatment of liver diseases, it will be important to establish clear therapeutic range of PRR.

In this present study, the liver protection of different doses of PRR by ig administering to alpha-naphthylisothiocyanate (ANIT)-induced cholestatic hepatitis rats was investigated. The general status of rats, histopathology of liver, biochemical parameters including ALT, aspartate aminotransaminase (AST), TBIL, direct bilirubin (DBIL), and alkaline phosphatase (ALP) levels were determined to evaluate the hepatoprotective effect of PRR, and cluster analysis and correspondence analysis were used to synthetically evaluate the possible relationships of dose and effect using PRR to treat liver injury with acute cholestasis in rats.

Materials and methods

Materials

PRR pieces used in the decoction were purchased from Lvye Co., Ltd. (Beijing, China) and were identified by Prof. XIAO Xiao-he, a taxonomist in China Military Institute of Chinese Materia Medica. ANIT was purchased from Sigma Chemical Co. (St. Louis, USA) and dissolved in olive oil to a final concentration of 2% before use. Kits for determining ALT, AST, TBIL, DBIL, and ALP were purchased from the Mindray Bio-medical Electronics Co., Ltd. (Shenzhen, China).

Animals

All the 90 male Wistar rats of approximately 200－220 g were obtained from Vital River Lab Animal Technology Co., Ltd. (Beijing, China) (License No. SCXK 2007-004). Rats were kept under controlled conditions as follows: light (12 h light/dark cycles), controlled temperature [(25 ± 2) ℃], humidity (60%－70%), draughty, free access to standard laboratory chow and water. Animal welfare and experimental procedures were carried out strictly in accordance with the guidelines approved by the Ministry of Science and Technology of China and related ethical regulations.

Water-extract preparation of PRR

PRR pieces were decocted twice with water for 1.5 h each. The resulting extracts were decanted, filtered by 6-layer gauze, and evaporated to dryness under reduced pressure. The extraction percentage of the prepared PRR extract was 34.95%. This dried extract was redissolved in water for ig administration to rats.

Main constituents present in the PRR preparation were determined by analysis using HPLC with spectrophometric detector. The procedures were as follows: PRR preparation (0.5 g) was extracted with methanol (25 mL) under ultrasonication for 20 min. The solution was filtrated and then submitted for HPLC analysis. The HPLC equipment was controlled with an HPLC pump (Agilent 1200, USA) using an Agilent Zorbax SB-C_{18} columm (250 mm × 4.6 mm, 5 μm), eluting with acetonitrile (A) and H_2O (B) (15:85). The flow rate was 1.0 mL/min. The detection wavelength was 230 nm. The results showed that PRR preparation contained peoniflorin (3.18 ± 0.03)%, albiflorin (0.85 ± 0.01)% (Fig. 1). The above quality control sample was used in this pharmacodymic evaluation.

Experimental procedure

All the 90 animals were randomly divided into nine groups with 10 animals in each group: normal group (N), ANIT model group (M), ANIT + A-dose

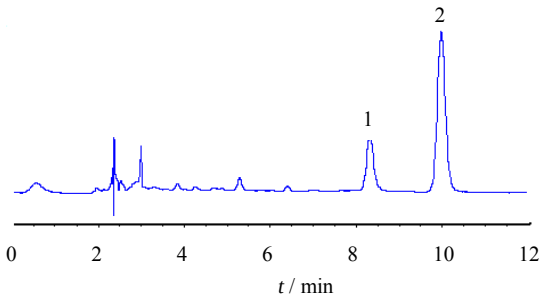

Fig. 1 HPLC chromatogram of main components in methanol extract of PRR

1: albiflorin 2: peoniflorin

PRR (A, 1 g/kg), ANIT + B-dose PRR (B, 9 g/kg), ANIT + C-dose PRR (C, 18 g/kg), ANIT + D-dose PRR (D, 36 g/kg), ANIT + E-dose PRR (E, 54 g/kg), ANIT + F-dose PRR (F, 72 g/kg), and ANIT + G-dose PRR (G, 81 g/kg) groups. (A-dose was according to *Chinese Pharmacopeia* (Pharmacopoeia Committee of P. R. China, 2010), and G-dose was maximally feasible dosage (81 g/kg in a 8 mL vehicle) of PRR. The maximally feasible dosage was defined as maximal amount of extract that could be dissolved in a vehicle to make the volume suitable to a rat's stomach (Shen and Sun, 2000). The experiment lasted for 5 d, twice a day. The cholestatic hepatitis model of rats (except the normal group which received an equal volume of olive oil) were induced by a solution of 2% ANIT (in olive oil, 1:50, 60 mg/kg) once after ig administration for 3 d with A－G doses. The rats (except the normal and model groups, which received an equal volume of distilled water) were ig administrated with A－G dosed after being treated with ANIT for 2 d. At the end of 4 d (when the rats have received ANIT for 24 h and PRR for 4 d) and 5 d, the rats were fasted for 12 h and had free access to water. Blood samples were collected by ophthalmic vein. Immediately after sacrifice, the livers obtained were stored at −80 ℃ until use.

Biochemical assays

The blood sample (2.0 mL) was centrifuged at 3000 × *g* for 10 min. Biochemical parameters, including serum ALT, AST, TBIL, DBIL, and ALP levels, were determined using a commercially available clinical test kit and a Mindray Clinical Analyzer BS300 (Mindray Bio-Medical Electronics Co., Ltd., China).

Histological assessment of liver damage

Necropsies were performed on all tested animals. Live tissue samples obtained from the same lobe of liver in each animal were immediately fixed and preserved in 10% neutral buffered formalin. After fixation for 48 h, the tissues were embedded in paraffin, cut into approximately 4－5 μm-thick sections, and stained with hematoxylin-eosin (HE). Histopathological observation of the liver injury was evaluated and analyzed by light microscopy for cell injury and necrotic changes, which were scored on a scale of 0 to 3:0 refers to no lesions; 1 refers to mild (less than 25% of the tissues were affected); 2 refers to moderate (25%－50% of the tissues were affected); 3 refers to severe (more than 50% of the tissues were affected).

Statistical analysis

All values are expressed as $\bar{X} \pm s$. Significant difference among groups was analyzed by Student's *t*-test. $P < 0.05$ was considered to be statistically significant in all cases. Cluster analysis and correspondence analysis were performed using the Windows SAS 9.1 software (SAS, USA).

Cluster analysis

Cluster analysis is a method of unsupervised learning, and a common technique for multivariate analysis, which is used to sort similar samples into different groups. An unsupervised classification procedure, involving the similarity between the objects to be clustered, was processed using this technique. The similarities or dissimilarities between samples (objects) are usually represented in a dendrogram for ease of interpretation (Kong *et al*, 2009). Objects in a cluster are similar to the others, but they are different from those outside the cluster, particularly objects in other clusters with respect to a predetermined selection criterion. The between-groups linkage method as the amalgamation rule was used to establish clusters (Kannel *et al*, 2007).

Correspondence analysis

Correspondence analysis (Pusha and Gudi, 2009; Kong *et al*, 2009) is an important multivariate statistical method which is used to study the relationship between investigated factor and multi-variables. Based on the idea of principal component analysis (PCA), correspondence analysis could express these multi-variables by two principal component variables *Z*1 and *Z*2 which have the main contributions to the total data set. The principal component variable aggregations [*Z*1 and *Z*2] of every valid point are calculated and depicted

by 2D-projection. So, the internal change regularity of investigated factor could be directly represented.

Results

General status

The general status of the rats deteriorated with increase in ANIT-intoxication. Compared with the healthy rats in the normal group, rats exposed with ANIT showed decreased body weight, less water-intake, withered and caducous fur, and preference of gathering and fatigue. Opposed to the model group, bright coat and enhanced moving activity, etc., were observed in the A—G group rats treated with PRR. There is no death found in all groups.

Biochemical analysis

The serum activities of ALT, AST, TBIL, DBIL, and ALP were used as biochemical markers for the acute liver injury, and the contents of these five markers in the nine groups at two different times are shown in Fig. 2. At the end of 24 h (when the rats had been treated with ANIT for 24 h and PRR for 4 d): compared with N group, the levels of ALT, AST, TBIL, DBIL, and ALP in the M group were significantly elevated ($P < 0.01$) [ALT from (59.36 ± 15.71) to (515.90 ± 171.46) U/L, AST from (202.21 ± 24.65) to (900.30 ± 304.54) U/L, TBIL from (7.52 ± 3.14) to (43.08 ± 13.76) μmol/L, DBIL from (7.52 ± 3.05) to (36.18 ± 9.05) μmol/L, ALP from (444.42 ± 63.53) to (693.14 ± 74.62) U/L], illustrating that the hepatocyte was damaged along with cholestasis. Compared with the corresponding levels in M group, the serum ALT, AST, TBIL, DBIL, and ALP levels in B—G dose groups were significantly decreased, and effects of PRR were enhanced with increasing the dose in a dose-dependent manner. However, the serum level in A-dose group was similar to that in M group, showing that A-dose PRR had few normalizing gallbladder for jaundice-curing effects on the ANIT-induced acute cholestatic hepatitis rats. At the end of 48 h (when the rats had been treated with ANIT for 48 h and PRR for 5 d): Compared with N group, the levels of ALT, AST, TBIL, DBIL, and ALP in M group were significantly elevated ($P < 0.01$) [ALT from (50.04 ± 7.98) to (1360.61 ± 486.45) U/L, AST from (242.41 ± 40.55) to (2039.22 ± 426.28) U/L, TBIL from (8.43 ± 2.37) to (49.12 ± 13.56) μmol/L, DBIL from (7.97 ± 4.43) to (42.62 ± 7.54) μmol/L, and ALP from (397.78 ± 49.45) to (636.63 ± 84.32) U/L], showing the notable hepatocyte damage with cholestasis. The results of our study showed that liver injury with cholestatis occurred 24 h after ANIT treatment and progressed at 48 h, which were consistent with the previous report (Yoshiji, 2007). Compared with M group at 48 h, the serum ALT, AST, TBIL, DBIL, and ALP levels in B—G dose groups were more significantly decreased than those at 24 h. But, the serum level in A-dose group was similar to the value in M group, showing that A-dose PRR had little hepato-

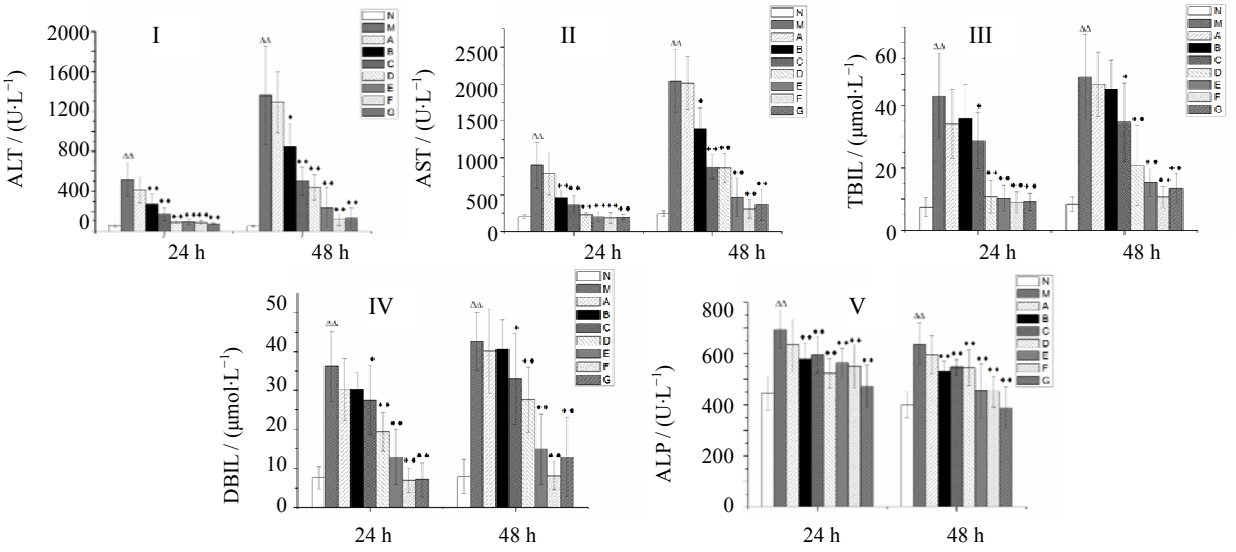

Fig. 2 Serum ALT, AST, TBIL, DBIL, and ALP levels in nine groups of rats treated with or without PRR

N: normal group; M: model group; A: 1 g·kg^{-1} PRR group; B: 9 g·kg^{-1} PRR group; C: 18 g·kg^{-1} PRR group; D: 36 g·kg^{-1} PRR group; E: 54 g·kg^{-1} PRR group; F: 72 g·kg^{-1} PRR group; G: 81 g·kg^{-1} PRR group; Fig.3 and Table 1 are same

I—V: serum ALT, AST, TBIL, DBIL, and ALP levels in nine groups at 24 h and 48 h, respectively

$^{\triangle}P < 0.05$ $^{\triangle\triangle}P < 0.01$ *vs* normal group; $^{*}P < 0.05$ $^{**}P < 0.01$ *vs* ANIT model group

protective effects on the ANIT-induced acute cholestatic hepatitis rats at 48 h.

Histopathological analysis

The liver sections stained by HE in ANIT-treated rats with or without PRR and untreated control rats were examined for necrosis at 48 h after the treatment. Typical histopathologic section photos were shown in Fig. 3. Hepatocytes in the untreated control group showed few histological changes (Fig. 3A). Hepatocytes in the group treated with ANIT alone presented necrotic and degenerative changes with severe inflammatory cell infiltration, ballooning, and mild fibroplasias and cholestasis were most noticeable. Hepatocytes were frequently found swollen and the cytoplasm was coarsely granular (Fig. 3B). By contrast, hepatocytes in the ANIT-treated group administered with PRR (1 g/kg) presented less necrotic and degenerative changes and less inflammatory cell infiltration. Perivascular spaces showed considerable eosinophils infiltration, and lymphocytes were often seen typically localized in the portal areas (Fig. 3C). In B-dose group, lobules of liver showed normal structure, but portal areas moderately infiltrated with lymphocytes were observed (Fig. 3D). Compared with the model group, photomicrographs of group C—G showed that the architecture of hepatic lobule was improved with the increasing of PRR dose; Normal contours of central vein were seen, but porta hepatis was infiltrated with a few lymphocytes (Fig. 3E—I). Liver pathological changes and scores of injury degree of rats in each group were shown in Table 1.

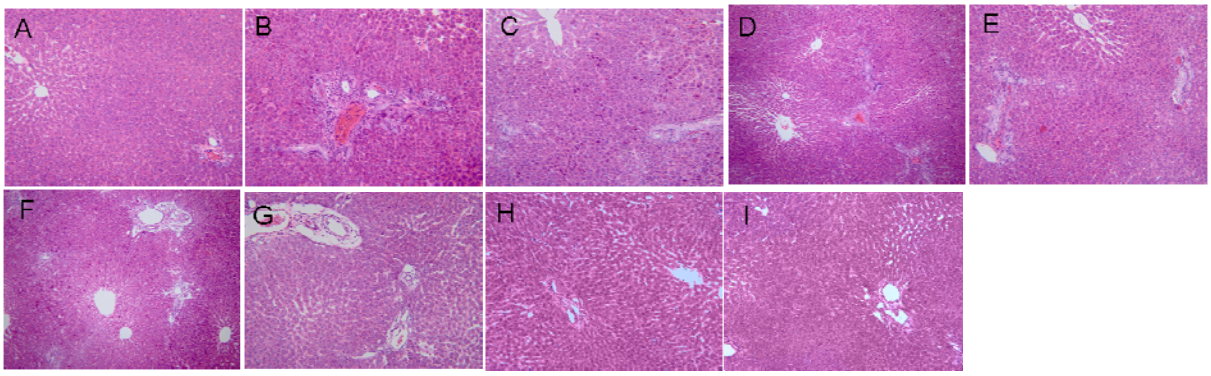

Fig. 3 Histopathologic changes in the liver tissue of nine groups of rats treated with or without PRR (HE stainning)

Table 1 Effects of PRR treatment on ANIT-induced liver necrosis in rats (n = 10)

Groups	Histopathlogy scores			
	0	1	2	3
N	10	0	0	0
M$^{\triangle\triangle}$	0	2	3	5
A	0	3	3	4
B*	0	3	5	2
C**	0	4	4	2
D**	0	5	3	2
E**	2	7	1	0
F**	4	5	1	0
G**	5	5	0	0

Cluster analysis

Serum biochemical indices (serum ALT, AST, TBIL, DBIL, and ALP levels) of hepatic cell damage, were very important. To analyze the "dose-effect" relationships of PRR on acute cholestatic hepatitis rats, cluster analysis was used in our study and the results were shown in Fig. 4.

It was clear that the samples could be divided into three clusters: (1) Group N24, D24, E24, F24, G24,

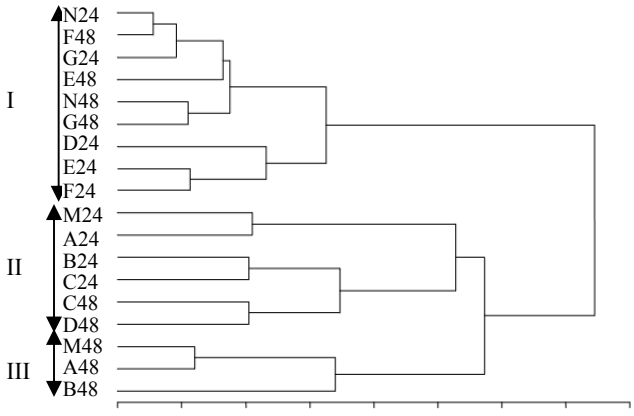

Fig. 4 Dendrogram for cluster analysis of investigated groups

N24, M24, A24, B24, C24, D24, E24, F24, and G24 represented the normal, model, low—high dose PRR groups at 24 h. N48, M48, A48, B48, C48, D48, E48, F48, and G48 represented the normal, model, low—high dose PRR groups at 48h

N48, E48, F48, and G48, namely normal group, E-, F-, and G-dose PRR groups at 24 and 48 h, and D-dose PRR group at 24 h, mixed into cluster I. And it indicated that different doses of PRR had different therapeutic actions on ANIT-induced acute cholestatic hepatitis of rats, but the therapeutic actions on D-dose PRR at 48 h will be weakened. (2) M24, A24, B24, C24, C48, and D48, which presented model group at 24 h, A-, B-, and C-dose PRR groups at 24 h, and C-, D-dose PRR groups at 48 h, gathered into cluster II. It showed that there had been few effects with A-, B-, and C-dose PRR at 24 h, and C-, D-dose PRR at 48 h. (3) M48, A48, and B48 in cluster III meant model group at 48 h, A-, B-dose PRR groups at 48 h. Model groups were not gathered together but clustered with other groups at each experimental time, respectively. The results showed that liver lesions induced by ANIT deteriorated with time while A- and B-dose PRR groups at 48 h had few effects on the degeneration of cholestatic hepatitis in model group.

Correspondence analysis

In this study, the "dose-effect-response" relationships of the liver protection of PRR on acute hepatic injury with chloestatis of rats were investigated by correspondence analysis on the biochemical markers (serum ALT, AST, ALP, TBIL, and DBIL levels) using SAS 9.1 software (SAS, USA) as shown in Fig. 5.

Each point, which was obtained by correspondence analysis on the five markers, was depicted in the rectangular coordinate system with *Z*1 as horizontal axis and *Z*2 as longitudinal axis to represent each investigated group. The point of each group was clearly distributed in this rectangular coordinate system based on the point of N24 (the normal group at the end of 24 h) as the zero. N24, M24, and A24－G24 represented the normal, model, and low-high-dose PRR groups at the end of 24 h and N48, M48, A48－G48 represented the normal, model, and low-high-dose PRR groups at the end of 48 h. The nine groups at the end of 24 h were shown with blue symbol, and the other nine groups at the end of 48 h with red symbol. The euclidean distances between each group were calculated and shown in Table 2.

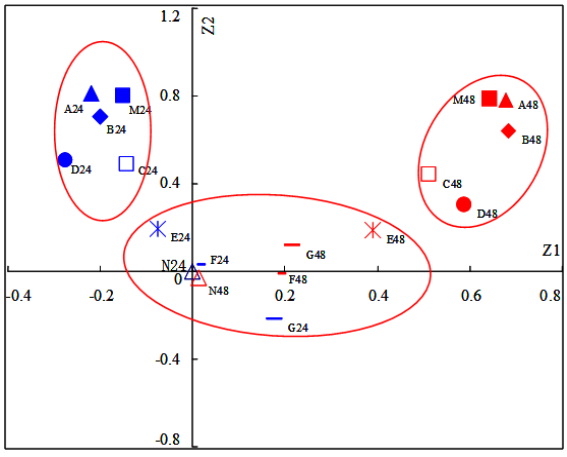

Fig. 5 Correspondence analysis plot of principal components 1 and 2 (*Z*1 and *Z*2) for distribution of each investigated group

This plot was obtained by correspondence analysis using SAS 9.1 software on biochemical markers (serum ALT, AST, TBIL, DBIL, and ALP levels) in each group. It could reflect the "dose-effect- response" relationships of PRR on hepatic injury rats

Table 2 Euclidean distances between each group

Group to Group	Euclidean distances	
	24 h	48 h
M to N	0.8144	1.0320
A to N	0.8035	1.0195
B to N	0.7327	0.9516
C to N	0.5099	0.6890
D to N	0.5749	0.6682
E to N	0.2109	0.4363
F to N	0.0308	0.1753
G to N	0.2805	0.2521
A to M	0.0723	0.0370
B to M	0.1082	0.1482
C to M	0.3112	0.3667
D to M	0.3209	0.4814
E to M	0.6082	0.6510
F to M	0.7883	0.9173
G to M	1.0689	0.7938

N, M, A, B, C, D, E, F, and G represented the normal, model, and low-high different doses of PRR groups, respectively

The distances among the groups reflected the discrepancy degree of these groups. From Fig. 5 and Table 2, the "dose-effect-response" relationships of PRR on acute hepatic injury rats could be found: (1) The distance of M48 and N48 (1.0320) was bigger than that of M24 and N24 (0.8144), showing that ANIT could successfully induce the cholestatic hepatitis of rats. (2) E24, F24, and N24 had the shortest distance,

while A24, B24, C24, D24, and N24 had the longest distances, which illustrated that therapeutic action of A-, B-, C-, and D-dose PRR on ANIT-induced cholestatic hepatitis of rats was weaker than that of E- and F-dose PRR at 24 h. (3) A24, B24, C24, D24 and M24, and A48, B48, C48, D48 and M48 had the shortest distance, illustrating A-, B-, C-, and D-dose PRR showing little therapeutical function on the ANIT-induced cholestatic hepatitis. (4) The distance of E24 and E48 was the longest among the distance of other points at 24 h and 48 h, showing that the therapeutic action of E-dose PRR on ANIT-induced cholestatic hepatitis of rats will be weakened with increasing of time.

Conclusion

ANIT is well known to cause cholestasis (Krell, Hoke, and Pfaff, 1982) and widely used to establish a model similar with human intrahepatic cholestasis. It has been reported that ANIT could produce cholangiolitic hepatitis in rats after a single ig administration (Ogawa *et al*, 2000; Plaa and Priestly, 1977), and was characterized by hepatocellular damage and marked neutrophil infiltration. Therefore, rats treated with ANIT were used as a common model of cholestasis since this compound produced cholestasis in a reproducible and dose-dependent manner (Goldfarb, Singer, and Propper, 1962; Vore, 1991).

Some reports have previously shown that TCM had complementary therapeutic effects on Western medicines although the dose-effect response has been unclear. Therefore, PRR, as a classical traditional Chinese drug, which exhibits the significant efficacy in the treatment of liver disease, should be followed with interest in the dose-effect relationship.

In this study, we employed the methods of examining common changes of rats, histopathology of liver, serum biochemical parameters levels to address the hepatoprotective effect of PRR on ANIT-induced cholestatic hepatitis rats. Analyses of the serum biochemical parameters and histopathology of rats showed that the biochemical parameters of rats under the action of PRR were significantly lower than those in the model rats and pathological changes of liver was markedly attenuated with the treatment of PRR dosage, which indicated that PRR could improve the cholestatic hepatitis of rats induced by ANIT with the addition and increasing doses of PRR. Administration of high dose PRR could exert an important role in hepatoprotective effect even treating cholestatis hepatitis.

In conclusion, the results showed that PRR could significantly ameliorate ANIT-induced acute cholestatic hepatitis of rats in a dose-dependent manner, and administration dose of PRR in clinical use should be added properly in order to gain the expectant and potential therapeutic effect, especially in the treatment of heavy acute cholestasis hepatitis.

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