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Toxicological risks of *Rhizoma Paridis* saponins in rats involved NF-κB and Nrf2 signaling

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Abstract

The aim of the study was to safety evaluate the long-term use of Rhizoma Paridis saponins (RPS). After 90-day administration of RPS in rats, it induced the liver and lung injury through the over-expression of reactive oxygen species (ROS) and pro-inflammatory cytokines, and down-regulating the levels of antioxidant and detoxified enzymes. Meanwhile, as the self-protection of rats, RPS treatment activated the transcription of Nrf2 and elevated the expression of GSH and HO-1 to inhibit tissues getting worse. After 30 days' recovery, abnormalities in liver and lungs disappeared accompanying with the levels of phase II enzymes, pro-inflammatory cytokine, and nuclear factors back to normal. In conclusion, 350 mg/kg/day of RPS inducing toxicity and detoxicity reaction involved NF-κB and Nrf2 signaling. Our work provided useful data for correct administration of RPS and minimizes the danger of toxic herbal product use.

Keywords: *Rhizoma paridis* saponins; oxidative stress; inflammation; self-protection; recovery

Abbreviations: ALT, alanine amino transferase; AST, aspartate amino transferase; BUN, Blood urea nitrogen; CAT, catalase; COX-2, cyclooxygenase-2; Cr, creatinine; ELISA, enzyme-linked immunosorbent assay; GSH, reduced glutathione; GSSG, oxidized glutathione; GST, glutathione-S-transferase; γ-GT, γ-glutamyl- transferase; HO-1, heme oxygenase-1; IL6, interleukin 6; MDA, malonaldehyde; Nrf-2, nuclear factor-2 erythroid related factor-2; 8-OHdG, 8-hydroxy-2'-deoxyguanosine; ROS, reactive oxygen species; RPS, Rhizoma Paridis saponins; RT-PCR, reverse transcription polymerase chain reaction; SOD, superoxide dismutase; TNF-α, tumor necrosis factor α.

Introduction

Paris polyphylla var. *yunnanensis* (Fr.) Hand.-Mazz. (PPY) as a traditional Chinese medicinal herb has an effect of heat-clearing and detoxicating, detumescence and acesodyne in folk for a long time [1]. It is reported that *Rhizoma Paridis* saponins (RPS) belonging to the steroidal saponins are the main and active components in *Paris polyphylla* [2-4]. In traditional clinical usage, excessive ingestion of RPS could cause side effects such as nausea, vomiting, diarrhea and even heart palpitations and convulsions. As RPS become more popular for their activity, there have been increasing concerns about safety and potential toxicity of RPS.

Our preliminary research revealed that the main kinds of components in RPS included diogeninyl and pennogenyl saponins [5]. Dioscin as one of the diogeninyl compounds containing in RPS showed slight gastro-intestinal tract distension during the treatment period and hemolytic anemia in the hematology assessment [6]. Meanwhile, RPS possessed sedative-hypnotic activity, gastric stimulus [7] and hepatotoxicity [8] side effects. However, scientific information on RPS regarding the toxic effects is limited. Taken into account these drawbacks, it is critical to understand the toxicological property associated with the long-term use of RPS.

The production of reactive oxygen species (ROS) has been identified to contribute to drug-induced liver, heart, renal and brain toxicity [9]. To manage oxidative stress, cells possess antioxidant protection mechanisms, which primarily consist of classical antioxidant enzymes such as superoxide dismutase (SOD), catalase (CAT), the

glutathione molecule (GSH), and of an additional group of enzymes, termed phase II detoxifying enzymes, that include glutathione-S-transferase (GST) and heme oxygenase-1 (HO-1) [10]. In this research, we focused on the long-term toxicity of RPS involving detoxifying enzymes and antioxidant enzymes.

Materials and methods

Drugs

The dried rhizoma of *Paris polyphylla* var. *yunnanensis* was collected in September 2010 from Lijiang, Yunnan Province, China, and identified by Professor Gao. A voucher specimen (GWCL201009) was deposited at the School of Pharmaceutical Science and Technology at Tianjin University, Tianjin, China. Rhizoma Paridis saponins (RPS) was prepared as previously described.

Experimental animals

Eighteen male Sprague-Dawley rats at six weeks of age were purchased from the Laboratory Animal Center of academy of Military Medical Sciences (Beijing, China quality certification number: SCXK (Jun) 2012-0004). All the experiments involving rats were approved by local Animal Ethics Committee and performed in strict compliance with the ethical guidelines issued by national legislations of China and local guidelines. After one week of acclimatization, the rats were housed in a room maintained at 24±1°C, relative humidity of 55±5%, artificial lighting from 8:00 to 19:00 and air-exchange rate of 18 times per hour. The animals were kept in stainless-steel wire-mesh cages and allowed free access to tap water and diet. Before carrying out the animal experiment, we measured the body weight of each rat. The rats were randomly allocated into three groups. The high dose of RPS (RPSH) was administrated orally 350 mg of RPS in 10 mL of 0.9%

sodium chloride per kg of body weight every day for 90 days. The lower dose of RPS (RPSL) was fed orally 50 mg of RPS in 10 mL of 0.9% sodium chloride per kg of body weight every day for 90 days. Normal control rats were administered appropriate vehicles. During the experiment, the body weight of each rat was measured every week. Food consumption, water consumption, urine volume, urine density and urine pH were monitored every other day during the course of the study. Blood samples (0.5 mL) were collected into heparinized tubes from each rat by the puncture of the retro-orbital sinus on the 30^{th}, 60^{th}, 90^{th} and 120^{th} days. Blood was immediately processed for plasma by centrifugation at 3500 g for 15 min. Plasma was frozen and maintained at -20°C until analysis. After the end of collection, all the rats were sacrificed. Autopsies were harvested. Portions of each tissue were fixed in 10% formalin (pH 7.4) for histology, snap frozen in liquid nitrogen for oxidative stress test.

Histopathological examination

For the histopathological examination, portions of the liver, kidney, heart, spleen, brain and lung tissues were fixed in 10% formalin, and after proper dehydration, the tissues were embedded in paraffin wax. Five-µm-thick sections were prepared and stained with hematoxylin and eosin. Every organ was randomly cut into 6 histological sections. Histopathology examination was completed using Nikon eclipse TE2000-U Microscope and performed by a pathologist who was unaware of whether tissues were treated.

Biochemical analyses

Serum levels of alkaline phosphatase (ALP), aspartate transaminase (AST), alanine transaminase (ALT), gamma glutamyl transpeptidase (γ-GT), blood urea nitrogen (BUN) and creatinine (Cr)

were measured by the detection kits based on the manufacturer's instructions obtained from Nanjing Jiancheng Institute of Biotechnology (Nanjing, China).

Oxidative stress in the liver tissues

Livers fixed for snap frozen in liquid nitrogen were rapidly washed and homogenized in ten volumes (v/w) of ice-cold saline solution. The homogenate was centrifuged at 3000 rpm for 10 min. Its supernatant was used as a total liver homogenized sample (10% homogenates). The levels of malondialdehyde (MDA), superoxide dismutase (SOD), reduced glutathione (GSH), oxidized glutathione (GSSG) and glutathione S-transferase (GST) enzyme activities were measured by spectrophotometry according to the manufacturer's instructions using commercially available kits (Nanjing Jiancheng Bioengineering Institute).

Reactive oxygen species (ROS) release assay

The tissue cell samples were prepared following the ROS assay kit manufacturer's instructions (Applygen Technologies Inc., Beijing, China). Intracellular ROS concentration was measured using the oxidant-sensitive fluorescent probe, 2,7-dichlorofluorescin (DCFH) diacetate (DA). Cells was mixed with 400 nM DCFHDA for 30 min and then washed with 1 x PBS. The oxidation product dichlorofluorescein (DCF) fluorescence was imaged C6 Soft ware.

Assay of nuclear 8-hydroxy-2-deoxyguanosine (8-OHdG) level

8-OHdG is produced by the oxidative damage of DNA and serves as an established marker of oxidative stress. Each urine sample was centrifuged at 3000 rpm for 20 min, and the supernatant was used for measuring 8-OHdG concentration. The level of 8-OHdG was determined by an

ELISA kit following manufacturer's instructions (Huiying Co., Shanghai, China).

Measurements of COX-2, TNF-α and IL-6 levels by ELISA assays

Plasma samples were analyzed for COX-2 (Huole Co., Shanghai, China), TNF-α and IL-6 (Suer Co., Shanghai, China) with rat ELISA kits by following the manufactures' instructions. Each sample was assayed in duplicate, and the values were within the linear portion of the standard curve.

RT-PCR analysis

Total RNA was isolated from rat liver and lung using TRIzol (Life Technologies Inc.) according to the manufacturer's instruction. The quality of RNA was assessed by the absorbance of the samples at 260 and 280 nm. cDNA synthesis was performed using RevertAidTM M-MuLV RT (Fermentas, Hanover, MD, USA) according to the supplier's protocol. Resulting reverse transcription products were stored at -80 □ until analysis. A series of cDNA was amplified in PCR reactions consisting of 1×Taq polymerase buffer with 1.5 mM of $MgCl_2$ (Promega, Madison, WI), 200 mM of each dNTP, 10 pmol of each primer pair (**Table S1**), and 1 U of Taq DNA polymerase (Promega). Polymerase chain reaction products were electrophorized on 3.0% agarose gel and visualized after ethidium bromide staining.

Western blot assay

Total proteins from livers and lungs were isolated using the tissue protein extraction kit (Bio-Rad, USA), and the obtained protein was quantified by the Bradford Assay Kit (BioRad, Hercules, CA). The protein samples (20-100 μg) were separated on 12% SDS-PAGE. Proteins were transferred to

PVDF membranes (Millipore Corp., USA) and probed with GST-α, GAPDH, HO-1 (Bioworld Technology, USA),.GST-μ, GST-π (Boster, China), and Nrf2, NF-κB (Santa Cruz Biotechnoligy, USA) antibodies followed by appropriate secondary antibody. Equal protein loading was checked by quantifying GAPDH. The relative optical densities of the bands were quantified using Kodak Imaging Program and Image-Pro Plus software.

Immunohistochemistry

Nrf2 in livers were detected by direct immunostaining. All the sections were examined and taken pictures under Nikon eclipse TE2000-U Microscope. Li J and Man SL examined the immunohistochemistry slides independently. Li J utilized an immunohistochemical score (IHS) to estimate each group's immunoreactivity.

Statistical Analysis

Statistical evaluation was conducted by using the SPSS 17.0 for Windows package software. Data have been expressed as the means ± standard error mean (SEM). One-way variance analysis and Duncan multiple range test were used to determine significantly different groups. P values less than 0.05 were considered as significant differences for all statistical calculations.

Results

General observations

None of the animals died during the study period. However, compared with the control group, the rats in RPS group exhibited general variations, such as body trembling, smaller body weight gain (**Figure 1**), less physical activity, diarrhea and

some altered feces. Meanwhile, the fur of the rats in the RPS treated group was not as smooth and glossy as the ones in the control group. However, no statistical differences were observed for the urinalysis data (**Table 1**).

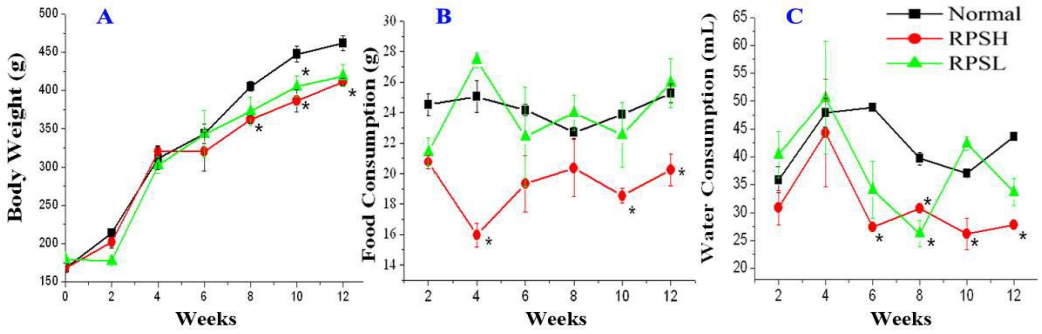

Figure 1 Body weight and food/water consumption changes of SD rats during the 90-day toxicological assessment. A, Body weight changes; B, Food consumption changes; C. Water consumption changes during the 90-day toxicological assessment. *p<0.05, compared with normal group.

Table 1 Urinalysis values of SD rats.

Groups	pH	Urine volume (mL/24 h)	Specific gravity (g/mL)	Urine Protein (mg/mL)
Normal	9.14±0.15	4.28±2.05	0.99±0.02	16.00±2.56
RPS H	8.91±0.56	5.38±1.54	0.99±0.03	17.66±3.10
RPS L	9.27±0.18	4.57±1.93	1.00±0.03	15.24±2.70

Hematology and clinical biochemistry

Selected hematology data were shown in **Table 2**. No significant changes were observed for any of the parameters examined. Clinical biochemistry data were shown in **Table 3**. After ninty-day administration of 350 mg/kg/day of RPS by oral, a significant increase in concentration of transaminases and alkaline phosphatase

indicated liver function failure. The levels of ALP, ALT and AST have been decreased after withdrawal of RPS for thirty days recovery. BUN and Cr levels were increased on the 30th day, while being returned to normal on the 90th day.

Table 2 Hematology values of male SD rats.

	WBC ($\times10^9$/L)	LY ($\times10^9$/L)	RBC ($\times10^{12}$/L)	HCT (%)	MCV (fL)	HGB (g/dL)	MCH (pg)	PDW (%)	MPV (fL)	PCT (%)	P-LCR (%)
Normal	10.2±0.5	9.5±0.3	9.3±0.1	60.7±0.4	65.0±0.4	167.5±0.9	18.0±0.3	7.2±0.0	15.7±0.4	0.9±0.1	65.0±2.2
RPS H	12.1±2.0	10.8±1.8	9.5±0.2	60.9±0.8	64.2±0.4	170.2±2.4	18.0±0.2	7.3±0.1	16.1±0.8	0.9±0.1	67.5±4.8
RPS L	10.8±0.4	9.3±0.0	9.3±0.1	60.2±0.5	64.7±0.4	163.0±2.0	17.5±0.2	7.2±0.0	17.8±0.1	0.6±0.0	76.8±0.6

Table 3 Clinical biochemistry values of SD rats during 90-day treatment and the recovery after 90-day long-term toxicity.

Parameters	Period	Normal	RPS H	RPS L
ALP (U/gprot)	30th day	16.40±0.85	25.32±1.91*	22.53±1.00*
	90th day	8.51±0.28	11.04±0.69*	8.51±0.78
	Recovery	2.38±0.39	2.92±0.55	2.64±0.15
ALT (U/gprot)	30th day	10.45±1.20	21.33±2.37*	21.52±3.94*
	90th day	12.24±4.75	41.29±8.23*	18.56±1.54
	Recovery	27.26±6.02	28.64±3.97	31.41±3.56
AST (U/gprot)	30th day	141.7±5.8	536.7±20.2*	475.9±5.8*
	90th day	141.7±15.5	644.7±3.4**	256.5±13.5*
	Recovery	163.0±0.6	174.8±1.8*	171.3±0.2*
γ-GT (U/L)	30th day	15.40±0.62	28.53±4.37*	25.27±4.21
	90th day	12.32±4.14	35.66±7.27*	16.44±3.27
	Recovery	23.41±1.50	47.91±4.70*	31.78±2.12
BUN (mmol/L)	30th day	467.0±143.1	615.8±147.0*	403.0±64.4
	90th day	638.4±97.1	691.2±75.1	563.1±51.2
	Recovery	492.0±6.9	447.8±8.1	477.4±7.6
Cr (μmol/L)	30th day	53.79±0.57	64.57±2.91*	54.16±6.00
	90th day	62.59±0.22	56.75±3.71	59.76±7.98
	Recovery	33.83±1.66	41.12±1.11	39.14±3.32

* $p < 0.05$, ** $p < 0.01$, compared with normal group.

Organ weight

Organ weight data were shown in **Table 4**. Absolute organ weight of the lung was significantly elevated in the RPS treated rats compared with the normal one ($p<0.05$). No statistically significant variations were detected in any other organs. Meanwhile, the lung weight of the recovery RPSH-treated rats was returned to normal.

Table 4 Absolute organ weights of the male SD rats (g).

Parameters	Normal	RPS H	RPS L
Brain	1.99±0.01	1.90±0.02	1.94±0.05
Heart	1.23±0.01	1.26±0.02	1.24±0.01
Lung	1.36±0.05	1.77±0.10*	1.58±0.11
Liver	11.10±0.42	10.18±0.13	10.86±0.48
Thymus	0.30±0.02	0.27±0.02	0.32±0.03
Spleen	0.52±0.03	0.55±0.06	0.51±0.03
Stomach	1.90±0.03	1.98±0.05	1.97±0.08
Kidney	2.48±0.09	2.26±0.05	2.50±0.09
Adrenals	0.08±0.00	0.07±0.00	0.07±0.01
Prostate	0.89±0.09	0.77±0.02	0.97±0.12
Spermatophore	1.15±0.04	1.06±0.26	1.20±0.06
Testes	3.12±0.08	3.32±0.09	3.23±0.14
Epididymide	0.56±0.01	0.52±0.02	0.57±0.01

*$p<0.05$, compared with normal group.

Histopathological examination

Microscopic examination of organs was performed on animals from different groups. Histopathological examinations of livers and lungs showed some abnormalities in RPSH groups. Liver section from control rats showed normal liver histologic architecture with the central vein and surrounding normal hepatocytes. However,

occasional binucleated cells, massive hepatocellular necrosis, diffuse necrotic cells, and infiltration of inflammatory cell were observed in the RPSH-treated livers probably due to drug toxicity (**Figure 2B1**). In addition, histopathology revealed mild chronic lymphocytic interstitial infiltrates and fibrinous exudate occupying the RPSH-treated alveolar spaces. The alveoli were lined by plump vacuolated pneumocytes (**Figure 2B2**). There were no abnormalities in other organs.

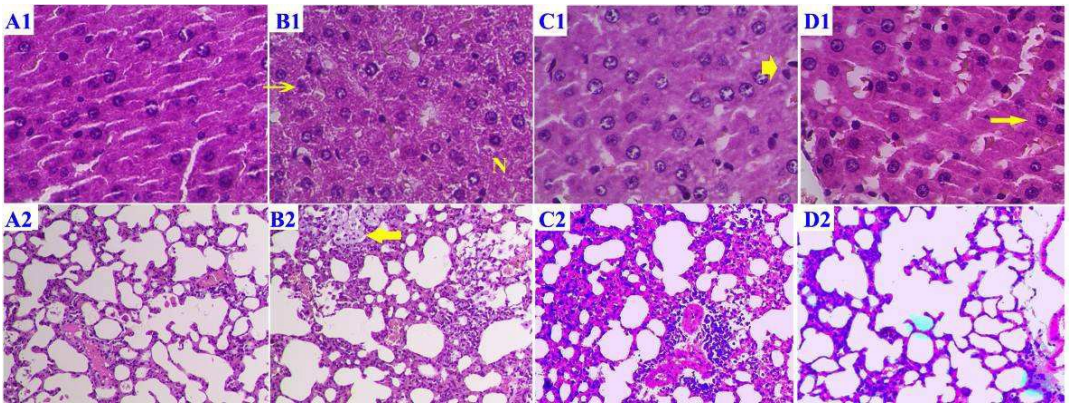

Figure 2 Representative sections (HE staining) of the rat livers and lungs (x 200). A, normal group, B, RPSH group on the 90th day, C, RPSL group, D, RPSH after recovery period. 1 means liver tissues, 2 means lung tissues. Pathological finding in RPSH treated liver is microfoci of necrosis (N) in B1. Arrow indicated different kinds of hepatocytes such as increased number of binucleate hepatocytes in B1, hypertrophy and hyperplasia of Kupffer cells in C1, single normal hepatocytes in D1. Arrow indicated intra-alveolar fibrin and mild chronic interstitial infiltrate in B2.

Oxidative stress on DNA and lipid

8-OHdG as a marker of oxidative DNA damage was significantly elevated in RPSH-treated rats (p<0.05) and returned to normal at recovery. MDA as a product of lipid

peroxidation was also remarkably increased in RPSH-treated groups (p<0.05), and reduced at recovery (**Figure 3**).

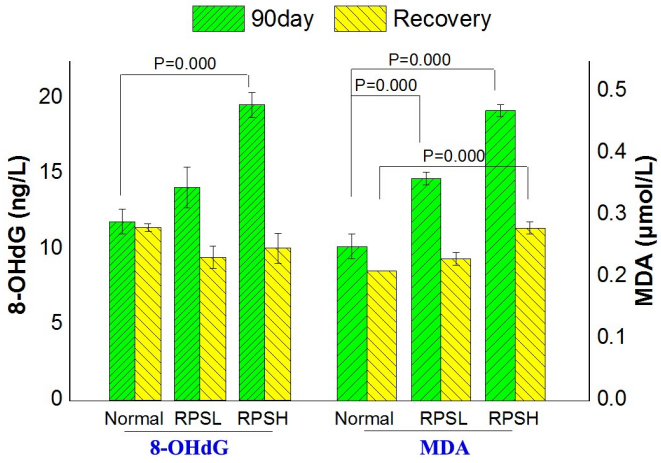

Figure 3 Effect of RPS on the levels of 8-OHdG and MDA in SD rats. Each value is expressed as mean ± S.E.M..

Effects of RPS on the oxidative stress of liver tissues

ROS generation was monitored through increases in fluorescence intensity of dichlorofluorescein. As shown in **Figure 4**, the levels of ROS were detected in liver cells with over-expressed under RPS treated group. Therefore, increasing evidence indicated that ROS was accumulated by RPS. The relative antioxidant enzymes including SOD, CAT and GSH were detected and shown in **Table 5**. Levels of SOD and CAT were significantly decreased in RPS-treated groups (p<0.05). In contrast, the level of GSH was remarkably increased (p<0.05), which may be related to the self-protecting mechanisms due to the liver oxidative stress induced by RPS.

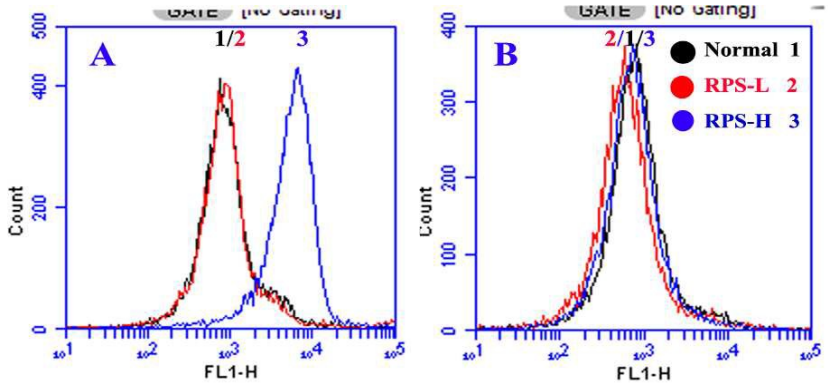

Figure 4 Effect of RPS on the production of cellular ROS in liver tissues. Liver cells were labeled with DCF-DA and examined for ROS production by flow cytometer. (A) 90-day (B) Recovery.

Table 5 Antioxidant index values of liver in male SD rats.

Parameters	90-days			Recovery		
	Normal	RPS-L	RPS-H	Normal	RPS-L	RPS-H
CAT (U/mgprot)	15.64±0.16^{a}	13.83±0.02^{b}	9.24±0.05^{c}	14.31±0.28^{a}	12.93±0.15^{ab}	9.71±1.84^{b}
SOD (U/mgprot)	1.18±0.03^{a}	0.64±0.06^{b}	0.51±0.03^{b}	1.00±0.00^{a}	0.73±0.02^{b}	0.63±0.01^{c}
GSH (mg/gprot)	27.95±0.78^{a}	46.64±1.21^{b}	55.21±0.98^{c}	27.43±1.11^{a}	37.29±1.05^{b}	39.90±0.22^{b}
GSH/GSSG	11.53±1.09^{a}	11.27±1.10^{a}	13.67±0.22^{b}	11.60±1.19^{a}	12.20±1.56^{a}	12.83±1.16^{a}

Different letters meant significant differences between two groups (p<0.05).

Effects of RPS on the expression of the pro-inflammatory cytokine

Exposure to RPS had a significant dose-response effect on markers of acute inflammation after 90 days administration. All these markers included IL-6, TNF-α, COX-2 and NF-κB (**Figure 5**). However, they returned to normal after the 30-day recovery.

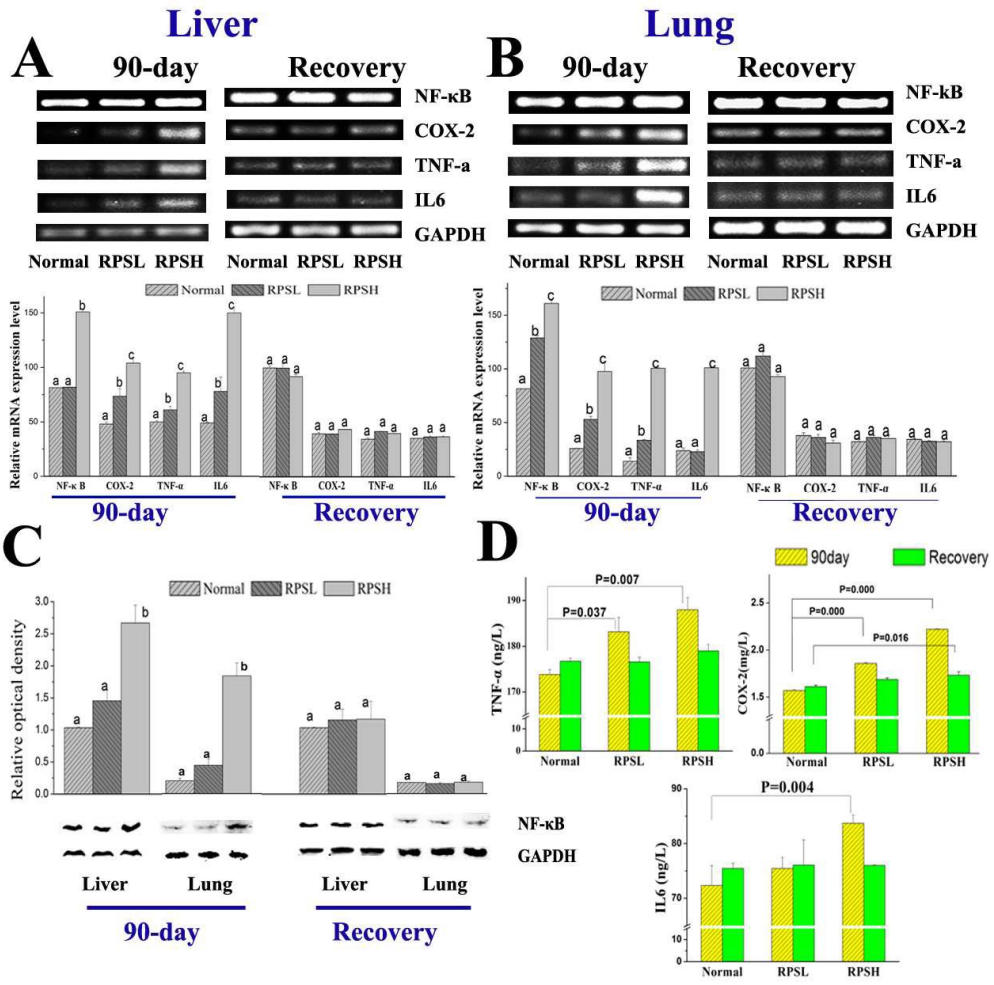

Figure 5 Effect of RPS treatment on the pro-inflammatory factor expression in rat (A) and (B) Relative expression of pro-inflammatory factor by RT-PCR assay. (C) Western blot evaluation of these pro-inflammatory factors for the liver and lung tissues treated with different doses of RPS. The protein expression levels of NF-κB were normalized against GAPDH. (D) Measurements of levels of COX-2, TNF-α and IL-6 by ELISA assay. Different letters meant significant differences between two groups ($p<0.05$).

Effects of RPS on the levels of hepatic phase II detoxification enzymes

Glutathione S transferases (GSTs) and heme oxygenase-1 (HO-1) as important

phase II enzymes display anti-oxidant, detoxification, anti-inflammatory and cytoprotective activities [11]. As **Figure 6** shown, rat receiving 50 mg/kg of RPS and 350 mg/kg of RPS showed significant inhibition of GSTs mRNA expressions compared with those treated with control diet. Meanwhile, RPSH significantly decreased the GSTα and GSTπ protein expression. In contrast, the mRNA and protein expressions of HO-1 which was another important anti-inflammatory and cytoprotective enzyme were up-regulated by RPSH administration. Nrf2 as a vital role during the activation of genes encoding antioxidant proteins and phase II detoxifying enzymes was also up-regulated. All the levels returned to normal after 30-day recovery. Immunostaining results showed a clear enrichment of Nrf2 protein both in the cytoplasmic and nuclear fraction in hepatocytes upon RPS treatment. Meanwhile, Nrf2 protein exhibited higher expression in the nuclear fraction of hepatocytes after 30-day recovery comparing with normal groups (**Figure 6C**).

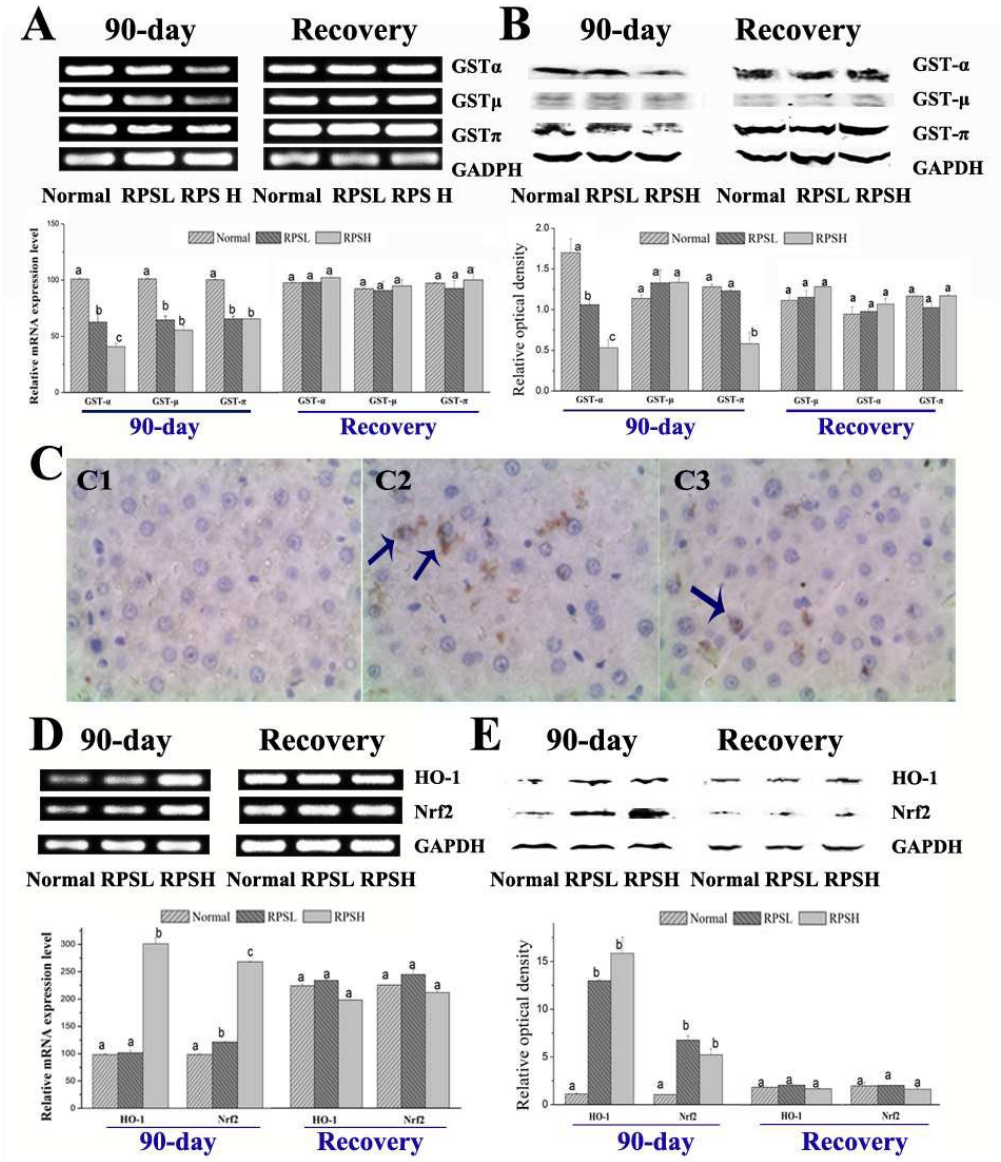

Figure 6 Effect of RPS on the mRNA and protein expression of detoxification enzymes in the liver tissues. (A) and (D) Relative mRNA expression of detoxification enzymes by RT-PCR assay. (B) and (E) Western blot evaluation of detoxification enzymes. The protein expression levels of GSTs, HO-1 and Nrf2 were normalized against GAPDH. (C) Immunohistochemical analyses of Nrf2 in liver tissues (X 400). Positive staining was observed as a dark brown color. (C1) Normal (C2) RPSH (C3) RPSH recovery groups. Arrow in C2 indicated nucleus and cytolymph-positive cells. Arrow in C3 indicated nucleus-positive cells. Different letters meant significant differences among

three groups (p<0.05).

Discussion

As our previous report, RPS as toxic nature produces are often used as anticancer drugs. Up to now, there are at least 120 species of poisonous natural products, of which more than half have been found to possess remarkable anticancer properties [12]. Due to the medicinal value and widespread use of RPS [1], it is critical to evaluate the toxicity of RPS with the long-term use.

The results from the 90-day long tern toxicity study showed changing trends on the individual body weight (**Figure 1**) due to their lower food and water consumption. This change may be related to the properties of saponins including gastric stimulus side effect [7]. Meanwhile, there were no significant change in the hematology data and urine analysis (**Table 1 and 2)**.

Clinical biochemistry data including the concentration of transaminases and alkaline phosphatase which were suggestive hepatic damage were significantly increased after ninety-day administration of RPS (**Table 3**). Although there were no changes in liver weight, several abnormalities appeared in RPSH liver tissues (**Figure 2B1**). These evidences were consistent with the clinical biochemistry data. In addition, the absolute weight of lung tissues was significantly increased (**Table 4**). Using the histopathological evaluation, several mild chronic lymphocytic interstitial infiltrates and fibrinous exudates were observed in the RPSH alveolar spaces (**Figure 2B2**). All these indicated that high dose of RPS induced liver and lung injury.

As we known, oxidative stress has been regarded as a direct and mechanistic indicator of hepatotoxic potential [13]. In our study, RPS induced overproduction of ROS in liver tissues (**Figure 4**) which may result in oxidant-antioxidant imbalance making cellular components like DNA and lipids damage [14]. Therefore, 8-OHdG as the marker of oxidative DNA damage and MDA as the product of lipid peroxidation were both significantly elevated in RPSH-treated rats ($p<0.05$) (**Figure 3**).

Oxidative stress also plays a critical role in the induction mechanisms of pro-inflammatory cytokines. Oxidative stress and inflammation may interact with each other to promote both DNA damage and activation of NF-κB, inducing many acute or chronic liver and lung injury [15]. In the experiment, RPS induced tissue injury accompanying with ROS-activated NF-κB and its oxidant products. NF-κB subsequently activated the expression of COX-2, TNF-α and IL-6 (**Figure 5**). Meanwhile, these pro-inflammatory factors enhanced oxidative stress. ROS generated by these pro-inflammatory cytokines further induced NF-κB activation and overproduce pro-inflammatory cytokines. Consequently, this interaction between oxidative stress and inflammation induced the liver and lung injury in the RPSH-treated group.

In the normal condition, ROS overproduction would activate Nrf2/ARE signaling to protect the body against oxidants [16]. In hence, Nrf2-regulated genes are responsible for increased cellular anti-oxidative or detoxification systems. However, some toxicants suppress the activation of Nrf2 and promote the transcription of NF-κB to induce the tissue injury. In this research, the inherent defensive enzymes such as CAT

and SOD as important anti-oxidant enzymes of liver, and GSTs as anti-oxidant and detoxification enzymes were decreased by high dose of RPS treatment ($p<0.05$). Fortunately, Nrf2 was activated by RPS administration accompanying with the levels of HO-1 elevated (**Figure 6C, 6D, 6E**), which as an inducible enzyme activated by Nrf2 played a central role against inflammation and oxidative stress [17]. The increased Nrf2 protein in both cytoplasm and nucleus suggested that not only the total Nrf2 protein levels, but also the activated Nrf2 are significantly induced by RPS, since translocation of Nrf2 protein into the nuclei is considered as the starting point of Nrf2 pathway activation [18]. In addition, GSH as an antioxidant and detoxification small molecule substance in livers [19] indirectly regulated by Nrf2 was significantly increased in RPSH groups (**Table 4**). The increased levels of GSH, HO-1 and Nrf2, as least in part, supported the self-protection of rats against RPSH treatment induced oxidative stress and inflammatory conditions. Furthermore, after 30 days' recovery, abnormalities in liver and lung tissues disappeared. The levels of ROS, phase II enzymes, pro-inflammatory cytokines, and nuclear factors such as NF-κB and Nrf2 were all returned to normal.

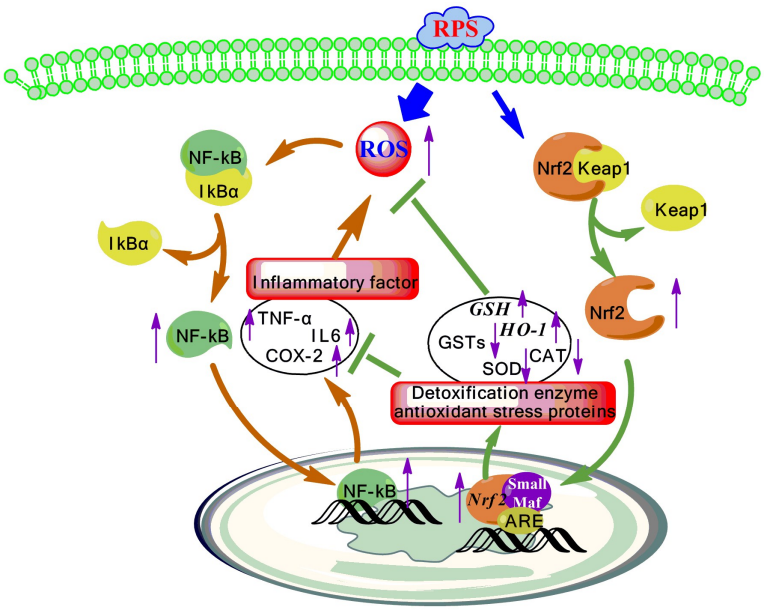

Figure 7 Proposed mechanism of toxic dose of RPS induced oxidant stress and inflammation disorders in rats

Our findings suggested the toxicity and detoxicity mechanisms induced by toxic dose of RPS shown schematically in **Figure 7**. In summary, RPS as a hypotoxic drug decreased body weight of rats and induced liver and lung injury through the over-expression of ROS and pro-inflammatory cytokines, and down-regulation of antioxidant and detoxified enzymes. Meanwhile, as the self-protection of rats, RPSH activated the transcription of Nrf2 and elevated the expression of GSH and HO-1. All these reduced the damage degree of liver and lung tissues. Fortunately, after 30 days' recovery, abnormalities in liver and lung tissues disappeared. The levels of ROS, phase II enzymes, pro-inflammatory cytokines, and nuclear factors were all returned to normal. The results of the study demonstrated for the first time that the toxicity and detoxicity mechanisms of RPS which were anticancer drugs isolated from traditional toxic Chinese medicines involved NF-κB and Nrf2 signaling. Our work provided useful data for correct administration of RPS and minimizes the danger of toxic herbal product use.

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