Evaluation of safety of Modified-Danggui Buxue Tang in rodents: immunological, toxicity and hormonal aspects

Jian-Hui Xie^{a,1}, Zhi-Wei Chen^{a,c,1}, Ya-Wei Pan^b, De-Min Luo^b, Zi-Ren Su^c, Hai-Ming Chen^a, Zhen Qin^d, Shui-Qing Huang^{e,*}, Gao Lei^{b,*}

^a *The Second Affiliated Hospital, Guangzhou University of Chinese Medicine, Guangzhou, 510006, PR China*

^b *Department of Orthopaedics, Dongguan Kanghua Hospital, Dongguan 523080, PR China*

^c *Dongguan Mathematical Engineering Academy of Chinese Medicine, Guangzhou University of Chinese Medicine, Dongguan 523000, PR China*

^d *Department of Pharmacology, Guiyang Medical University, Guiyang 550025, PR China*

^e *School of Continuing Education, Guangzhou University of Chinese Medicine, Guangzhou 510006, PR China*

* Corresponding author: Gao Lei, E-mail: lei.chenggao@163.com;

Tel: +86 20 3935 8517. Fax: +86 20 3935 8390.

* Corresponding author: Shui-Qing Huang, E-mail: hsq@gzucm.edu.cn.

¹These authors contributed equally to this work.

Abbreviations

DBT, Danggui Buxue Tang; RRF, A DBT-derived herbal recipe; RA, Radix Astragali; RAS, Radix Angelicae Sinensis; FE, Folium Epimedii; DTH, delayed type hypersensitivity; PR, prednisone acetate; LE, levamisole hydrochloride; CMI, cell-mediated immune response; DNFB, 1-fluoro-2,4-dinitrobenzene; CRBC, chicken red blood cells; NOAEL, No Observed Adverse Effect Level; RBC, red blood cell; HGB, hemoglobin; HCT, hematocrit; MCV, mean corpuscular volume; MCH, mean corpuscular hemoglobin; MCHC, mean corpuscular hemoglobin concentration; RDW, red cell distribution width; HDW, hemoglobin distribution width; PCT, plateletocrit; PLT, platelets; MPV, mean platelet volume; MPC, mean platelet component; PDW, platelet distribution width; WBC, white blood cell counts, RET, reticulocytes; NE, neutrophils; LYM, lymphocytes; MON, monocytes; EOS, eosinophils; BAS, basophils; PT, prothrombin time; AST, aspartate aminotransferase activity; ALT, alanine aminotransferase activity; ALP, alkaline phosphatase activity; ALB, albumin; TP, total protein; GLU, glucose; CHOL, total cholesterol; CPK, creatine phosphokinase activity; T-BIL, total bilirubin; CRE, creatinine; TG, triglycerides; Ure, urea; Na⁺, sodium ions; K⁺, potassium ions; Cl⁻, chloride ions; T, testosterone; E2, estradiol; P, progesterone; FSH, follicle stimulating hormone; LH, luteinizing hormone; PRL, prolactin.

ABSTRACT

Ethnopharmacological relevance: Radix Astragali (RA), Radix Angelicae Sinensis (RAS) and Folium Epimedii (FE) are three of the extensively applied herbs among traditional Chinese medicines for gynecological disorders and osteoporosis. A derivative herbal formula—RRF, consisting of the three medicines with a weight ratio of 5:1:5, is derived from a famous Chinese herbal formula—Danggui Buxue Tang (DBT). RRF has shown noteworthy perimenopause ameliorating effect in both ovariectomized rats and natural aging female rats, which might represent a promising candidate for the treatment of perimenopausal disorders. The aim of this study was to evaluate its immunological potential, chronic toxicity and reproductive effects by 26-week repeated daily administration in female rats, in order to optimize its safe use.

Materials and methods: The effect of RRF on immunological function was studied by macrophage phagocytosis, immune organ index, serum immunoglobulin level as well as delayed type hypersensitivity (DTH) in mice. For toxicity assessment, acute toxicity study was performed according to fixed dose procedure with a single oral administration of RRF to mice. In the oral chronic toxicity, 120 female rats were administrated RRF orally in 0, 1100, 4400, or 8800 mg/kg/day doses for 26 weeks. Clinical signs, mortality, body weights, feed consumption, haemato-biochemical parameters, organ weights, histopathology and reproductive hormone profiles were examined at the end of the 13- and 26-week dosing period, as well as after the 4-week recovery period.

Results: Oral administration of RRF at three doses (282, 564 and 1128 mg/kg) significantly increased the indices of phagocytosis K , as compared with prednisone acetate (PR) group ($p < 0.05$ or 0.01). Exposure of RRF dose-dependently boosted circulating serum IgM level (all $p < 0.01$) in response to CRBC in PR-induced mice. Furthermore, RRF treatment elicited a significant increment (all $p < 0.01$) in

DNFB-induced DTH response and the immune organ indices in a dose-dependent manner in mice, in parallel to DNFB-induced group. In the single dose acute toxicity and repeated dose 90-day chronic toxicity investigations, no toxic signs/mortality were observed. RRF treatment did not cause any toxicologically significant changes in clinical signs, food consumption, body weight, relative organ weight, haematological parameters, clinical chemistry, gross pathology and histopathology between treatment and control groups. No treatment related gross/histopathological lesions were observed and no target organ was identified. Long-term repeated administration of RRF exerted a significant promotion on serum level of steroid hormone estradiol, progesterone and testosterone release, along with decrease of circulating pituitary follicle stimulating hormone, luteinizing hormone, and prolactin levels in female rats. The No Observed Adverse Effect Level (NOAEL) of RRF was determined to be over 8800 mg/kg/day for elderly female rats, a dose that was equivalent to 50 times of human dose.

Conclusion: The present investigation demonstrated that RRF possessed appreciable immunopotentiating activity and had a relatively wide margin of safety. Long-term treatment of RRF exhibited estrogenic properties, and retarded certain age-associated degenerations. RRF might have the potential for further development as a safe and effective alternative/complementary to conventional medication in relieving perimenopausal symptoms.

Keywords: Danggui Buxue Tang; Modified Herbal formula; Immunological; Chronic toxicity; Reproductive effect

Introduction

Herbal medicines have been widely applied in the world for thousands of years and are generally considered to be safe on the basis of experience evidence. The

worldwide public is using increasing numbers of herbal remedies in conjunction with or as replacements for conventional medicine (Saad et al., 2006). However, along with increase of herbal medicine use worldwide, concerns have been raised over the lack of modern scientific evidence regarding the efficacy and safety of herbal products (Firenzuoli and Gori, 2007). Hence, there is a need to assure the safety of herbal formulations in order to maximize their benefits even though these have been proven to be efficacious in pharmacological studies or by clinical evaluation.

In China, there is a long history of treating gynaecological disorders/conditions with traditional Chinese medicine (TCM). Among the famous TCM formulas, Danggui Buxue Tang (DBT), first described by Li Dongyuan in *Neiwaishang Bianhuo Lun* in AD 1247, has been applied in gynaecology, especially for women with menopausal symptoms for hundreds of years. Previous pharmacological results suggested that DBT, composed of well-documented Radix Astragali (root of *Astragalus membranaceus* (Fisch.) Bge. var. *mongholicus* (Bge.) Hsiao, Leguminosae, RA) and Radix Angelicae Sinensis (root of *Angelica sinensis* (Oliv.) Diels, Apiaceae, RAS), had the potential to promote hematopoietic function, prevent osteoporosis, stimulate cardiovascular circulation, and stimulate immune response (Dong et al., 2006; Gao et al., 2006, 2007a, 2007b, 2007c, 2008).

Herbal recipes from TCM are prepared with a specific combination of different herbs as formulae by a unique methodology, which, if properly prepared, would synergistically deliver multiple bioactive effects and compromise potential toxicity. In our previous investigation, we found that a derivative herbal formula (named RRF) from DBT, which was made up of RA, RAS, and Folium Epimedii (leaves of *Epimedium brevicomum* Maxim., FE) with a weight ratio of 5:1:5, exerted a potential benefit in relieving perimenopause symptom both in ovariectomy (OVX) rats as well as natural aging female rat model (Su et al., 2013; Xie et al., 2013). RRF had a

potential efficacy on relieving sexual hormonal imbalance and promoting bone formation. RA, RAS and FE are three of the extensively applied herbal remedies with well-established histories of use among TCMs for age-related diseases, gynecological disorders and osteoporosis, representing a promising candidate for the treatment of perimenopausal disorders (Yang et al., 2002; Qin et al., 2005). Modern researches suggested that these three herbs not only have exhibited appreciable immunomodulatory effects either used as a single herb or as a formula in combination with other Chinese medicine (Zhang et al., 1999; Zhao et al., 2012; Zheng et al., 2015), but also proved to exert positively protective effect against reproductive toxicity (Wan et al., 2010; Kim et al., 2012; Yuan et al., 2014).

Despite preliminary evidence of its therapeutic benefits and the traditional use of RA, RAS and FE, the scientific validation of the systematic safety of RRF has not been established so far. In this follow-up study, we endeavored to evaluate the possible immunomodulatory potential, acute and chronic oral toxicity, as well as reproductive effects of RRF by 26-week repeated daily administration in female rats, in order to optimize its safe use.

2 Materials and methods

2.1. Plant materials and preparation of RRF

Plant materials Radix Astragali (RA), the root of *Astragalus membranaceus* (Fisch.) Bge. var. *mongholicus* (Bge.) Hsiao (Leguminosae) and Radix Angelicae Sinensis (RAS), the root of *Angelica sinensis* (Oliv.) Diels (Apiaceae) were purchased from Shanxi Hunyuan wangsheng Astragalus development Co., Ltd (Lot. 070519) and Guangxi Xinlong Pharmaceutical Co., Ltd (Lot. 070615), respectively. Folium Epimedh (FE), the leaves of *Epimedium brevicornum* Maxim (Berberidaceae) was obtained from Longxi County in Gansu province (Lot. 070715), all of which are

authenticated by Prof. Karl W.K. Tsim of Hong Kong University of Science and Technology. The authenticated voucher specimens (Voucher # 07-05-25 for RA, Voucher # 07-06-20 for RAS and Voucher # 07-07-20 for FE) were kept in School of Chinese Materia Medica, Guangzhou University of Chinese Medicine. Assurance of quality control for all the materials was validated according to Chinese Pharmacopeia (China Pharmacopoeia Committee, 2010).

Preparation of RRF RRF was prepared as follows: Amounts of RA, RAS, and FE were weighed according to a ratio of 5 : 1 : 5. RAS and RA were ground and then mixed by vortex. The extraction was performed in 8 times of boiling water (800 mL per 100 g mixture) for 2 h twice. This extraction followed the ancient preparation and was shown to be the best extracting condition (Song et al., 2004). For second extraction, the residue from the first extraction was filtered, and the same extracting condition was applied. Thereafter RAS-RA extract was concentrated to a relative density (RD) of 1.18~1.22 (60 °C), centrifuged for 0.5 h at 15000 rpm, and the supernatant obtained was subjected to reconcentration (RD 1.35~1.40) and then dried under vacuum to get RA-RAS extractum. The dried and powered aerial part of EF was thoroughly extracted with 7 times of 70% ethanol (700 mL per 100 g powder) for 1 h twice. The ethanol extract was pooled and concentrated to a relative density of 1.10~1.15 (60 °C), centrifuged for 0.5 h at 15000 rpm. The resulting supernatant was reconcentrated to RD 1.35~1.40 under vacuum, and dried out by vacuum drying technique. The above dry extracts were blended thoroughly with an appropriate amount of microcrystalline cellulose and crosslinking polyvinylpyrrolidone (PVP) to produce RRF samples for each experiment. HPLC assays as described previously had been employed for assurance of quality control of the typical chemicals of the three herbs, astragaloside IV for RA, ferlic acid for RAS, and icariin for FE (Xie et al., 2013). RRF was stored at 4 °C and diluted to the desired concentrations in distilled

water at the time of administration.

2.2. *Experimental animals and maintenance*

Sprague–Dawley (SD) rats (180~220 g of either sex; 11-month elderly females, 390~410 g), and Kun-ming (KM) mice of both sexes (18 ± 2 g) at the initiation of treatment were obtained from the Medical Experimental Animal Center of Guangzhou University of Chinese Medicine. Licences for rats and mice were SCXK (YUE) 2008–0020. For the experiment, the animals were transferred to the laboratory and submitted to adaptation by period of 7 days. They were acclimatized under controlled temperature ($25 \pm 2^{\circ}\text{C}$) and humidity (50-70%) on a 12-h light/12-h dark cycle (artificial lighting from 08:00 to 20:00) and had free access to standard chow and drinking water. In all experiments, the animals were kept in cages with raised, wide-mesh floors to prevent coprophagy. The experimental protocols involved were in accordance to the rules and guidelines of the Experimental Animal Center of Guangzhou University of Chinese Medicine and, approved by the Animal Care and Use Ethics Committee of our institution, with reference to European Community guidelines for the use of experimental animals.

2.3. *Macrophage phagocytosis assay*

For assay of phagocytic activity *in vivo* a colloidal carbon clearance test was performed following the method of Hudson and Hay (1980), with some modifications. Sixty female mice were divided into six groups of 10 each, i.e., control group, prednisone acetate group (PR group), RRF treated groups (282, 564 and 1128 mg/kg) and levamisole hydrochloride group (LE-treated group). They were pretreated with prednisone acetate (PR, 10 mg/kg, *p.o.*) daily for 4 consecutive days prior to drug treatment except for the control group. RRF-treated groups were administered RRF at doses of 282, 564 and 1128 mg/kg, respectively, and LE-treated group was administered LE at a dose of 60 mg/kg. RRF-treated groups and LE-treated group

were administered the respective doses via oral gavage once daily for 7 consecutive days from the 5th day. Control group and PR group were given equivalent volume of olive oil throughout the experimental period. On the 11th day, all animals were injected colloidal carbon ink intravenously (1 mL/100 g) 1 h after the last administration. After injection of carbon suspension at 2 min (t₁) and 12 min (t₂), 20 µL aliquots of blood samples were obtained from the retroorbital venous plexus of individual mice with a heparinized microhematocrit glass capillary, and immediately lysed with 2 mL 0.1% Na₂CO₃. The absorbances were measured spectrophotometrically at 576 nm. The rate of carbon clearance, termed as phagocytic index (*K*), was determined following the formula: $K = (\ln OD_1 - \ln OD_2) / (t_2 - t_1)$, where OD₁ and OD₂ are the optical densities at time t₁ and t₂ respectively.

2.4. Determination by antibody titration (humoral-mediated response)

Humoral immune response assay was carried out according to the method of Duan et al. (2010) with some modifications. Fresh blood was collected from white leghorn chickens in freshly prepared sterile Alsever's solution. Chicken red blood cells (CRBC) were washed three times in pyrogen-free, sterile saline and centrifuged at 3000 rpm for 10 min. The supernatant was removed and the sediment was re-suspended in normal saline to a concentration of 5% by improved Neubaur chamber for immunization and challenge. Animal grouping and drug treatment were the same as described in section 2.3. On the 9th day, 1 h after administration, except the control group, mice were immunized with suspensions of CRBC, by intraperitoneal injection at a dose of 20 mL/kg body weight. On the 11th day, 1 h after the final oral administration, all mice blood samples were collected from the retro-orbital venous plexus with heparinized microhematocrit glass capillary. After standing at room temperature for 30 min, samples were centrifuged at 3000 rpm for 10 min for serum preparation. For IgM assay, 10 µL aliquots of above mentioned

serum were diluted by normal saline to a final volume of 4 mL. Afterwards, 0.5 mL of 5% CRBC, 0.5 mL 10% guinea pig plasma and 0.5 mL normal saline were added to 4 mL diluted sera in sequence. For control group, the complement was substituted with normal saline. The mixed samples for IgM assay were incubated for 30 min at 37 °C, and then immersed in an ice-water bath to terminate the reaction. Each sample was centrifuged at 3000 rpm for 15 min, and the absorbances of supernatant were measured by spectrophotometry at 540 nm to represent the serum level of IgM.

2.5. Delayed-type hypersensitivity response (DTHR) for cell-mediated immunity

Measurement of sensitizing agent 1-fluoro-2,4-dinitrobenzene (DNFB)-induced DTH was carried out following the method of Ma et al. (1989) with slight modifications. Sixty female Kunming mice were randomly assigned to six groups of 10 each. On the 1st day, mice were initially sensitized by applying 50 µL of 1% DNFB in mixed acetone-olive oil (4:1, v/v) to the abdomen skin of mice depilated with barium sulphide and, the challenge was repeated through the same route the next day. After second sensitization, RRF (282, 564 and 1128 mg/kg/d, *p.o.*) and the reference drug LE (60 mg/kg/d, *p.o.*) were administered respectively for 7 successive days from the 3rd day, while DNFB-induced group was given equivalent volume of olive oil during this period. On the 9th day, all mice were subjected to contact hypersensitivity with 10 µL of 1 % DNFB on both surfaces of right ear of mice, 60 min after the last dose. While the left ear was treated with olive oil alone. Twenty four hours after the final DNFB application, all mice were sacrificed by cervical dislocation, and biopsy specimens of both ears were removed by a specific 8-mm punch. Ear swelling was evaluated by measuring the weight difference between the right and left ears, as an indicator of immunogen-elicited DTH. The immune organs including spleen and thymus gland were harvested and, weighed for calculation of immune organ indexes.

2.6. Acute toxicity test of RRF

Acute oral toxicity study was performed according to fixed dose procedure in rats and mice. Two experimental groups of rats (9 rats of each sex in each group) were treated orally with a single dose of 0 and 4400 mg/kg body weight (equivalent to 200 times of the normal human dose in clinical prescription). For mice (10 mice of either sex in each group), the maximal administration dose was 8800 mg/kg body weight (equivalent to 400 times of the clinical application dosage). Animals receiving the vehicle (saline) served as control. Animals were observed individually at least once during the first 30 min after administration, periodically during the first 24 h (with special attention during the first 4 h) and daily thereafter for a period of 14 days. Food and water were provided throughout the experiment. For 14 days the animals were weighed and the number of deaths noted.

2.7. Experimental schedule for general toxicity of RRF

To generally detect potential long-term, repeat-dose toxicity of RRF and thereby define the characteristic, extent, dose and time-dependent relationship, as well as target organs and tissues of potentially toxic effects, 120 elderly female SD rats were assigned randomly and evenly to 4 experimental groups. The animals were separated and housed 5 in each cage. Group I animals (control) were orally administered with distilled water throughout the course of the study. Animals in Groups II (1100 mg/kg body weight/day), III (4400 mg/kg body weight/day), IV (8800 mg/kg body weight/day) received orally administered RRF dissolved in distilled water once-daily for a period of 13 or 26 weeks. The dosages selected in the present study were based on existing data on the effective dose, results of acute toxicity study of RRF in mice and rats, and suggested human dose (equivalent to 10, 40, and 80 times of the normal human dose in clinical prescription, respectively). Clinical signs (general behavior, fur condition, breathing and nose conditions, eyes and oral secretions, urine and

faeces), toxic reactions, and mortality were monitored daily after the initiation of RRF treatment. Body weight, food and water consumption were recorded once a week. After 13 weeks, 10 rats in each group were sacrificed. Another 10 rats in each group were sacrificed at the end of the 26th week. The remaining rats were sacrificed after 4 weeks of recovery. The animals of the recovery groups were observed for reversibility, persistence and delayed occurrence of toxic effects. All examinations were conducted as described above.

At the end of the tested period, all animals were sacrificed and subjected to hematology and clinical chemistry assays, necropsy examination, organ weighing, and histopathologic examination. This toxicity study was carried out in compliance with *Drug Registration Regulation* issued by China Food and Drug Administration (CFDA).

2.8. Clinical observation and feed consumption

The animals were observed daily prior to and following administration for signs of toxicity and mortality throughout the experimental period. Detailed clinical signs were assessed and recorded, including changes in skin and fur, eyes and mucous membranes, manure, psyche states and behavior patterns, etc. The body weight was measured at the initiation of treatment and once a week during the treatment period thereafter. The amounts of feed were weighed before they were supplied to each cage and their remnants were measured the next day. The differences were calculated and regarded as daily feed consumption (g/animal/day).

2.9. Haematological and biochemical parameters

Samples of blood were obtained from all animals on Week 13 and Week 26 of the investigation, and after recovery. All rats were fasting but allowed access to water *ad libitum* for more than 14 h prior to blood sample collection. Collected from the abdominal artery of each animal under anesthesia with isoflurane, blood samples were

collected into two tubes: (1) heparinized centrifuge tubes and (2) dry non-heparinized centrifuge tubes. The heparinized blood was used for a hematological determination, which include red blood cell (RBC), hemoglobin (HGB), hematocrit (HCT), mean corpuscular volume (MCV), mean corpuscular HGB (MCH), mean corpuscular HGB concentration (MCHC), red cell distribution width (RDW), platelets (PLT), mean platelet volume (MPV), platelet distribution width (PDW), white blood cell (WBC) counts, reticulocytes (RET), neutrophils (NE), lymphocytes (LYM), monocytes (MON), eosinophils (EOS), and basophils (BAS) with according to the manufacturer's operator manual. Plasma was isolated and used to determine the prothrombin time (PT).

The non-heparinized blood was allowed to coagulate before being centrifuged and the serum separated. Serum was analyzed for changes in biochemistry using an automatic biochemical analyzer (RA-1000, Technico, USA), which measured aspartate aminotransferase activity (AST), alanine aminotransferase activity (ALT), alkaline phosphatase activity (ALP), albumin (ALB), total protein (TP), total cholesterol (CHOL), creatine phosphokinase activity (CPK), total bilirubin (T-BIL), creatinine (Cre), triglycerides (TG), urea (Ure) and glucose (GLU). The concentrations of sodium ions (Na^+), potassium ions (K^+) and chloride ions (Cl^-) were measured with an electrolyte autoanalyzer (EasyLyte $\text{Na}^+/\text{K}^+/\text{Cl}^-$ Analyzer, MEDICA, USA). All parameters of blood chemistry and hematology were measured following standard procedures.

2.10. Organ weights, gross necropsy and histopathological examination

All animals were fasted overnight before scheduled necropsies. A complete necropsy was conducted on all animals and consisted of an external examination, including the identification of all clinically recorded lesions, and a detailed internal examination. The rats were anesthetized with diethyl ether and sacrificed by

decapitation after blood collection from the abdominal aorta. After dissection to remove fat and connective tissue, the following organs were carefully dissected out and weighed: brain (cerebrum, cerebellum and brainstem), thymus glands, heart, lungs, liver, spleen, kidneys, adrenal glands, ovary and uterus. The relative organ weight was calculated based on the terminal body weight before fixation.

The following organs of all animals were fixed in 10% neutral formalin: brain, thoracic spinal cord, thyroid glands, Gley's glands, pituitary gland, thymus, adrenal glands, esophagus, salivary glands, stomach, small intestine, large intestine, pancreas, spleen, lungs, trachea, kidneys, liver, heart, aorta, bladder, ovaries, uterus, vagina, mammary gland, sternum, sub-mandibular lymph nodes, bone marrow.

The fixed organs of the vehicle control and high dose group and any organs were subjected to histopathological examination. These samples were paraffin-embedded, microsectioned at a nominal thickness of 4 μ m and then stained with hematoxylin and eosin for histopathological examination under optical microscopy (OLYMPUS BX50).

2.11. Radioimmunoassays for reproductive hormone

Testosterone (T), estradiol (E₂), progesterone (P), follicle stimulating hormone (FSH), luteinizing hormone (LH), and prolactin (PRL) were measured by standard radioimmunoassay (RIA) and reagents, respectively. The results were expressed as ng/mL, pmol/mL, ng/mL, IU/L, IU/L and ng/mL, accordingly, with standards calibrated against reference preparation. All procedures of RIA were performed as described in the kit manuals.

2.12. Statistical analysis

The data obtained by the various parameters was statistically evaluated by Student's *t*-test, and presented as means $\pm$ standard deviation (S.D.) for the indicated number of independently performed experiments. Significant differences (as shown in

the plots) were classified as: *for $p < 0.05$; more significant **for $p < 0.01$. All analyses were performed using version 10.1 of the SPSS program (SPSS Inc., Chicago, IL, USA).

3. Results

3.1. Effect of RRF on the macrophage phagocytosis

As displayed in Fig. 1, administration of RRF at three doses potentiated the indices of phagocytosis K with respect to PR group (0.017 ± 0.005) by rapid removal of carbon particles from the blood stream. Treatment with 564 mg/kg and 1128 mg/kg RRF had significantly greater carbon clearance ability (0.025 ± 0.003 ; 0.024 ± 0.007 , both $p < 0.01$) than lower dose (0.021 ± 0.003 , $p < 0.05$), as indicated by a significant enhancement in phagocytic index.

Fig. 1. Macrophage phagocytic activity data for PR-induced mice treated with RRF. PR group (grey bars), Control group (open column), LE-treated group (60 mg/kg/d, dark-grey column) and RRF-treated groups (282, 564, and 1128 mg/kg/d, solid-dark bars). Vertical bars represent standard deviation (SD, $n = 11$). Asterisks designate significant differences: * $p < 0.05$ and ** $p < 0.01$ versus PR group; # $p < 0.01$ versus control group.

3.2. Antibody titration test

The results are shown in Fig. 2. Administration of RRF at three dosages (282, 564 and 1128 mg/kg) dose-dependently boosted the production of circulating serum IgM (1.357 ± 0.471 , 1.017 ± 0.404 and 1.007 ± 0.113 , all $p < 0.01$) respectively, in response to CRBC in PR-induced mice (0.313 ± 0.192). RRF pretreatment even achieved a more significant increment in IgM level than those of control and LE group, with the optimum effect observed at 282 mg/kg/d.

Fig. 2. Effect of RRF on serum IgM levels in PR-induced mice. PR group (grey bars), Control group (open columns), LE-treated group (60 mg/kg/d, dark-grey columns) and RRF-treated groups (282, 564, and 1128 mg/kg/d, solid-dark bars). Vertical bars represent standard deviation (SD, $n = 10$). Asterisks denote significant differences: * $p < 0.05$ and ** $p < 0.01$ versus PR group; # $p < 0.01$ versus control group.

3.3. The delayed-type hypersensitivity reaction

The results of the effect of RRF on cell-mediated immune response (CMI) through DTH are summarized in Fig. 3. Oral administration of RRF at three tested doses (282, 564 and 1128 mg/kg) displayed promotive effect (0.68 ± 0.33 mg, 0.71 ± 0.36 mg and 0.75 ± 0.29 mg, respectively; all $p < 0.01$) in DNFB-induced DTH response in a dose-dependent manner, in comparison to DNFB-induced group (0.31 ± 0.23 mg). Treatment with positive control PR elicited a significant decrease (0.90 ± 0.55 mg, $p < 0.05$).

The effects on mice immune organs (weight divided by body weight) of RRF treatment are represented in Fig. 4. It is displayed that RRF administration evoked significant increase in thymus of mice (0.5498 ± 0.1021 , 0.5592 ± 0.0909 and 0.5839 ± 0.0745 , respectively; all $p < 0.01$) in a dose-related fashion, in parallel to PR group (0.1663 ± 0.0451). Similarly, treatment with RRF resulted in obvious recovery in spleen (0.3828 ± 0.1424 ; 0.3508 ± 0.0946 ; 0.3710 ± 0.1028 , respectively; all $p < 0.01$) with respect to PR group (0.1766 ± 0.0401), which was superior to the LE group (0.2915 ± 0.672).

Fig. 3. Effect of RRF treatment on cell-mediated immune response by DTH in DNFB-induced mice. Control group (open bars), DNFB-induced group (grey bars), LE-treated group (60 mg/kg/d, dark-grey columns) and RRF-treated groups (282, 564, and 1128 mg/kg/d, solid-dark bars). Vertical bars represent standard deviation (SD, $n = 12$). Asterisks denote significant differences: * $p < 0.05$ and ** $p < 0.01$ versus DNFB-induced group. # $p < 0.01$ versus control group.

Fig. 4. Effect of RRF on immune organs-thymus and spleen in PR-induced mice. The results are expressed as the organ indexes using the formula: weight of spleen/thymus (mg)/body weight (g). PR group (grey bars), Control group (open columns), LE treated group (60 mg/kg/d, dark-grey columns) and RRF-treated groups (282, 564, and 1128 mg/kg/d, solid-dark bars). Vertical bars represent standard deviation (SD, $n = 10$). Asterisks designate significant differences: * $p < 0.05$ and ** $p < 0.01$ versus PR group; ## $p < 0.01$ versus control group.

3.4. Acute toxicity evaluation

In this study, a single oral dose safety on RRF was conducted as the first step of safety evaluation, which may provide preliminary safety information (OECD, 2001). RRF was administered once daily by gavage to 18 SD rats and 20 KM mice of both sexes at dose levels of 2200 and 4400 mg/kg/day respectively for 14 days. Animals did not manifest any significant abnormal signs and alternations, behavioral changes, water or food consumption, body weight variations, or macroscopic findings at any time of observation. No deaths of both rats and mice occurred during the 14 days of study. Autopsy results exhibited no significant change or lesion in the viscera of any animal.

3.5. Mortality and Clinical observations

The clinical symptom is one of the major important observations to indicate the

side effects on organs (Jothy et al., 2011). All groups treated with 26-week RRF did not show any significant symptoms or abnormal alteration on the behavior patterns compared to their respective controls, and no mortality was observed due to gavage of RRF during the study period, indicating that the administration of RRF at the doses tested has a negligible level of toxicity on the growth of animals.

3.6. Body weights, organ weights and feed consumption

Variations of body weight have been deemed as an important indicator of adverse effects of substances (Hilaly et al., 2004). Body weight of all the animals were measured every week throughout the study. Fig. 5 shows a graphical representation of the percent changes in body weight following treatment of rats with RRF. There were significant increases ($p < 0.05$) in body weights over the experimental period for all treatment groups. In all groups, body weight gradually increased from the 1st week to the 30th week, and body weight of animals in three treatment group showed no significant differences compared with that of control. In addition, as depicted in Figs. 6 (A&B), there was no consistent effect of food intake and water consumption by repeated oral administration of RRF (1100, 4400 and 8800 mg/kg/day) for female SD rats in comparison with the control and treatment group regardless of recovery group.

Organ-to-bodyweight ratio (relative organ weight) is another sensitive index for toxicity evaluation (Rosidah et al. 2009). Relative organ weights of 26-week treated rats are shown in Table 1. The data obtained show that no statistically significant difference was detected in the organ-to-bodyweight ratios between all the treatment groups and their control counterparts.

Fig.5. Mean body weight of female rats dosed with RRF.

Fig.6. Mean food (A) and water (B) consumption by female SD rats treated with RRF.

3.7. Hematological parameters

The hematopoietic system is one of the most sensitive targets for toxic chemicals and serve as important index of overall health status for both humans and animals (Rosidah et al., 2009). The effect of RRF on the hematological parameters of the experimental and control rats is presented in Table 2. The results indicate that all hematological parameters measured remained within the physiological range throughout the 30-week experimental period. There were no significant changes in white blood cell, neutrophils, lymphocytes, monocytes, eosinophils, basophils,

neutrophils, lymphocytes, red blood cell, hemoglobin, hematocrit, mean corpuscular volume, mean corpuscular HGB concentration, red cell distribution width, hemoglobin distribution width, platelets, mean corpuscular HGB, mean corpuscular volume, red cell distribution width, platelets, plateletocrit, platelet distribution width, reticulocytes, prothrombin time between the control and treated animals (main groups). Despite a significant change was observed in some parameters (mean platelet volume and mean platelet component), the above parameters remained within the range of normal physiological variation and there was no dose response relationship.

3.8. Blood biochemistry

The data for the biochemical parameters in the treated and control rats are presented in Table 3. All the parameters remained within the physiological range throughout the 30-week experimental period. No statistically significant differences were observed amongst the blood chemistry, including aspartate aminotransferase activity, alanine aminotransferase activity, albumin, total protein, total bilirubin, total cholesterol, creatinine, sodium ions, potassium ions, chloride ions between the control and treated animals. Furthermore, administration of 1100 mg/kg RRF could decrease the glucose concentration at the three tested periods ($p < 0.01$; $p < 0.01$ and $p < 0.05$, respectively). Low and high doses of RRF reduced the triglycerides level at 13-week administration ($p < 0.01$ and $p < 0.05$, respectively).

3.9. Histopathological findings

Histopathological examination is an important aspect of safety assessments (Greaves, 2007). In the histopathological examination, no noteworthy histopathological lesions of internal organs of the female rats in the control group as well as in rats treated with RRF were observed, though some abnormalities were found. The major pathological findings from the histopathological examination included minimal inflammatory cell foci and vacuolar degeneration in the liver, portal

myocardial inflammatory cell infiltration in the heart, interstitial inflammation in the lung, tubule calcium deposition and interstitial inflammation, distal convoluted tubule cortical vacuolar degeneration and protein cast in the kidneys, inflammatory cell infiltration in small and large intestine, stomach and adrenal gland. However, these pathological findings were noted spontaneous or incidental in a manner not dependent on dose. These symptoms are part of chronic progressive nephropathy often observed in female rats of old age. The high-dose group (8800 mg/kg) did not differ significantly from the control group in any organ. Overall, the histopathological observations were non-significant and not related to the treatment administered.

3.10. Effect of RRF on reproductive hormone profile

In this study, serum levels of reproductive hormone, namely sturgeon homologues of follicle stimulating hormones (FSH), luteinizing hormone (LH), prolactin (PRL), testosterone (T), estradiol (E₂) and progesterone (P), were dissected out by radioimmunoassay (RIA) before and after administration of RRF. Results indicated that RRF of three tested doses significantly increased circulating concentrations of T, E₂, and P, and decreased levels of LH, prolactin and FSH throughout the tested period. At three months, RRF of all doses significantly promoted E₂ production (all $p < 0.01$) and decreased FSH (all $p < 0.01$), LH ($p < 0.01$, $p < 0.01$ and $p < 0.05$, respectively) and PRL ($p < 0.05$, $p < 0.01$ and $p < 0.05$, respectively) levels as compared to controls. T concentration in middle- and high-dose group ($p < 0.05$ and $p < 0.01$) and circulating P level in high- dose group ($p < 0.01$) markedly ascended in parallel to control counterpart. At 6 month post administration, serum E₂ level (all $p < 0.01$), P concentration ($p < 0.05$, $p < 0.05$ and $p < 0.01$, respectively) of three dose groups, as well as T content in middle- and high-dose groups (both $p < 0.01$) increased substantially. While LH (all $p < 0.01$), PRL levels of all RRF-treated group ($p < 0.05$, $p < 0.01$ and $p < 0.05$, respectively) and

FSH of middle- and high- dose groups ($p < 0.05$ and $p < 0.01$, respectively) diminished obviously with respect to control counterparts. After the 4-week recovery period, E_2 level of three doses and T concentration in middle- and high- dose groups ($p < 0.05$ and $p < 0.01$) maintained a relatively higher level. LH ($p < 0.01$, $p < 0.05$ and $p < 0.01$, respectively) and FSH ($p < 0.05$, $p < 0.01$ and $p < 0.01$, respectively) levels of three doses experienced varied decrements in parallel to controls. As indicated by the data, long-termed RRF exposure might have the potential to inhibit PRL, FSH and LH releases and stimulate circulatory E_2 , T and P productions towards a more physiologically favorable balance, indicating possible positive regulation of endocrine imbalance caused by degradation of ovary function in female senescence rats.

Fig. 7. Serum circulating levels of estradiol (A), progesterone (B), testosterone (C), follicle stimulating hormones (D), luteinizing hormone (E) and prolactin (F) from rats exposed to 0, 1100, 4400, and 8800 mg/kg/day for 13, 26 weeks and 4 weeks of recovery. Levels of these parameters were measured by radioimmunoassay (RIA) and values are mean $\pm$ S.D. ($n = 10$ (13 weeks), 10 (26 weeks) or recovery (30 weeks)/dose). * $p < 0.05$ & ** $p < 0.01$ compared with normal group.

4. Discussion

Traditional use of plant-based remedies for treatment of diseases is widely practiced around the globe. It is estimated that approximately 60% of the world's population relies on herbal medications. Despite the worldwide use of traditional medicine, there have been concerns over the lack of safety information. Indeed, many researchers have undertaken to establish scientific evidences of the efficacy of herbal medicines, but few have initiated studies addressing their safety.

RRF is a botanical drug product that are intended to treat gynaecological disorders and that is now waiting for the approval of the CFDA of China for clinical trials. RRF was formulated with three well-documented herbs containing well-known bioactive constituents, which have been used traditionally as uterine tonics in treating many gynaecological ailments like infertility and menstrual disorders like menorrhagia and dysmenorrhoea. Besides the historical and experienced applications,

a toxicity assessment of this medicinal herbal product should be performed to optimize its safe application. In the present work, its immunologic effect and toxicity-related preclinical evaluation of RRF was carried out.

The immune system is an excellent marker of health, and immunopotentiating effect plays a substantial role for relief of perimenopausal disorders and promotion of health status in midlife women (Anderson, 1997). Danggui Buxue Tang (DBT), on which the formula RRF is based, was demonstrated to stimulate the immune response by inducing T-lymphocytes proliferation, interleukin-2 secretion and boosting the phagocytosis of macrophages *in vitro* (Gao et al., 2006; Gao et al., 2007). The results of the present work indicate an improvement in the innate immunity in mice administered RRF, as evidenced by significant elevation in phagocytic capability, serum IgM levels, immune organ index, and promoted DTH reaction. Herewith, this finding suggests that RRF has appreciable immunopotentiating property *in vivo*, which specifically acted by activating the function of the mononuclear phagocytic system, augmenting humoral immune response, and stimulating cellular immune response.

In the oral acute toxicity study, no significant mortality or alteration on the behavior patterns of the treated rats were observed as compared to their respective controls for experimental period of 14 days. At autopsy, not any gross lesions of internal organs were found. This suggests that RRF did not induce acute toxicity at the dose tested.

In the chronic oral toxicity assessment, there were no deaths or any gross signs of toxicity observed after oral administration of RRF up to the highest dose tested (8800 mg/kg/day) until the scheduled necropsy. No significant differences were observed in feed consumption, body weights and organ-to-weight ratios of the three experiment in parallel to the control group. Liver and kidney are the first organs to

show toxicity when they are exposed to potential toxic substances. From the results obtained, there were no significant impact on the usual biochemical markers of liver and kidney toxicity (the serum levels of transaminases ALT and AST, bilirubin as well as urea and creatinine) after RRF administration, it could be inferred that RRF might not induce significant alternation on hepatic and renal function. And no significant changes were observed in the electrolytes levels, suggesting that RRF did not affect the function and equilibrium of body fluid. It is noteworthy that the levels of blood glucose and triglyceride markedly decreased in the dose groups, compared to respective controls, indicating that RRF might exert hypoglycemic and lipid-modulating activity, which in some extent corresponded with previous reports of similar pharmacological activities of DBT (Kea et al., 2012; Zhang et al., 2007). These effects might be due to the presence of certain hypoglycemic and hypolipidemic chemicals in RRF. However, indepth investigation was merited. Some variations in other hematology and serum biochemistry parameters were considered to be not dose-dependent and treatment-related, and within the range of normal biological variation. Therefore, these were not considered to be elicited by RRF.

The toxicology profile of RRF was further studied by histopathological analysis on those isolated organs from both treatment and control rats. There were no significant changes or damages observed in the morphology of all isolated vital and reproductive organs, either from the rats ingested with RRF or normal rats. In the microscopic examination, some rats presented with anomalous pathologies but no consistent treatment-related histopathological abnormalities were observed in rats. These findings are readily detected in normal female rats of the age. Thus, the observations occurred sporadically in the treatment groups without a dose–response relationship were not considered to be changes attributable to repeated exposure of RRF, according to the comparison with the recovery and control group. No delayed

toxicity and abnormality related to the treatment were observed. This again suggests that daily oral administration of RRF for 26 weeks did not cause significant detrimental changes or morphological disturbances to the rats or to their vital organs.

Reproductive toxicity study is regarded as part of safety evaluation process which supplements toxicological systemic evaluations (Dalsenter et al., 2004). Therefore, this study evaluated some well-validated perimenopausal-associated pituitary–ovarian parameters in reproductive toxicology. The most pronounced endocrine changes for women during the perimenopause are decreased ovarian production of E₂, P, T, and increased pituitary production of FSH and LH (Jaffe, 1991). As a main hormone secreted from ovary, E₂ plays a crucial role in gonad and skeleton developments. LH and FSH are the predominant hypothalamic signals responsible for the modulation of synthesis and secretion of pituitary gonadotropins and are essential for steroidogenesis and follicular development in the ovary (Rubin, 2000). The dramatic descension of E₂ and elevation in FSH and LH are characteristics of a dampening of the positive hypothalamic-pituitary feedback response to estrogen (Ottolenghi et al., 2004), finally causing a panel of perimenopause syndromes (Hall, 2007). The findings outlined suggest that long-term administration of RRF exerted a significant promotion on serum level of steroid hormone E₂, T and P releases, along with remarkable decrease of circulating pituitary hormone PRL, FSH and LH levels in female rats. It might be inferred that RRF exhibit estrogenic properties, and appeared to retard certain age-associated degenerations, shifting the endocrine dynamic towards a more physiologically favorable balance. The finding was in concert with our previous investigation on the ovariectomy middle-aged female rats as well as natural aging female rat model (Xie et al., 2013; Su et al., 2013). Hence, RRF may be endowed with potential beneficial effects in relieving perimenopausal symptoms, ameliorating deleterious degeneration in aspects of perimenopausal-associated

pituitary-ovarian hormones.

In conclusion, RRF possessed immunopotentiating activity and had a relatively favorable safety profile under the study conditions. However, further investigations including genotoxicity, carcinogenicity and teratogenicity studies are desired further to support the safe therapeutic use of RRF. Also, experiments should be performed in a suitable non-rodent model before extrapolating the results in humans.

5. Conclusion

The present investigation demonstrated that RRF possessed appreciable *in vivo* immunopotentiating activity and had a relatively wide margin of safety, as it did not cause any lethality nor produced any remarkable physical, haematological, biochemical and histopathological adverse effects both in acute and chronic toxicity studies in rodents. Long-term treatment of RRF might exhibit estrogenic properties, and retard certain age-associated degeneration. RRF might have the potential for further development as a safe and effective alternative/complementary to conventional medication in relieving perimenopausal symptoms.

Acknowledgments

This work was supported by Grants from the Guangdong International Cooperation Projects in 2009 (No. 2009B050200004), National Science Foundation of China (No. 30973895 & 81503202), Science and Technological Program for Dongguan's Health Care Institutions (No. 2012105102012), Science and Technology Planning Project of Guangdong Province (No. 2014A020221042) and Natural Science Foundation of Guangdong Province (No. 2015A030310217).

Reference

Anderson, D.J., 1997. Interrelationship Between Endocrine and Immunologic Phenomena During the

- Perimenopause. Perimenopause, 78-86.
- China Pharmacopoeia Committee, 2010. Pharmacopoeia of the People's Republic of China, vol. 1. Chemical Industry Press, Beijing.
- Dalsenter, P.R., Cavalcanti, A.M., Andrade, A.J., Araújo, S. L., Marques, M.C., 2004. Reproductive evaluation of aqueous crude extract of *Achillea millefolium* L. (Asteraceae) in Wistar rats. Reproductive Toxicology 18(6), 819-842.
- Dong, T.T.X., Zhao, K.J., Gao, Q.T., Ji, Z.N., Zhu, T.T., Li, J., Duan, R., Cheung, A.W., Tsim, K.W.K., 2006. Chemical and biological assessment of a Chinese herbal decoction containing Radix Astragali and Radix Angelicae sinensis: determination of drug ratio in having optimized properties. Journal of Agricultural and Food Chemistry 54, 2767–2774.
- Duan, B.W., Li, Y., Liu, X., Yang, Y.J., 2010. Effect of polysaccharides immunologic function of immunosuppression mice. Chinese Journal of Chinese Material Medica 35(11), 1466-1469.
- Firenzuoli, F., Gori, L., 2007. Herbal medicine today: clinical and research issues. Evidence-based Complementary and Alternative Medicine 4 (S1), 37–40.
- Gao, Q.T., Cheung, J.K.H., Li, J., Chu, G.K.Y., Duan, R., Cheung, A.W.H., Zhao, K.J., Dong, T.T.X., Tsim, K.W.K., 2006. A Chinese herbal decoction, Danggui Buxue Tang, prepared from Radix Astragali and Radix Angelicae sinensis stimulates the immune responses. Planta Medica 73, 1227–1231.
- Gao, Q.T., Choi, R.C.Y., Cheung, A.W.H., Zhu, J.T., Li, J., Chu, G.K.Y., Duana, R., Cheunga, J.K.H., Jiang, Z.Y., Dong, X.B., Zhao, K.J., Dong, T.T.X., Tsim, K.W.K., 2007a. Danggui Buxue Tang—a Chinese herbal decoction activates the phosphorylations of extracellular signal-regulated kinase and estrogen receptor alpha in cultured MCF-7 cells. FEBS Letters 581, 233–240.
- Gao, Q.T., Li, J., Cheung, J.K., Duan, J., Ding, A., Cheung, A.W.H., Zhao, K., Li, W.Z., Dong, T.T.X., Tsim, K.W.K., 2007b. Verification of the formulation and efficacy of Danggui Buxue Tang (a decoction of Radix Astragali and Radix Angelicae sinensis): an exemplifying systematic approach to revealing the complexity of Chinese herbal medicine formulae. Chinese Medical Journal 2,

12–22.

- Gao, Q.T., Cheung, J.K., Li, J., Jiang, Z.Y., Chu, G.K., Duan, R., Cheung, A.W., Zhao, K.J., Choi, R.C., Dong, T.T., Tsim, K.W., 2007c. A Chinese herbal decoction, Danggui Buxue Tang, activates extracellular signal-regulated kinase in cultured T-lymphocytes. *FEBS Letter* 581(26), 5087-93.
- Gao, Q.T., Cheung, J.K.H., Choi, R.C., Cheung, A.W.H., Li, J., Jiang, Z.Y., Duan, R., Zhao, K.J., Ding, A.W., Dong, T.T.X., Tsim, K.W.K., 2008. A Chinese herbal decoction prepared from *Radix Astragali* and *Radix Angelicae sinensis* induces the expression of erythropoietin in cultured Hep3B cells. *Planta Medica* 74, 392-395.
- Greaves, P., 2007. *Histopathology of preclinical toxicity studies* (3rd edn). Academic Press: Amsterdam; 1.
- Hall, J. E., 2007. Neuroendocrine changes with reproductive aging in women. *Seminars in Reproductive Medicine* 25(5), 344-351.
- Hilaly, J.E., Israili, Z.H., Lyouss, B., 2004. Acute and chronic toxicological studies of *Ajuva Iva* in experimental animals. *Journal of Ethnopharmacology* 91, 43-50.
- Hudson, L., Hay, F.C., 1980. *Practical immunology*. 2nd ed. London: Blackwell, 73-92.
- Jaffe, R.B., 1991. The menopause and perimenopause period. In: Yen S, Jaffe R, eds. *Reproductive endocrinology*, 3rd ed. Philadelphia: WB Saunders, 389-393.
- Jothy, S.L., Zakaria, Z., Chen, Y., Lau, Y.L., Latha, L.Y., Sasidharan, S., 2011. Acute oral toxicity of methanolic seed extract of *Cassia fistula* in mice. *Molecules* 16(6), 5268-82.
- Kea, H.L., Zhanga, Y.W., Zhou, B.F., Zhen, R.T., 2012. Effects of Danggui Buxue Tang, a traditional Chinese herbal decoction, on high glucose-induced proliferation and expression of extracellular matrix proteins in glomerular mesangial cells. *Natural Product Research* 26,1022-6.
- Kim, W., Kim, S.H., Park, S.K., Chang, M.S., 2012. *Astragalus membranaceus* ameliorates reproductive toxicity induced by cyclophosphamide in male mice. *Phytotherapy Research* 26(9), 1418-21.
- Ma, S.P., Qu, R., Hang, B.Q., 1989. Immunologic inhibition and antiallergic action of *Cryptotympana*

- atrata* Fabricius. Chinese Journal of Chinese Material Medica 14(8), 490-493.
- Ottolenghi, C., Uda, M., Hamatani, T., Crisponi, L., Garcia, J.E., Ko, M., Pilia, G., Sforza, C., Schlessinger, D., Orabosco, A., 2004. Aging of oocyte, ovary, and human reproduction. Annals of the New York Academy of Sciences 1034, 117-131.
- Qin, L., Zhang, G., Shi, Y.Y., Lee, K.M., Leung, P.C., 2005. Prevention and treatment of osteoporosis with traditional herbal medicine. In: Deng, H.W. (Ed.), Current Topics of Osteoporosis. World Scientific Publisher, Singapore, 513-531.
- Rosidah, Yam, M.F., Amirin, S., Mariam, A., Gabriel, A.A., Zaini Mohd., A., 2009. Toxicology evaluation of standardized methanol extract of *Gynura procumbens*. Journal of Ethnopharmacology 123, 244-249.
- Rubin, B.S., 2000. Hypothalamic Alterations and Reproductive Aging in Female Rats: Evidence of Altered Luteinizing Hormone-Releasing Hormone Neuronal Function. Biology of reproduction 63, 968-976.
- Saad, B., Azaizah, H., Abu-Hijleh, G., Said, O., 2006. Safety of traditional arab herbal medicine. Evidence-based Complementary and Alternative Medicine 3(4), 433-9.
- Song, Z.H., Ji, Z.N., Lo, C.K., Dong, T.T., Zhao, K.J., Li, O.T., Haines, C.J., Kung, S.D., Tsim, K.W., 2004. Chemical and biological assessment of a traditional Chinese herbal decoction prepared from Radix Astragali and Radix Angelicae sinensis: orthogonal array design to optimize the extraction of chemical constituents Planta Medica, 70(12), 1222–1227.
- Su, J.Y., Xie, Q.F., Liu, W.J., Lai, P., Liu, D.D., Tang, L.H., Dong, T.T., Su, Z.R., Tsim, K.W., Lai, X.P., Li, K.Y., 2013. Perimenopause Amelioration of a TCM Recipe Composed of Radix Astragali, Radix Angelicae Sinensis, and Folium Epimedii: An *In Vivo* Study on Natural Aging Rat Model. Evidence-based Complementary and Alternative Medicine. doi: 10.1155/2013/747240.
- The Organization of Economic Co-operation and Development (OECD), 2001. The OECD guideline for testing of chemicals: 420 Acute oral toxicity. Paris: OECD.

- Wan, J., Tian, G., Tian, Y., Gao, M., Ai, Q., Li, Z., Mei, L., 2010. Investigation on the effect of angelica injection injection therapy via Guanyuan acupoint on mice with premature ovarian failure induced by tripterygium glycosides. *sJournal of Dalian Medical University* 32 (3), 265-269.
- Xie, Q.F., Xie, J.H., Dong, T.T., Su, J.Y., Cai, D.K., Chen, J.P., Liu, L.F., Li, Y.C., Lai, X.P., Tsim, K.W., Su, Z.R., 2012. Effect of a derived herbal recipe from an ancient Chinese formula, Danggui Buxue Tang, on ovariectomized rats. *Journal of Ethnopharmacology* 144 (3), 567-75.
- Yang, Q., Populo, S.M., Zhang, J., Yang, G., Kodama, H., 2002. Effect of *Angelica sinensis* on the proliferation of human bone cells. *Clinica Chimica Acta* 324, 89-97.
- Yuan, D., Wang, H., He, H., Jia, L., He, Y., Wang, T., Zeng, X., Li, Y., Li, S., Zhang, C., 2014. Protective effects of total flavonoids from *Epimedium* on the male mouse reproductive system against cyclophosphamide-induced oxidative injury by up-regulating the expressions of SOD3 and GPX1. *Phytotherapy Research* 28(1), 88-97.
- Zhao, X.F., He, H.G., Zhang, J.P., Shi, L.Y., 2012. Effects of *Astragalus* on the Cyclophosphamide-induced immune compromised mice. *Journal of Zhejiang University of Traditional Chinese Medicine* 6, 749–751.
- Zhang, Y.F., Yu, Q.H., 1999. The Immunoregulatory Effect of Total flavones *Epimedium*. *Journal of Shenyang Pharmaceutical University* 16(3), 182-184.
- Zhang, H., Chen, S., Deng, X., Yang, X., Huang, X., 2007. The effects of Danggui-Buxue-Tang on blood lipid and expression of genes related to foam cell formation in the early stage of atherosclerosis in diabetic GK rats. *Diabetes Research and Clinical Practice* 77(3), 479-81.
- Zheng, S.H., Ren, W.G., Huang, L.F., 2015. Geoherbalism evaluation of *Radix Angelica sinensis* based on electronic nose. *Journal of Pharmaceutical and Biomedical Analysis* 105, 101–106.

Table 1 Relative organ weights in rats orally treated with RRF.

Parameter	Dose (mg/kg)											
	0 mg/kg			1100 mg/kg			4400 mg/kg			8800 mg/kg		
	13 wk	26 wk	Recovery	13 wk	26 wk	Recovery	13 wk	26 wk	Recovery	13 wk	26 wk	Recovery
Brain	0.653±0.148	0.595±0.131	0.518±0.120	0.648±0.117	0.559±0.163	0.550±0.136	0.651±0.128	0.606±0.161	0.562±0.153	0.632±0.162	0.590±0.153	0.579±0.160
Liver	3.051±0.172	2.456±0.152	2.878±0.574	2.085±0.381	2.542±0.287	2.641±0.337	3.048±0.373	2.476±0.206	2.626±0.320	2.860±0.291	2.768±0.488	2.662±0.456
Heart	0.303±0.065	0.276±0.033	0.401±0.202	0.328±0.029	0.282±0.014	0.307±0.032	0.317±0.048	0.324±0.094	0.288±0.030	0.318±0.032	0.281±0.043	0.286±0.042
Lung	0.444±0.058	0.371±0.071	0.410±0.074	0.452±0.047	0.443±0.112	0.449±0.075	0.471±0.096	0.439±0.120	0.384±0.078	0.447±0.073	0.401±0.068	0.364±0.058
Spleen	0.194±0.024	0.186±0.066	0.164±0.025	0.206±0.032	0.158±0.042	0.154±0.021	0.177±0.032	0.173±0.019	0.157±0.033	0.197±0.031	0.189±0.033	0.182±0.027
Kidney	0.642±0.106	0.588±0.076	0.601±0.126	0.671±0.049	0.583±0.035	0.611±0.049	0.660±0.063	0.574±0.033	0.609±0.105	0.647±0.031	0.637±0.042	0.640±0.117
Adrenal	0.019±0.006	0.017±0.007	0.012±0.004	0.021±0.010	0.015±0.005	0.015±0.007	0.017±0.009	0.016±0.005	0.014±0.008	0.020±0.011	0.019±0.007	0.015±0.007
grand												

Thymus	0.077±0.023	0.051±0.015	0.069±0.048	0.099±0.029	0.041±0.026	0.056±0.015	0.097±0.035	0.044±0.020	0.052±0.009	0.084±0.023	0.059±0.022	0.078±0.037
Uterus	0.317±0.131	0.267±0.052	0.408±0.084	0.334±0.104	0.283±0.054	0.435±0.073	0.363±0.194	0.323±0.069	0.500±0.164	0.335±0.082	0.334±0.112	0.391±0.183
Ovary (Lt.)	0.062±0.009	0.045±0.008	0.054±0.014	0.070±0.012	0.051±0.003	0.045±0.014	0.065±0.010	0.049±0.009	0.047±0.008	0.054±0.011	0.052±0.009	0.051±0.016

Note: The relative organ weight was calculated based on the terminal body weight before fixation Values are mean ± S.D. ($n = 10$ (13 weeks), 10 (26 weeks) or recovery (30 weeks)/dose). * $p < 0.05$ & ** $p < 0.01$ compared with normal group.

Table 2 Hematological values in rats orally treated with RRF.

Parameter	Unit	Hematological values											
		0 mg/kg			1100 mg/kg			4400 mg/kg			8800 mg/kg		
		13 wk	26 wk	Recovery	13 wk	26 wk	Recovery	13 wk	26 wk	Recovery	13 wk	26 wk	Recovery
WBC	10 ⁹ /L	6.62±2.04	3.68±1.58	4.24±1.85	5.14±1.84	3.45±1.08	3.98±1.97	5.50±1.10	4.24±1.82	2.80±1.22	6.13±2.30	3.83±1.13	3.01±1.38
NEUT	%	19.11±7.78	6.55±3.54	5.41±2.43	20.21±9.04	8.70±6.98	5.52±2.19	19.21±13	8.34±4.89	5.31±1.81	23.34±8.3	7.91±2.88	7.44±2.45
								.21			5		
LYM	%	60.49±7.03	43.38±16.16	40.12±20.31	66.56±10.49	43.10±11.85	39.90±21.02	68.41±13	39.93±13.90	44.96±10.1	58.56±7.0	43.41±9.61	42.37±12.2
								.55		9	6		2
MONO	%	7.73±5.06	27.11±7.67	34.00±16.49	4.70±2.70	30.94±11.21	35.52±14.95	4.84±4.2	29.84±5.51	32.54±5.98	7.35±3.55	30.26±7.52	33.83±10.5
								4					9
BAS	%	0.30±0.11	0.21±0.11	0.24±0.11	0.23±0.11	0.27±0.16	0.18±0.09	0.21±0.1	0.21±0.11	0.23±0.11	0.24±0.12	0.15±0.11	0.16±0.08
								2					
EOS	%	1.90±0.94	3.09±1.79	2.24±1.21	1.85±0.57	2.45±1.91	2.23±1.19	2.06±1.0	2.61±1.28	2.40±1.19	2.02±0.94	2.37±1.04	2.36±1.07

2													
NE	10 ⁹ /L	1.25±0.61	0.21±0.06	0.21±0.10	1.06±0.59	0.27±0.17	0.20±0.10	1.11±0.9	0.40±0.37	0.14±0.05	1.43±0.82	0.31±0.17	0.22±0.11
1													
LYM	10 ⁹ /L	3.99±1.27	1.73±1.10	1.99±1.70	3.43±1.39	1.54±0.78	1.70±1.51	3.69±0.7	1.66±0.73	1.23±0.53	3.54±1.28	1.65±0.59	1.27±0.65
9													
MON	10 ⁹ /L	0.52±0.36	0.90±0.19	1.22±0.30	0.28±0.15	1.02±0.36	1.34±0.82	0.41±0.5	1.31±0.82	0.93±0.47	0.49±0.40	1.15±0.44	0.88±0.63
0													
EOS	10 ⁹ /L	0.12±0.06	0.11±0.08	0.09±0.06	0.16±0.22	0.08±0.05	0.09±0.05	0.11±0.0	0.11±0.06	0.07±0.05	0.17±0.18	0.10±0.06	0.06±0.03
6													
RBC	10 ¹² /L	8.17±0.70	8.25±0.81	7.61±0.62	8.26±0.97	8.26±0.44	7.85±0.69	8.34±0.63	8.28±0.70	7.92±1.02	8.22±0.60	8.57±0.69	7.53±0.37
HGB	g/L	150.50±10.3	144.90±10.2	134.40±8.53	148.00±11.6	144.60±5.13	137.60±7.89	151.40±7.93	146.40±7.18	139.50±8.8	150.70±8.	148.70±7.56	134.30±8.7
271547													
HCT	%	45.40±3.60	45.60±3.41	41.80±2.82	44.60±4.17	44.50±1.78	42.60±2.59	45.90±3.00	45.60±2.37	43.90±3.14	45.30±3.1	47.10±2.51	41.60±1.84

3

MCV	fL	55.56±1.51	55.42±2.14	55.15±1.94	54.09±1.84	53.98±1.78	54.52±1.97	55.09±1.51	55.30±2.51	55.72±4.51	55.11±1.3	55.21±2.20	55.33±1.45
											1		
MCH	pg	18.48±0.58	17.61±0.98	17.68±0.58	18.01±0.95	17.53±0.76	17.60±0.79	18.17±0.63	17.72±0.86	17.78±1.60	18.36±0.5	17.39±0.81	17.85±0.90
											4		
MCHC	g/L	332.60±6.31	318.10±8.77	320.80±9.25	332.40±7.92	324.90±4.84	322.50±4.50	330.20±8.32	320.70±5.14	319.00±4.9	333.30±7.	315.00±4.35	322.50±9.7
										4	38		1
RDW	%	13.23±2.26	12.97±2.59	11.72±1.46	13.08±1.73	12.42±0.89	11.51±0.89	14.00±3.37	12.16±0.84	11.83±1.03	12.06±1.4	12.22±0.63	11.50±0.89
											5		
HDW	%	27.75±1.74	23.83±2.99	20.81±2.56	28.71±1.68	23.59±2.36	21.15±1.87	28.61±2.73	23.50±1.96	20.93±1.81	27.04±1.6	22.84±1.16	20.95±1.45
											7		
PLT	10 ⁹ /L	980.40±134.	979.40±220.	853.20±103.	1076.60±96.	918.40±82.1	814.70±83.4	1029.30±13	901.30±106.	832.70±105	967.00±9	867.50±96.1	733.50±176
		99	00	90	43	8	9	3.26	07	.29	0.84	7	.49
MPV	fL	9.84±0.36	8.45±0.48	8.37±0.29	9.34±0.28**	8.55±0.14	8.57±0.44	9.59±0.41	8.52±0.14	8.59±0.41	9.76±0.36	8.60±0.20	8.43±0.43
PCT	s	0.97±0.17	0.81±0.18	0.72±0.08	1.01±0.10	0.78±0.06	0.70±0.08	0.99±0.15	0.77±0.10	0.72±0.10	0.95±0.11	0.75±0.09	0.62±0.14

PDW	%	34.83±1.27	40.22±1.27	40.16±3.05	35.29±1.22	39.85±1.08	37.94±1.02	35.54±1.48	39.29±1.22	38.21±2.08	35.80±1.0	40.00±1.33	39.43±2.21
											5		
MPC	(g/d)	172.50±4.90	180.40±9.01	178.60±2.88	180.10±4.93	179.70±5.29	175.70±3.23	174.30±5.62	175.40±3.17	175.30±4.4	173.60±3.	177.70±4.30	173.30±6.2
					**		*			7	92		0
PT	s	69.20±53.48	70.20±41.99	79.40±45.89	149.80±120.	42.40±27.93	44.70±20.50	134.70±88.4	39.00±24.43	60.70±20.7	134.70±7	63.90±34.06	53.80±30.0
					96			2		2	7.88		9
RET	%	2.33±0.74	3.13±0.63	2.97±0.50	2.97±0.82	2.75±0.33	2.85±0.29	2.32±0.66	3.37±0.64	3.20±1.04	2.75±0.75	3.63±0.57	3.18±0.59

Note: Red blood cell (RBC), hemoglobin (HGB), hematocrit (HCT), mean corpuscular volume (MCV), mean corpuscular HGB (MCH), mean corpuscular HGB concentration (MCHC), red cell distribution width (RDW), hemoglobin distribution width (HDW), platelets (PLT), plateletocrit (PCT), mean platelet volume (MPV), mean platelet component (MPC), platelet distribution width (PDW), white blood cell (WBC) counts, reticulocytes (RET), neutrophils (NE), lymphocytes (LYM), monocytes (MON), eosinophils (EOS), and basophils (BAS) prothrombin time (PT). Values are mean ± S.D. ($n = 10$ (13 weeks), 10 (26 weeks) or recovery (30 weeks)/dose). $*p < 0.05$ & $**p < 0.01$ compared with normal group.

Table 3 Serum biochemical values in rats orally treated with RRF.

		Dose (mg/kg)											
Parameter		0 mg/kg			1100 mg/kg			4400 mg/kg			8800 mg/kg		
Unit		13 wk	26 wk	Recovery	13 wk	26 wk	Recovery	13 wk	26 wk	Recovery	13 wk	26 wk	Recovery
ALT	U/L	29.50±7.50	52.00±25.15	56.00±44.34	28.40±12.89	47.60±18.51	76.20±68.38	30.10±11.66	33.70±14.24	66.1±60.59	27.20±7.93	54.20±22.58	58.50±45.01
AST	U/L	127.60±22.24	189.40±86.08	157.20±135.66	124.30±36.34	159.70±42.71	161.80±119.89	118.50±21.69	130.80±41.77	154.20±107.39	140.80±19.81	171.20±46.15	126.70±60.59
TP	g/L	66.71±3.12	59.80±10.26	65.60±5.56	68.62±4.18	62.79±2.33	67.70±6.75	67.53±2.77	64.86±4.01	67.03±8.40	68.63±4.22	62.41±4.69	64.32±7.71
ALB	g/L	41.40±2.45	36.82±6.68	42.05±5.99	42.27±3.00	38.45±4.07	42.89±5.97	41.09±2.32	39.64±3.45	43.65±7.45	42.28±3.90	38.83±4.58	41.43±7.87
T-BIL	μmol/L	1.61±0.35	1.83±0.72	1.62±0.42	1.64±0.39	1.95±0.63	2.23±0.92	1.55±0.28	1.57±0.60	1.68±0.70	1.54±0.32	1.68±0.31	1.50±0.37
CHOL	mmol/L	1.45±0.31	1.49±0.39	1.83±0.32	1.57±0.23	1.37±0.21	2.12±0.95	1.49±0.28	1.46±0.49	1.90±0.62	1.58±0.29	1.33±0.56	1.87±0.60
TG	mmol/L	0.73±0.34	0.62±0.23	0.62±0.22	0.35±0.05**	0.44±0.06*	0.71±0.24	0.46±0.28	0.47±0.10	0.75±0.29	0.42±0.16*	0.66±0.38	0.60±0.15

CPK	μmol/ L	1330.10±430. 69	1816.40±311.20	1095.90±987.03	1425.70±862.68	1316.10±691. 43	1222.00±698.98	1346.50±553. 11	1418.20±987. 72	1658.90±602.03	1491.00±462. 39	1862.80±739. 88	1591.30±1207.0 3
	mmol /L	141.30±2.75	142.90±2.23	140.60±0.97	141.60±0.52	143.30±1.16	140.00±0.94	141.90±1.20	144.00±0.67	141.10±1.37	141.70±0.67	142.20±1.03	140.40±1.17
K ⁺	mmol /L	4.90±0.73	5.15±0.24	4.35±0.61	4.71±0.55	4.92±0.71	4.27±0.35	4.80±0.44	4.80±0.61	4.45±0.67	4.62±0.47	4.92±0.44	4.66±0.68
	mmol /L	103.27±1.46	103.76±1.44	101.67±1.88	104.59±0.86	104.31±1.27	103.07±0.89	104.55±1.15	104.28±1.25	103.30±3.01	104.16±1.43	103.27±1.18	103.11±1.46
GLU	mmol /L	12.56±1.37	8.78±1.39	10.11±1.66	8.56±1.94**	8.98±1.08	10.19±1.37	8.91±2.37**	8.17±1.09	9.95±2.46	10.17±3.09*	8.62±1.18	11.28±1.75
	μmol/ L	42.00±6.13	47.90±20.85	48.40±7.55	45.10±6.51	44.40±4.58	43.30±7.12	42.80±4.13	52.80±5.29	44.50±8.90	43.20±4.47	56.90±12.74	52.40±11.12

Note: Aspartate aminotransferase activity (AST), alanine aminotransferase activity (ALT), albumin (ALB), total protein (TP), glucose (GLU), total cholesterol (CHOL), creatine phosphokinase activity (CPK), total bilirubin (T-BIL), creatinine (Cre), triglycerides (TG), sodium ions (Na⁺), potassium ions (K⁺), chlorideions (Cl⁻). Values are mean ± S.D. (*n* = 10 (13 weeks), 10 (26 weeks) or recovery (30 weeks)/dose). **p* < 0.05 & ***p* < 0.01 compared with normal group.

Graphical Abstract