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Research Article

A Traditional Chinese Medicine Shaoyao Ruangan Heji Ameliorates Carbon Tetrachloride-induced Liver Injury Through Multiple Stress and Toxicity Pathways

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Abstract

Shaoyao Ruangan Heji (SYRG), a liquid pharmaceutical formulation of traditional Chinese medicine, has been used clinically for the prevention and treatment of chemotherapy-induced liver injury in Zhejiang Cancer Hospital (China) for 37 years. However, its underlying therapeutic mechanism is still unclear. Because oxidative stress is a crucial etiological factor implicated in the pathology of chemotherapy-induced liver injury, in this study, the protective effect of SYRG on carbon tetrachloride (CCl_4)-induced oxidative stress and liver injury in mice were evaluated and its mechanisms were investigated by Mouse Stress and Toxicity Pathway Finder PCR Array. The results showed that pretreatment with SYRG significantly attenuated CCl_4-induced liver damage in mice, evidenced by decreased serum enzyme activities of ALT and AST, which was also supported by the histopathological examination of the mice liver. The SYRG also showed a significant protective effect against CCl_4-induced hepatic MDA elevation and depletion of T-AOC, SOD and GSH content. Furthermore, SYRG regulated 25 genes related to six stress and toxicity pathways (Oxidative stress, hypoxia, cell necrosis, inflammatory response, DNA damage signaling and heat shock proteins/unfolded protein response) in the impaired liver induced by CCl_4. These results indicated that SYRG has protective effects against CCl_4-induced liver injury and its underlying mechanisms involve the modulation of multiple stress and toxicity pathways. These findings would be beneficial for understanding of the therapeutic effects of SYRG in treatment of chemotherapy-induced liver injure in clinic.

Key words: Traditional Chinese medicine, chemotherapy, liver injure, oxidative stress, carbon tetrachloride, toxicity pathways

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Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Therapeutic effectiveness of many chemotherapeutic agents is associated with varieties of liver injury when used alone or in combination in cancer patients. The appearances of symptoms include hepatocellular injury, cholestasis steatosis, veno-occlusive disease (VOD), fibrosis etc. Hepatocellular injury which may range from asymptomatic elevations of aminotransferases, increases in total bilirubin and sometimes increases in alkaline phosphatase levels, to signs of overt liver failure with hepatocellular necrosis occurring is relatively frequent in cancer chemotherapy patients (Rodriguez-Frias and Lee, 2007a, b; King and Perry, 2001). Although, the mechanisms involved in chemotherapy-induced hepatocellular injury are not fully characterized, many evidences indicate that oxidative stress is a crucial etiological factor implicated in this pathologic progression. Under normal circumstances, Reactive Oxygen Species (ROS) which are constantly generated during intracellular metabolism are tightly controlled by sophisticated defense systems. This defense system includes antioxidant enzymes such as superoxide dismutase (SOD), catalase and glutathione peroxidase and a number of non-enzymatic antioxidants and small proteins including glutathione (GSH). Low levels of ROS are also involved in intracellular signal transduction for cell homeostasis (Leonarduzzi *et al*., 2010). However, ROS are often over-produced in hepatocytes under many chemotherapy treatments, resulting in reduction-oxidation imbalance and damage of lipids, proteins and DNA. Such oxidative stress can subsequently leads to the apoptosis or necrotic death of hepatocytes (Jaeschke and Ramachandran, 2011; Lear *et al*., 1992; Kebieche *et al*., 2009; Pratibha *et al*., 2006).

Shaoyao Ruangan Heji (SYRG) which is also known as Zhonggan Heji, is a liquid pharmaceutical formulation prepared from nineteen Chinese materia medicas as specified in Table 1. This prescription is utilized for the treatment of liver diseases due to its traditional Chinese medicine effects of detoxicating. The SYRG has been used clinically for the prevention and treatment of chemotherapy-induced liver injure in Zhejiang Cancer Hospital (China) since 1977. In long clinical practice, it has been reported that SYRG could effectively ameliorate liver injury in chemotherapy treated cancer patients (Wang *et al*., 2006; He and Zhong, 1998). However, there is no further pharmacological evidence of SYRG on liver injury treatment and the underlying therapeutic mechanism of SYRG is still unclear.

It is hypothesized that SYRG may ameliorate chemotherapy-induced liver injure through anti-oxidative stress. Therefore, the present study was designed to evaluate the protective effect of SYRG on carbon tetrachloride (CCl_4)-induced oxidative stress and liver injury in mice. Meanwhile, its mechanisms were also investigated by Mouse Stress and Toxicity PathwayFinder PCR Array.

MATERIALS AND METHODS

Preparation and quality control of SYRG: The SYRG was supplied by the pharmaceutical preparation department of Zhejiang Cancer Hospital (China). As shown in Table 1, SYRG

Table 1: Nineteen Chinese materia medicas prepared for SYRG

Latin name (Chinese name)	Used part	Place of origin	Voucher numbers	Amount (g)
Hedyotis diffusa Willd (Bai Hua She She Cao)	Whole herb	Zhejiang	S20130101	86
Scutellaria barbata D. Don (Ban Zhi Lian)	Whole herb	Shandong	S20121101	86
Crataegus pinnatifida Bunge (Shan Zha)	Fructus	Shandong	S20120401	86
Imperata cylindrica Beauv.var.major (Nees) C.E.Hu (Bai Mao Gen)	Rhizoma	Zhejiang	S20121201	86
Lysimachia christinae Hance (Jin Qian Cao)	Whole herb	Zhejiang	S20130101	86
Ardisia japonica (Thumb.) Blume (Ai Di Cha)	Whole herb	Zhejiang	S20120326	86
Solanum lyratum Thumb. (Bai Mao Teng)	Whole herb	Zhejiang	S20121101	86
Agrimonia pilosa Ledeb. (Xian He Cao)	Whole herb	Zhejiang	S20121101	86
Liquidambar formosana Hance (Lu Lu Tong)	Fructus	Zhejiang	S20121101	34
Tetrastigma hemsleyanum Diels et Gilg (San Ye Qing)	Radix	Guangxi	S20121201	34
Paeonia lactiflora Pall. (Bai Shao)	Radix	Zhejiang	S20130202	34
Paris yunnanensis Franch. (Chong Lou)	Rhizoma	Guizhou	S20120701	29
Citrus reticulate Blanco (Qing Pi)	Frustus	Zhejiang	S20120401	26
Citrus reticulate Blanco (Chen Pi)	Pericarpium	Fujian	S20130301	26
Curcuma wenyujin Y.H. Chen et C. Ling (Yu Jin)	Radix	Sichuan	S20121201	26
Sparganium stoloniferum (Graebn.) Buch.-Ham. (San Leng)	Rhizoma	Zhejiang	S20121201	26
Curcuma phaeocaulis Val. (E Zhu)	Rhizoma	Guangxi	S20120401	26
Gardenia jasminoides Ellis (Zhi Zi)	Fructus	Zhejiang	S20121101	26
Gallus gallus domesticus Brisson (Ji Nei Jin)	Endothelium corneum gigeriae galli	Zhejiang	S20130301	26

was prepared from 18 medicinal herbs and one animal-derived material which complied with Chinese pharmacopoeia standards. All of these materials as 1001 g of total weight were boiled with distilled water (10 L) for 2 h and filtered. After being repeated twice, the mixture of filtrate was concentrated to 1000 mL. Then, 3 g sodium benzoate and 186 g candy sugar were added and finally, SYRG was sterilized by vapor sterilization (heat sterilization).

To guarantee the quality of SYRG, HPLC were employed to detect its main components paeoniflorin (0.245 mg mL^{-1}) and geniposide (0.423 mg mL^{-1}), respectively (Lu *et al*., 2001). In brief, 5 mL SYRG was extracted using 5 mL aether for three times. The water layer was discarded and the aether phase was evaporated to dryness. The residue was dissolved in 10 mL of mobile phase and the supernatant was filtered through a 0.45 μm PTFE filter for HPLC analysis. The analysis was performed using Shimadzu LC-4A HPLC system and Turner Packing Kromasil C18 column (200×4.6 mm, ID 5 μm). The mobile phase was 15% acetonitrile-water (0.1% phosphoric acid) solution, the flow rate was 1.0 mL min^{-1}. The effluent was monitored at 230 nm by Shimadzu SPD-10A UV detector.

Chemicals and reagents: The detection kits for protein content, SOD and GSH were purchased from Beyotime Institute of Biotechnology (Haimen, Jiangshu, China). The detection kits for total antioxidative capacity (T-AOC), malondialdehyde (MDA), aspartate aminotransferase (AST), alanine aminotransferase (ALT) were purchased from Nanjing Jiancheng Bioengineering Institute (Nanjing, Jiangshu, China). Trizol, RNase-free DNase and superscript III RNase reverse transcriptase were purchased from Invitrogen (Carlsbad, CA, USA), Mouse Stress and Toxicity PathwayFinder PCR Array, 2×Super Array PCR master mix, RNeasy® MinElute™ Cleanup Kit was purchased from Qiagen (Valencia, CA, USA), PCR primers and other PCR reagents were purchased from Shanghai Sangon Biological Engineering Technology and Services Co., Ltd. (Shanghai, China). All other reagents were of analytical grade and made in China.

Experimental animals: Six weeks old male ICR mice were purchased from Zhejiang Experimental Animal Center (Certificate No. SCXK 2008-0033, Hangzhou, Zhejiang, China). Rodent laboratory chow and tap water were provided *ad libitum* and maintained under controlled conditions: Temperature 24±1°C, humidity 50±10%, 12 h light/12 h dark cycle. All the procedures were in strict accordance with the P.R. China legislation on the use and care of laboratory animals and with the guidelines established by the Institute for Experimental Animals and were approved by the Institutional Animal Care and Use Committee in Zhejiang Academy of Medical Sciences, China.

CCl_4-induced liver injury in mice and experimental design: The mice were randomly divided into six groups, each consisting of ten mice. These groups are the normal group, the model control group, the positive control group and the three test groups. Mice in the positive control group were treated with Silymarin at a dose of 20 mg kg^{-1} b.wt., p.o., once per day), while mice in normal group and model group were treated with physiological saline. The dose of SYRG was decided based on clinical use. In general, 60 kg adult takes 90 mL of SYRG daily. According to the conversion factor between mice and human based on body surface area ($d_{mouse} = d_{human} \times 12.33$) (Reagan-Shaw *et al*., 2008), the common dose of SYRG for mice should be 18.5 mL kg^{-1} per day. In the present study, the mice in the three test groups were treated with SYRG at a dose of 5, 10 and 20 mL kg^{-1} per day, respectively. After seven days of treatment, the mice were injected with CCl_4 (10 mL kg^{-1} i.p., of 0.125% CCl_4 solution in olive oil). One hour after the seventh administration, except for the normal group, which was given only olive oil injection. The animals were sacrificed 24 h after the CCl_4 treatment. Blood was collected, allowed to clot and serum was separated for assessment of enzyme activity. Parts of the livers tissue were immediately transferred into 10% formalin for histopathological analysis and the other were collected and stored at -80°C for pending tests.

Measurement of serum biochemical markers: Collected blood samples were placed at 4°C for 2 h and centrifuged at 3000 rpm for 5 min to obtain the serum. The level of serum ALT and AST activities were determined by assay kits according to the manufacturer's instructions (Chen *et al*., 2014).

Histopathological studies: Liver slices were fixed with 10% formalin in phosphate buffered saline for 24 h and embedded in paraffin. Sections of 5 μm thickness were made using a microtome and stained with haematoxylin-eosin and observed under microscope to observe histopathological changes in the liver. Photographs of each of the slides were taken at 100×magnification.

Measurement of GSH, SOD, T-AOC, MDA and protein concentrations in liver homogenate: Liver samples were homogenized in phosphate buffer (5 mM, pH 7.4) to give a

10% (w/v) liver homogenate and then centrifuged at 5000 rpm for 3 min at 4°C. The supernatant of the liver homogenate was used for the estimation of GSH, SOD, T-AOC, MDA levels and protein concentrations by assay kits according to the manufacturer's instructions (Chen *et al*., 2014).

PCR array analysis: Mouse Stress and Toxicity PathwayFinder PCR Array which contains a panel of primer sets for a thoroughly researched set of 89 relevant genes (including 5 housekeeping genes: GUSB, HPRT1, HSP90AB1, GAPDH and β-Actin) was detected as mentioned previously (Chen *et al*., 2012a). In brief, 50 mg of each individual liver sample from model control group or SYRG 20 mL kg^{-1} group were pooled together, respectively. These two pooled samples were lysed with trizol reagent and the total RNA was isolated according to the manufacture's protocol. RNA was treated with RNase-free DNase and cleanup using RNeasy® MinElute™ Cleanup Kit, concentration was determined by spectrophotometry at 260 nm. Aliquots of RNA (1.5 μg) were converted into first strand cDNA using superscript III RNase reverse transcriptase. Real-time PCR was performed using an ABI PRISM® 7900 Detection System (Applied Biosystems, USA) in a 96-well plate format. There were 102 μL cDNA, 550 μL 2×SuperArray PCR master mix and 448 μL dd H_2O mixed and loaded 10 μL mixture to each well. The general PCR condition profile was as follows: Polymerase activation at 95°C for 10 min, followed by 40 cycles of denaturing at 95°C for 15 sec, annealing and extension at 60°C for 60 sec. Calculate the threshold cycle (Ct) for each well using the instrument's software. Calculate the ΔCt for each gene in each treatment group, ΔCt = average Ct-average of housekeeping genes' Ct. Calculate the ΔΔCt for each gene across two groups, ΔΔCt = ΔCt (group 2)-ΔCt (group 1), where, group 1 is the control and group 2 is the experimental. Calculate the fold-change for each gene from group 1 to group 2 as $2^{-\Delta\Delta Ct}$.

Real-time RT-PCR verification: Each individual liver sample from normal group, model control group and SYRG 20 mL kg^{-1} group were isolated and reverse-transcribed into cDNA. Real-time PCR was performed using the SYBR Green PCR master mix (Chen *et al*., 2015). The primer pairs used in real-time PCR were the following: Hmox1, 5'-AGGCCACCAAGGAGGTACAC-3', 5'-AGGAAGCCATCACCAG CTT-3'; Gstp1, 5'-CAGAACCAGGGAGGCAAAG-3', 5'-GAGCC ACATAGGCAGAGAGC-3'; Parp1, 5'- CCGCCTACTCTATCCTCA GC-3', 5'-GGGCTTCTTCATTCCAAAGTC-3'; IL1β, 5'-TGGGCTGG ACTGTTTCTAATG-3', 5'-GGTTTCTTGTGACCCTGAGC-3'; Cdkn1a, 5'-TCCAGACATTCAGAGCCACA-3', 5'-TCAAAGTTCCAC CGTTCTCG-3'; Bid, 5'-AGCCCTTGATGAGGTGAAGA-3', 5'- AGC AAAGATGGTGCGTGACT-3'; GAPDH, 5'- GGTTGTCTCCTGCGA CTTCA-3', 5'-TGGTCCAGGGTTTCTTACTCC-3'; GAPDH was used as an endogenous control. Primer amplification efficiency and specificity were verified for each set of primers. The mRNA levels of the tested genes relative to GAPDH mRNA were determined using the $2^{-\Delta\Delta Ct}$ method and as fold induction.

Statistical analysis: The data were expressed as Mean±Standard Deviation (SD) and examined for their statistical significance of difference with ANOVA and the standard's t-test. The p-values less than 0.05 were considered to be statistically significant.

RESULTS

SYRG protected against CCl_4-induced liver injury of mice: The ALT and AST are enzymes found in the highest amounts in the liver. Injury to the liver results in release of these substances into the blood. As shown in Fig. 1, CCl_4 administration (model group) at day 6 resulted in significant elevation of serum ALT and AST activity, compared to the control group. Besides, histopathological examination of livers challenged with CCl_4 showed pericentral necrosis with hydropic degeneration of hepatocytes and dense infiltration of inflammatory cells in the portal areas. Oral administration of SYRG at the doses of 10 and 20 mL kg^{-1} or silymarin at the dose of 20 mg kg^{-1} b.wt., prior to CCl_4 treatment significantly restored the activities of AST and ALT ($p<0.05$) (Fig. 1a). The histological observations basically supported these results. The SYRG and silymarin were able to prevent the development of histopathological changes, which exhibited areas of normal liver architecture and patches of inflammatory infiltration and necrotic hepatocytes (Fig. 1b). In addition, no signs of toxicity were observed in the SYRG and silymarin-treated mice on the basis of body weight and macroscopic examination of individual organs (Data no shown). These results suggested that SYRG protected against CCl_4-induced liver injury of mice.

SYRG reduced CCl_4-induced oxidative stress in liver of mice: The MDA is the product of lipid peroxidation and served as the marker for oxidative stress. The SOD catalyzes the dismutation of superoxide (O^{2-}) into oxygen and hydrogen peroxide, thus served as one of the major endogenous antioxidant enzymes. GSH is the major endogenous antioxidant which participates directly in the neutralization of free radicals and reactive oxygen compounds such as superoxide anions and hydrogen peroxide, maintenance of membrane protein thiols. Therefore, beside T-AOC, the values of MDA, SOD and

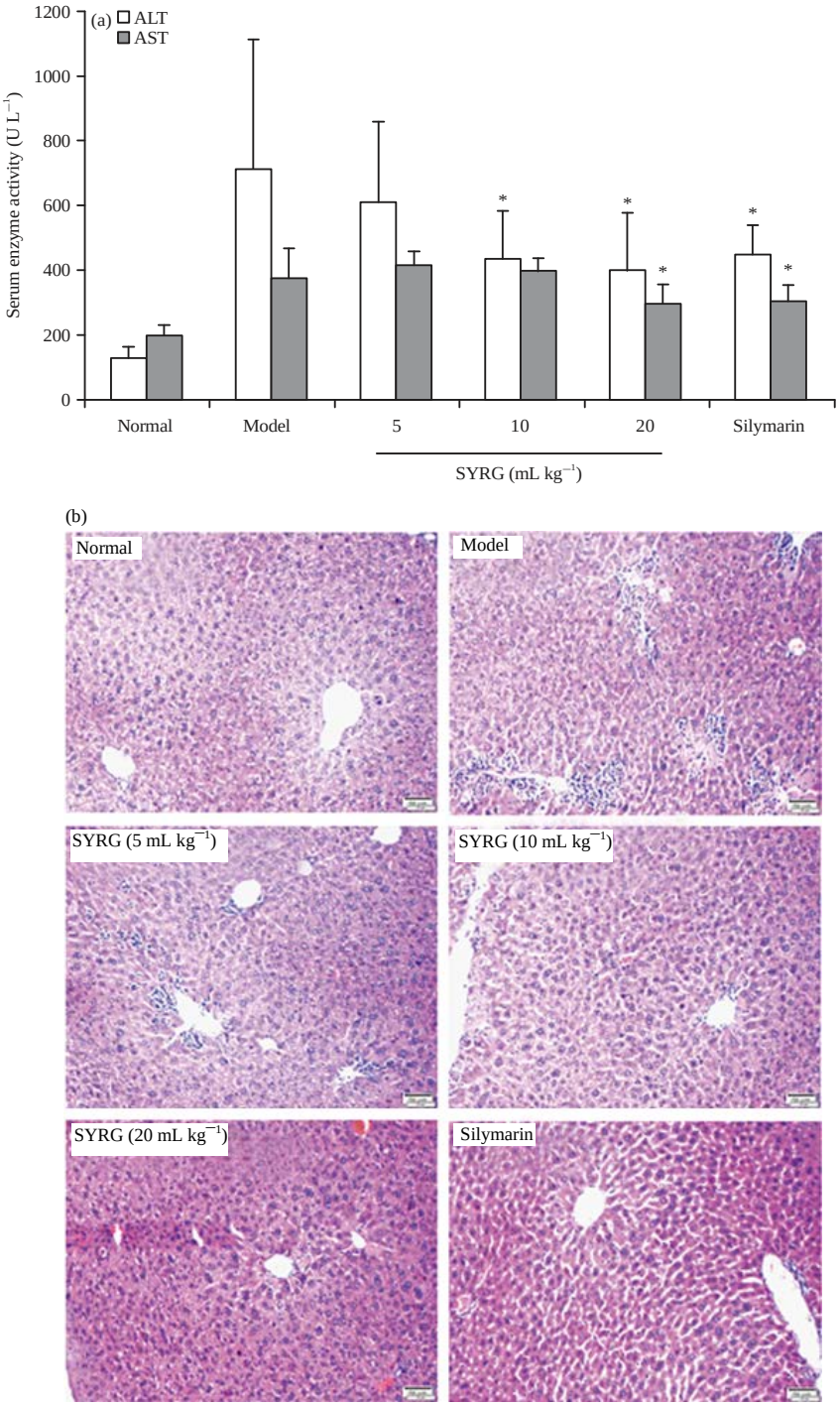

Fig. 1(a-b): SYRG protect against CCl_4-induced acute liver injury of mice. The ICR mice in SYRG group, positive control group, normal and model group were treated with SYRG, silymarin and physiological saline, respectively. After seven days of treatment, the mice were injected with CCl_4 (10 mL kg^{-1} i.p., of 0.125% CCl_4 solution in olive oil) except for the normal group, which was given only olive oil injection. The animals were sacrificed 24 h after the CCl_4 treatment. (a) Blood was collected and serum was separated for assessment of enzyme activity of ALT and AST. (b) Slices of liver were stained with hematoxylin and eosin for histopathological analysis. The values were presented as Mean±SD (n = 10). Significant differences with model group were designated as *p<0.05

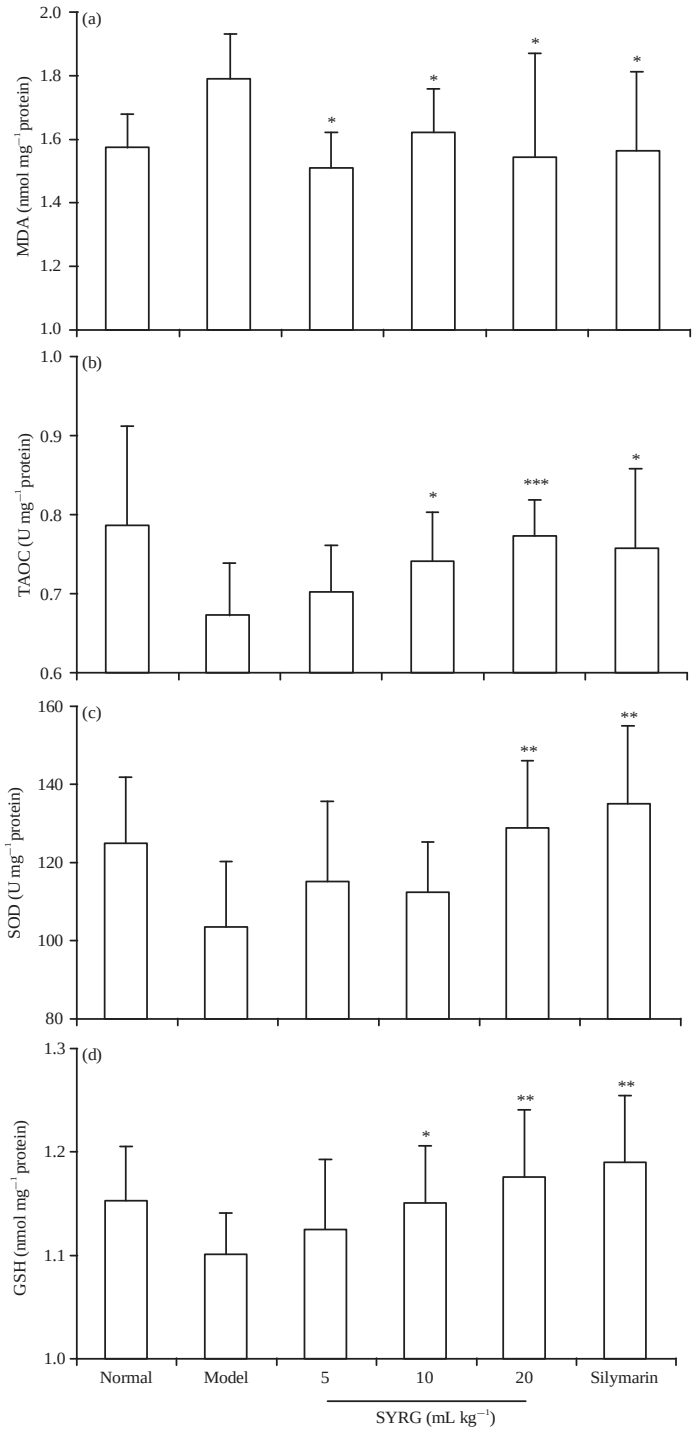

Fig. 2(a-d): SYRG protect against CCl_4-induced oxidative stress in liver of mice. Liver samples were homogenized and then centrifuged. The GSH, SOD, T-AOC, MDA levels and protein concentrations in the supernatant of the liver homogenate were determined by commercial assay kits. The values were presented as Means±SD (n = 10). Significant differences with model group were designated as *p<0.05, **p<0.01 and ***p<0.001

GSH were selected to evaluate the levels of oxidative stress in mice liver tissue. The results were shown in Fig. 2. The CCl_4 significantly induced oxidative stress in the liver as showed by significant increase level of MDA and decrease

Table 2: SYRG regulated expressions of genes associated with stress and toxicity pathways in injured liver of mice induced by CCl_4

Pathway	Gene	Description	RefSeq	Fold difference
Oxidative stress	Gstp1	Glutathione S-transferase, pi 1	NM_013541	-2.0
	Hmox1	Heme oxygenase (decycling) 1	NM_010442	1.9
	Prdx1	Peroxiredoxin 1	NM_011034	-3.0
	Txn1	Thioredoxin 1	NM_011660	-3.7
Hypoxia	Car9	Carbonic anhydrase 9	NM_139305	-2.6
	Mmp9	Matrix metallopeptidase 9	NM_013599	-1.6
	Serpine1	Serine (or cysteine) peptidase inhibitor, clade E, member 1	NM_008871	1.9
Cell necrosis	Parp1	Poly (ADP-ribose) polymerase family, member 1	NM_007415	-1.5
	Pvr	Poliovirus receptor	NM_027514	-2.1
	Ripk3	Receptor-interacting serine-threonine kinase 3	NM_019955	-2.2
Inflammatory response	IL1β	Interleukin 1 beta	NM_008361	-2.7
	IL6	Interleukin 6	NM_031168	-2.3
DNA damage signaling	Atm	Ataxia telangiectasia mutated homolog (human)	NM_007499	-2.7
	Atr	Ataxia telangiectasia and rad3 related	NM_019864	-9.1
	Cdkn1a	Cyclin-dependent kinase inhibitor 1A (P21)	NM_007669	-2.1
	Chek2	CHK2 checkpoint homolog (*S. pombe*)	NM_016681	-1.9
	Gadd45α	Growth arrest and DNA-damage-inducible 45 alpha	NM_007836	-1.7
	Gadd45γ	Growth arrest and DNA-damage-inducible 45 gamma	NM_011817	-10.8
	Hus1	Hus1 homolog (*S. pombe*)	NM_008316	-1.6
	Rad51	RAD51 homolog (*S. cerevisiae*)	NM_011234	-3.1
Heat shock proteins/Unfolded protein response	Bid	BH3 interacting domain death agonist	NM_007544	-1.8
	Calr	Calreticulin	NM_007591	-1.9
	Xbp1	X-box binding protein 1	NM_013842	-2.2
	Hsp90β1	Heat shock protein 90, beta (Grp94), member 1	NM_011631	-1.8
	Hspα4	Heat shock protein 4	NM_008300	-1.7

levels of T-AOC, SOD and GSH in the liver of model group compared to that of the normal group. Interestingly, oral administration of SYRG at the doses of 5-20 mL kg^{-1} significantly decreased CCl_4-induced MDA elevation ($p<0.05$); SYRG at 10 and 20 mL kg^{-1} significantly restored CCl_4-induced T-AOC and GSH descent ($p<0.05$, $p<0.01$ or $p<0.001$); while SYRG at 20 mL kg^{-1} restored CCl_4-induced SOD descent ($p<0.01$). Positive control silymarin also significantly reversed the CCl_4-induced MDA elevation and T-AOC, SOD, GSH descent ($p<0.05$ or $p<0.01$). These findings indicated that SYRG protected against CCl_4-induced oxidative stress in liver of mice.

SYRG regulated expressions of genes associated with multiple stress and toxicity pathways in injured liver of mice induced by CCl_4: To determine the mechanism involved in the protective effects of SYRG on liver, the expression of 84 genes associated with nine stress and toxicity pathways, including oxidative stress, hypoxia, osmotic stress, cell death (apoptosis, autophagy and necrosis), inflammatory response, DNA damage signaling and heat shock proteins/unfolded protein response in the pooled liver sample from model control group and SYRG treated group were first examined using PCR arrays. A complete list of genes contained in the array can be viewed on the following link: http://www.sabiosciences. com /rt_pcr_product/HTML/PAMM-003Z.html. The list of genes with expression fold change ≥1.5 or ≤-1.5 were shown in Table 2. The PCR Array analysis showed that SYRG regulated 25 of the 84 genes related to stress and toxicity pathways in the impaired liver induced by CCl_4. These 25 altered genes relate to oxidative stress (Gstp1, Hmox1, Prdx1 and Txn1), hypoxia (Car9, Mmp9 and Serpine1), cell necrosis (Parp1, Pvr and Ripk3), inflammatory response (IL1β and IL6), DNA damage signaling (Atm, Atr, Cdkn1a, Chek2, Gadd45α, Gadd45γ, Hus1 and Rad51) and heat shock proteins/unfolded protein response (Bid, Calr, Hsp90β1, Hspα4 and Xbp1).

To confirm the validity of the PCR array results, 6 putative differentially expressed genes including Hmox1, Gstp1, Parp1, IL1β, Cdkn1a and Bid were determined by Real-time RT-PCR in each individual liver sample from normal group, model control group and SYRG 20 mL kg^{-1} group. The results were shown in Fig. 3. Even though the abundance relative expression values of the genes detected by above two methods were not all the same, expression trends were generally consistent, suggesting that the results of PCR array detection were reliable.

DISCUSSION

Liver is the major organ for metabolism of foreign substances and functionally interposed between the site of

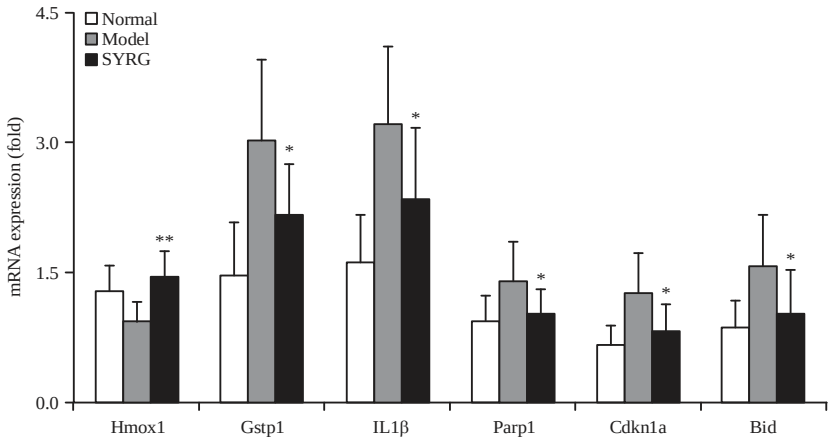

Fig. 3: Real-time RT-PCR validation of PCR array results. Six putative differentially expressed genes were determined by Real-time RT-PCR in each individual liver sample from normal group, model control group and SYRG 20 mL kg^{-1} group. The mRNA levels of the tested genes relative to GAPDH mRNA were determined using the $2^{\Delta\Delta Ct}$ method and as fold induction. The values were presented as Means±SD (n = 10). Significant differences with model group were designated as *p<0.05 and **p<0.01

resorption and the systemic circulation. These conditionsrender the liver not only the most important organ for detoxification of foreign substances but also a major target of their toxicity. Many chemotherapeutic agents, including conventional cytotoxic chemotherapies or latest molecular targeting inhibitors, are inevitably associated with hepatotoxicity (Teo *et al*., 2013). These serious side effects may limit the application of chemotherapeutic drug in clinic. Fortunately, some synthetic compounds (Iseri *et al*., 2007), trace elements (Liao *et al*., 2008) and natural products (Gaona-Gaona *et al*., 2011; Zarei and Shivanandappa, 2013) have proved to be helpful to attenuate chemotherapy-induced liver injure. However, majority of these evidences were only from laboratory studies, their effects and safety were not evaluated in clinical patients. The SYRG, a hepatoprotective traditional Chinese medicine, has been used clinically for prevention and treatment of chemotherapy-induced liver injure in Zhejiang Cancer Hospital (China) more than 37 years. In this study, experimental evidences were provided to demonstrate the protective effect of SYRG against oxidative stress-induced liver injury for the first time.

To evaluate the protective effect of SYRG on oxidative stress-induced hepatocellular injury *in vivo*, the effect of SYRG on CCl_4 induced oxidative liver injure in mice was assessed. The CCl_4 is metabolized by hepatic microsomal cytochrome CYP2E1, CYP2B1 or CYP2B2 to form a reactive trichloromethyl ($CCl_3^{\bullet}$) radical. This radical can further react with oxygen to form the trichloromethylperoxy radical $CCl_3OO^{\bullet}$, a highly reactive species. These radicals can bind to cellular molecules (nucleic acid, protein and lipid), impair crucial cellular processes, damage antioxidant enzymes such as SOD and deplete the levels of GSH which have a key role in coordinating innate antioxidant defense mechanisms and eventually leading to lipid peroxidation, cell necrosis and the leakage of the marker enzymes such as AST and ALT into serum (Weber *et al*., 2003). In this study, pretreatment with SYRG significantly attenuated CCl_4-induced liver damage in mice, evidenced by decreased serum enzyme activities of ALT and AST, which was also supported by the histopathological examination of the mice liver (Fig. 1). The SYRG also showed a significant protective effect against CCl_4 induced hepatic MDA elevation and depletion of T-AOC, SOD and GSH content (Fig. 2). These findings indicated that SYRG could ameliorate oxidative stress-induced hepatocellular injury *in vivo*.

In the process of tissue oxidative damage, ROS directly targets polyunsaturated fatty acids, cysteine residues, DNA bases and ion channels (such as calcium channels), leading to lose the integrity of subcellular structures, such as plasma membrane, endoplasmic reticulum, mitochondria and Golgi apparatus. Furthermore, ROS affects many redox-sensitive kinases, phosphatases, cytokines and transcription factors, which may alter signaling transduction pathways and eventually lead to cell apoptosis or necrosis. These changes in cellular physiology are often characterized by cascades of gene expression changes. It was reported that some natural non-enzymatic antioxidants exerted effects not simply by quenching ROS generation and propagation but also by intercepting reactive species at the level of critical signaling pathways (Leonarduzzi *et al*., 2010; Jaeschke and Ramachandran, 2011). Therefore, in order to elucidate some

of the underlying mechanisms involved in the protective effects of SYRG on oxidative stress-induced hepatocellular injury, the expressions of 84 genes associated with nine stress and toxicity pathways were detected by PCR array and the reliability of the detection results were confirmed by real-time PCR. The results showed that pretreatment with SYRG regulated 25 genes related to six stress and toxicity pathways in the impaired liver induced by CCl_4 (Table 2 and Fig. 3). Most of these genes had been reported earlier in CCl_4-induced acute hepatic failure in rat (Xu *et al*., 2013). The SYRG seems to reverse the regulation of CCl_4 on these genes.

Five important antioxidant genes relate to oxidative stress pathway were regulated by SYRG. Of them, three genes (Gstp1, Prdx1 and Txn1) which are normally very low in liver, could be strongly stimulated by CCl_4 (Xu *et al*., 2013; Koo *et al*., 1994). However, the mRNA level of Hmox1, an important target gene of antioxidant Nrf2 pathway, was down-regulated by CCl_4. This rate-limiting enzyme protects mammalian cells from oxidative stress by degrading toxic heme into free iron, carbon monoxide and biliverdin/bilirubin (Esmaeili and Alilou, 2014). Gong *et al*. (2002) found that antioxidant tea polyphenols and tea pigments could inhibit mRNA expression of Gstp1. Esmaeili and Alilou (2014) reported that antioxidant Naringenin could attenuate the decrease of liver Hmox1 expression in rats induced by CCl_4. Those results are compatible with our finding.

Two genes (Car9 and Mmp9) and gens Serpine1 relate to hypoxia pathway were down and up-regulated by SYRG, respectively. Although, the mechanism of CCl_4-induced hypoxia is yet unclear, some researches mentioned that hypoxia enhanced CCl_4 toxicity in whole animals, in perfused rat liver and in isolated hepatocytes (Weber *et al*., 2003). Matrix metalloproteinases (MMPs) are a family of proteinases that are capable of degrading extracellular matrices (ECM), which are essential to maintain the architecture of normal tissues. Many MMPs are not expressed in resting, healthy tissues but are promptly induced in response to tissue injury caused by trauma, infection and toxin. It was reported that in CCl_4 induced-liver injury, activated Kupffer cells produce inflammatory cytokines, which incite hepatic stellate cells to express MMP9 and thereafter ECM degradation may provoke the collapse of sinusoids and create hypoxia, leading parenchymal cell apoptosis and necrosis (Yan *et al*., 2008). On the contrary, Serpine1, also known as plasminogen activator inhibitor-1 (PAI-1), inhibits the activity of matrix metalloproteinases and maintains hepatocyte division and therefore, plays protective role in CCl_4-induced liver damage and fibrosis (Von Montfort *et al*., 2010).

Inflammation is another important pathological mechanism propagating CCl_4-induced liver injury. Excessive free radicals probably activate Kupffer cells, the resident macrophages of liver, which can mediate hepatic inflammation process by producing pro-inflammatory cytokines, such as TNFα, NO, COX-2, IL-1β, IL-6 and IL-10 (Weber *et al*., 2003). In this study, the mRNA expressions of IL-1β and IL-6 were down-regulated by SYRG, which were in agreement with the histopathological observations that SYRG decreased infiltration of inflammatory cells in the liver (Fig. 3). These results supported that SYRG inhibited inflammatory responses.

The majority of genes which were regulated by SYRG belong to DNA damage signaling pathways. The DNA damage from normal metabolic processes induces cell cycle arrest in eukaryotic cells via checkpoint activation or DNA repair machineries to repair the damaged DNA. However, when DNA damage is too severe to repair, the damaged cells undergo apoptosis cell death to avoid passing on the potentially lethal errors in DNA to their daughter cells. These damage responses including cell cycle checkpoint, DNA repair and DNA damage-induced apoptosis (Jun *et al*., 2012). The Atm and Atr are serine/threonine kinase members of the phosphoinositide 3-kinase-like family, which initiate cell cycle arrest via Chek1 and Chek2 and facilitate DNA repair complex activation to preserve DNA integrity (Smith *et al*., 2010). The Cdkn1a binds to and inhibits the activity of cyclin-CDK2, -CDK1 and -CDK4/6 complexes and functions as a regulator of cell cycle progression at G1 and S phase (Gartel and Radhakrishnan, 2005). The Gadd45 family members function as stress sensors, which are rapidly induced by genotoxic stress agents as well as by terminal differentiation and apoptotic cytokines (Liebermann and Hoffman, 2008). In this study, the mRNA expressions of Atm, Atr, Cdkn1a, Gadd45α, Gadd45γ as well as checkpoint protein Hus1 and Rad51 (one of the key components of the homologous repair pathway) were down-regulated by SYRG, supporting that SYRG reduced CCl_4-induced DNA damage responses.

The Endoplasmic Reticulum (ER) is the site where unfolded state proteins are translocated into and catalysted to fold to attain their final appropriate conformation. However, a variety of insults that disrupt protein-folding reactions in the ER may activate an adaptive signaling cascade known as the Unfolded Protein Response (UPR). These include changes in intralumenal calcium, alteration in the redox status and energy (sugar/glucose) deprivation. Persistent protein misfolding initiates apoptotic cascades that are now known to play fundamental roles in the pathogenesis of multiple human diseases including metabolic disease and neurodegenerative diseases (Malhotra and Kaufman, 2007). In this study, the mRNA expressions of Bid (a pro-apoptotic member of the Bcl-2

protein family), Calr (a Ca^{2+} reservoir that prevents misfolded proteins from being exported from the endoplasmic reticulum), Xbp1 (a transcription factor in UPR), two heat shock protein Hsp90β1 and Hspα4 (serve as chaperone proteins in protein-folding reactions) were down-regulated by SYRG, suggesting that SYRG reduced CCl_4-induced ER oxidative damage and unfolded protein response.

Three hallmarks of necrosis pathway were also down-regulated by SYRG. Of them, Parp1 is a nuclear enzyme which is activated following DNA damage and leads to cell death by at least two pathways. In the first pathway, the hyper-activation of Parp1 induces a form of cell death termed parthanatos that signals through mitochondrially regulated caspase-independent pathways. In another way, the hyper-activation of Parp1 results in the depletion of cytosolic nicotinamide adenine dinucleotide (NAD^+) reserves resulting in a dramatic reduction of cellular ATP levels and necrosis (Elkholi and Chipuk, 2014). The Ripk3 is a mediator of necroptosis that functions as an intermediary in TNFα signaling pathway (Yu *et al*., 1999). It was reported that absence of Ripk3 prevents ethanol-induced liver injury (Roychowdhury *et al*., 2013). Upon these results, it is hypothesized that SYRG could regulate multiple stress and toxicity pathways.

The SYRG was prepared from aqueous extracts of 18 medicinal herbs and one animal-derived material. Of these components, the extracts of *Hedyotis diffusa* Willd (Bai Hua She She Cao), *Scutellaria barbata* D. Don (Ban Zhi Lian), *Imperata cylindrica* Beauv.var.major (Bai Mao Gen), *Paeonia lactiflora* Pall. (Bai Shao), *Crataegus pinnatifida* Bunge (Shan Zha), *Ardisia japonica* (Thumb.) Blume (Ai Di Cha). *Solanum lyratum* Thumb. (Bai Mao Teng), *Agrimonia pilosa* Ledeb. (Xian He Cao), *Tetrastigma hemsleyanum* Diels et Gilg (San Ye Qing), *Curcuma wenyujin* Y.H. Chen et C. Ling (Yu Jin), *Curcuma phaeocaulis* Val. (E Zhu) and *Gardenia jasminoides* Ellis (Zhi Zi) have been reported to posses free radical scavenging capacities and antioxidant activities (Lin *et al*., 2004; Ohsugi *et al*., 1999; Newell *et al*., 2010; Kuo *et al*., 2009; Sun *et al*., 2013; Zhao *et al*., 2010; Uddin *et al*., 2014). Moreover, the extract of *Hedyotis diffusa* Willd (Bai Hua She She Cao), *Lysimachia christinae* Hance (Jin Qian Cao), *Paeonia lactiflora* Pall. (Bai Shao), *Agrimonia pilosa* Ledeb. (Xian He Cao), *Paris yunnanensis* Franch. (Chong Lou) and *Gardenia jasminoides* Ellis (Zhi Zi) showed hepatoprotective activities *in vivo* (Lin *et al*., 2002; Wang *et al*., 2012; Sun *et al*., 2007; Park *et al*., 2004; Chen *et al*., 2012b; Man *et al*., 2014). In addition, peoniflorin and geniposide, two main constituents of SYRG, also showed antioxidant and hepatoprotective activities (Kim and Ha, 2010; Kim *et al*., 2013; Ma *et al*., 2011). It has been reported that the free radical scavenging capacity and antioxidant activity of majority plant extracts are associated with their phenolic, flavonoid, proanthocyanidin and polysaccharide contents (Chen *et al*., 2014). The phenolic hydroxyl groups in the structure of these ingredients can serve as electron donor to terminate the radical chain reaction (Rice-Evans *et al*., 1996; Pietta, 2000; Santos-Buelga and Scalbert, 2000; Xu *et al*., 2011). Therefore, although SYRG may contain hundreds of different chemical compounds and its active ingredients responsible for the antioxidant and liver protective activities are still unclear, those published researches mentioned above could explain SYRG's activities partly. In general, it is considered that the therapeutic effects of traditional Chinese medicine are base on their multi-components and multiple targets or network targets interaction (Li and Zhang, 2013). The findings are accordance with these characteristics.

D'Andrea (2005) pointed out that antioxidants may interfere with the mechanism of action of chemotherapy and subsequently decrease its efficacy. However, others argue that antioxidant supplements are beneficial to patients undergoing chemotherapy because they alleviate toxic side effects of the chemotherapy as well as enhance the efficacy, allowing patients to tolerate chemotherapy for the full course of treatment and lessen the need for dose reduction (Conklin, 2004). Although, SYRG has not been found any obviously antagonistic effect on the treatment of chemotherapy in clinical yet, further systematically experimental study focusing on excluding this unfavorable effect is warranted.

CONCLUSION

In summary, it was concluded that a traditional Chinese medicine SYRG has protective effects against oxidative stress-induced liver injury and its underlying mechanisms involve the modulation of multiple stress and toxicity pathways. These findings would be beneficial for us in further understanding of the therapeutic effects of SYRG in treatment of chemotherapy-induced liver injure in clinic.

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