

Anti-Apoptosis Effects on Hearts of SHSST Cyclodextrin Complex in a Carbon Tetrachloride-Induced Cirrhotic Cardiomyopathy Rat Model

Cheng-Hsun Yang^{1, 2}, Wei-Jen Ting³, Pei-Ying Pai^{4, 5}, Sheng-Huang Chang⁶,
Tsung-Jung Ho^{2, 7}, Jing-Ying Lin⁸, Fuu-Jen Tsai², Viswanadha Vijaya Padama⁹,
Yuhsein Tsai^{1, 2}, and Chih-Yang Huang^{2, 3, 10}

¹Graduate Institute of Chinese Medicine, China Medical University, Taichung 40402

²School of Chinese Medicine, China Medical University, Taichung 40402

³Graduate Institute of Basic Medical Science, China Medical University, Taichung 40402

⁴Division of Cardiology, China Medical University Hospital, Taichung 40402

⁵Graduate Institute of Clinical Medical Science, China Medical University, Taichung 40402

⁶Department of Laboratory, Tsaotun Psychiatric Center, Ministry of Health and Welfare, Nantou 54249

⁷Chinese Medicine Department, China Medical University Beigang Hospital, Yunlin 63244

⁸Department of Medical Imaging and Radiological Science, Central Taiwan University of Science and Technology, Taichung 40601

⁹Department of Biotechnology, Bharathiar University, Coimbatore 641046, India
and

¹⁰Department of Health and Nutrition Biotechnology, Asia University, Taichung 41354
Taiwan, Republic of China

Abstract

Cirrhotic cardiomyopathy (CCM) is a common cardiac dysfunction in patients waiting for orthotopic liver transplantation (OLT). Carbon tetrachloride (CCl₄) intraperitoneal (IP) injection has been reported as successful in a cirrhosis-induced CCM model. In this work, we used the same assay for CCM induction using CCl₄ (0.2 mg/kg) IP injection twice per day for 14 days during the cardiac protection drugs treatment process. The cardiac protection drugs were silymarin (100 mg/kg/day), baicalein (30 mg/kg/day), San Huang Shel Shin Tang (SHSST, 30 mg/kg/day) and β -cyclodextrin modified SHSST (SHSSTc, 30 mg/kg/day and 300 mg/kg/day). After 4 weeks of treatment, the SHSSTc cardiac protection effects were determined through activation of the IGF1R cell survival pathway and inhibition of Fas-FADD death domain induced-apoptosis. SHSSTc cardiac protection was enhanced through β -cyclodextrin modification, which increased bio-availability, and displayed stronger protective effects than silymarin and baicalein, both of which are well-known liver protection drugs. Thus, SHSSTc might provide the best therapeutic benefit in CCM treatment.

Key Words: baicalein, beta-cyclodextrin modified SHSST, cirrhotic cardiomyopathy, silymarin

Corresponding authors: [1] Chih-Yang Huang, Ph.D., Graduate Institute of Basic Medical Science, China Medical University and Hospital, Taichung, Taiwan, No. 91, Hsueh-Shih Rd., Taichung 40402, Taiwan, R.O.C. Tel: +886-4-22053366 ext. 3313, Fax: +886-4-22032295, E-mail: cyhuang@mail.cmuh.edu.tw; and [2] Yuhsein Tsai, Ph.D., School of Chinese Medicine, China Medical University, Taichung, Taiwan, No. 91, Hsueh-Shih Rd., Taichung 40402, Taiwan, R.O.C. Tel: +886-4-22053366 ext. 1019, Fax: +886-4-22032295, E-mail: yhtsai@mail.cmuh.edu.tw
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Introduction

Some patients with liver cirrhosis suffer from subtle cardiac structure or functional abnormalities, defined as cirrhotic cardiomyopathy (CCM) (33). Typical characteristic of CCM is blunted contractile responsiveness to stress. The prevalence of CCM incidences has recently been estimated at about 24.3%, which included 85 cases of patients waiting for orthotopic liver transplantation (OLT) in 15 patients diagnosed in France, and 50% in USA (5, 38). Left ventricular diastolic dysfunction (DDF) is prevalent in advanced cirrhosis. In fact, CCM diagnosis is not easy. The current main determination factor for CCM is DDF (22).

CCM was reported to cause 7% to 21% of deaths after OLT resulting from overt heart failure (27, 35). There is no standard treatment for CCM because it does not have a solidly established diagnosis, and many cases are diagnosed based on high clinical suspicion (20). The standard treatment for noncirrhotic heart failure, preload reduction, bed rest, oxygen and diuretics, also works for CCM. *k*-Canrenoate, an aldosterone antagonist, was shown to decrease left vesicular (LV) wall thickness at 6 months of treatment, but it fails owing to no change in diastolic dysfunction (28).

We used silymarin and a traditional Chinese herbal medicine formula, San Huang Shel Shin Tang, or SHSST, and its water-soluble β -cyclodextrin (CD) complex, SHSSTc, in this work to treat CCM in induced rats using a carbon tetrachloride (CCl_4) intraperitoneal injection-induced cirrhosis model (29, 30, 37). Silymarin is a promising drug against cirrhosis consisting of a cocktail-like herbal liver-protective drug made with four flavonolignan isomers, silybin, silychristin, silydianin and isosilybin (1, 23). SHSST is a cocktail-like traditional herbal decoction used for liver and heart protection in China. SHSST is composed of 50% *Rheum officinale* Baill, 25% *Scutellaria baicalensis* Geprgi and 25% *Coptis chinensis* Franch in weight (6, 32). Rheum was reported to have a liver protection effect that protects the liver in CCl_4 -induced injury in rats. Scutellaria and Coptis were also discovered to exhibit similar liver protection effects in acute hepatotoxicity (3, 21, 36). Similar liver protection effects of Rheum, Scutellaria and Coptis chinensis are due to identical bioactive compounds, a combination of flavonoids, including baicalein (12, 17, 31).

Both SHSST and silymarin are promising liver protection drugs, but both present poor water solubility and bioavailability. A formulation approach is necessary to increase the solubility of these liver protection drugs. Beta-CD modification can increase the solubility and spectral properties of guest molecules, especially hydrophobic drugs, without changing their intrinsic properties to permeate the cell mem-

branes (11, 35). Thus, SHSST was modified into SHSST- β -CD-complex (SHSSTc) in this study, and was evaluated for its therapeutic effects in a CCM animal model.

Materials and Methods

Preparation of SHSST- β -CD Complex and Drugs

SHSST- β -CD complex was formed using a co-precipitation synthesis process. β -CD (70.0 g) was dissolved in 85 ml pure water at 70°C for 1 h. Twenty gram SHSST in 30 ml alcohol was slowly added to the β -CD solution with continuous agitation, stirring for 6 h. Forty ml of alcohol was then added to regulate the solubility of the hydrophobic solute in β -CD solution and then refrigerated overnight at 4°C. The precipitated SHSSTc (SHSST: β -CD = 1: 9 in weight) was filtered and washed with alcohol to remove unencapsulated SHSST. This residue was dried and stored at 4°C until use. The silymarin (Sigma, Missouri, USA.), baicalein (Sigma), SHSST and SHSSTc stock solutions were prepared by dissolving in distilled deionized water at 100 mg/ml each. CCl_4 was dissolved in olive oil at 4% v/v concentration.

Animal Model

Forty-two Sprague-Dawley rats (SD rats) were purchased from BioLASCO Taiwan Co., Ltd., Taipei, Taiwan, ROC. The 42 rats were divided into 7 groups, $n = 6$ in each group. The animal experimental protocol was in accordance to our previous published paper (35). The protocol was also approved by the Institutional Animal Care and Use Committee (IACUC) of China Medical University (No.100-3-B) (35). The CCM induction model involved intraperitoneal (IP) injection of CCl_4 into rats for induction of cirrhosis, applied twice at 0.2 mL/kg dosages in the first and fourteenth days. After CCM induction, 4 more weeks of drug treatments were applied through gavage assay to each rat. Groups were designated the normal control group, CCl_4 -induced CCM group, silymarin (100 mg/kg/day) treatment group, baicalein (30 mg/kg/day) treatment group, SHSST (30 mg/kg/day) treatment group, SHSSTc (30 mg/kg/day) treatment group and SHSSTc (300 mg/kg/day) treatment group.

DAPI and TUNEL Staining

Terminal deoxynucleotidyl transferase dUTP-mediated nick-end labeling (TUNEL) assay sections were incubated with proteinase K, washed in PBS, incubated with permeabilization solution, blocking buffer and then washed two times with PBS. The terminal deoxynucleotidyl transferase and fluorescein isothio-

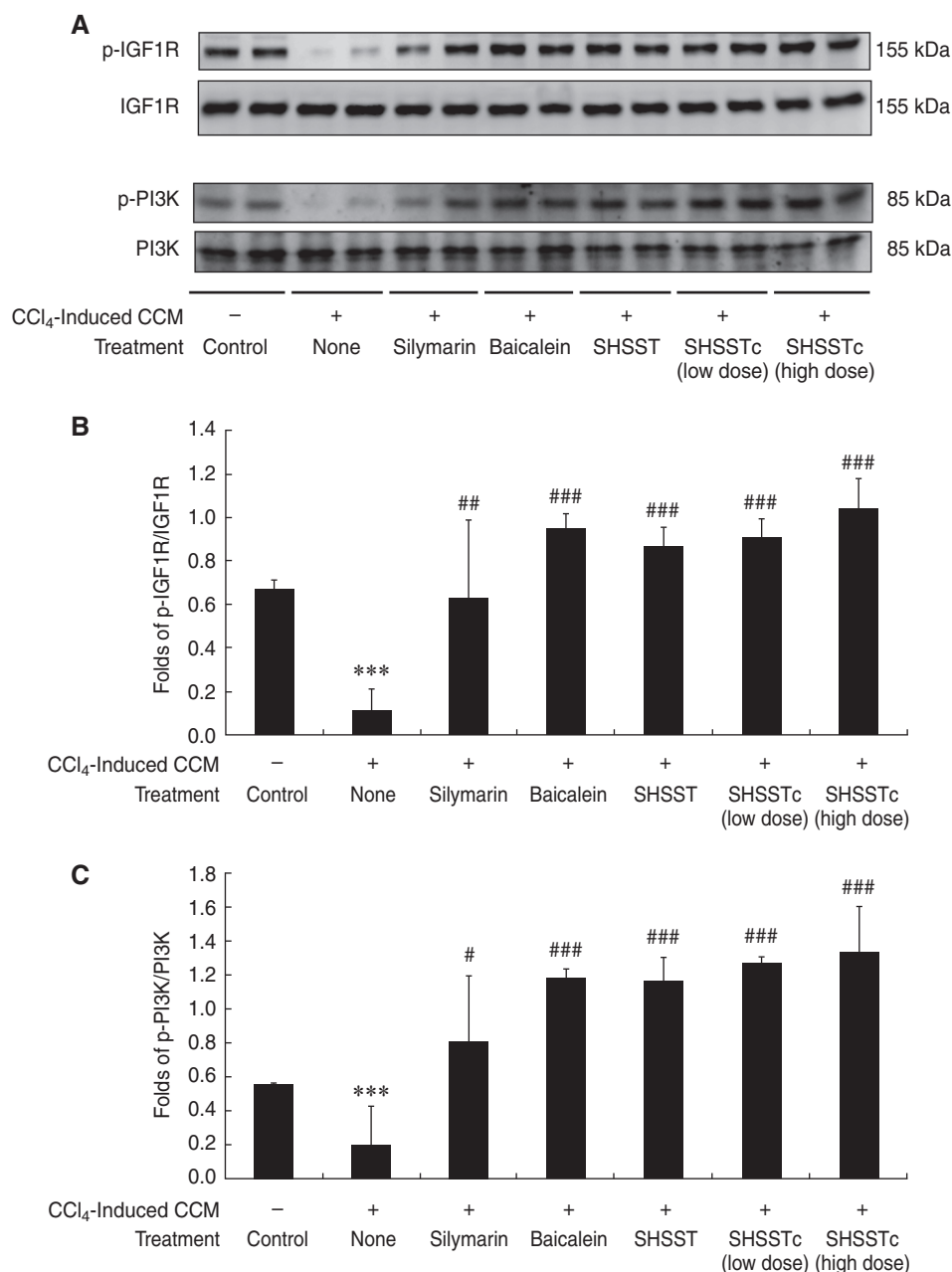


Fig. 1. (A) Analysis of expression of IGF1R and PI3K phosphorylation. (B-C) Normalized protein expression levels of p-IGF1R/IGF1R (B) and p-PI3K/PI3K (C). *** $P < 0.001$ compared with the control; ## $P < 0.01$ compared with CCl₄-induced CCM; ### $P < 0.001$ compared with CCl₄-induced CCM.

cyanate-dUTP were applied for 60 min at 37°C from an apoptosis detection kit (Roche Applied Science, Indianapolis, IN, USA). TUNEL-positive nuclei, indicating fragmented DNA, were fluoresced using bright green light at 460 nm. 4,6-diamidino-2-phenylindole (DAPI) was dissolved in PBS at 0.1 µg/ml and added to the slides for 5 min. The nucleus position was fluoresced using blue light at 454 nm. Photomicrographs were obtained using Zeiss Axiophot microscopes.

Tissue Protein Extraction

Heart tissue extracts from 6 rats in each group were obtained by homogenizing at a ratio of 100 mg tissue per 1 ml lysis buffer (0.05 M Tris-HCl, pH 7.4, 0.15 M NaCl, 0.25% deoxycholic acid, 1% NP-40, 1 mM EDTA). The homogenates were placed at 4°C and then centrifuged at 13,000 rpm for 40 min. The supernatants were stored at -80°C for further experiments.

Western Blot Assay

Protein samples were separated in 12% SDS poly-

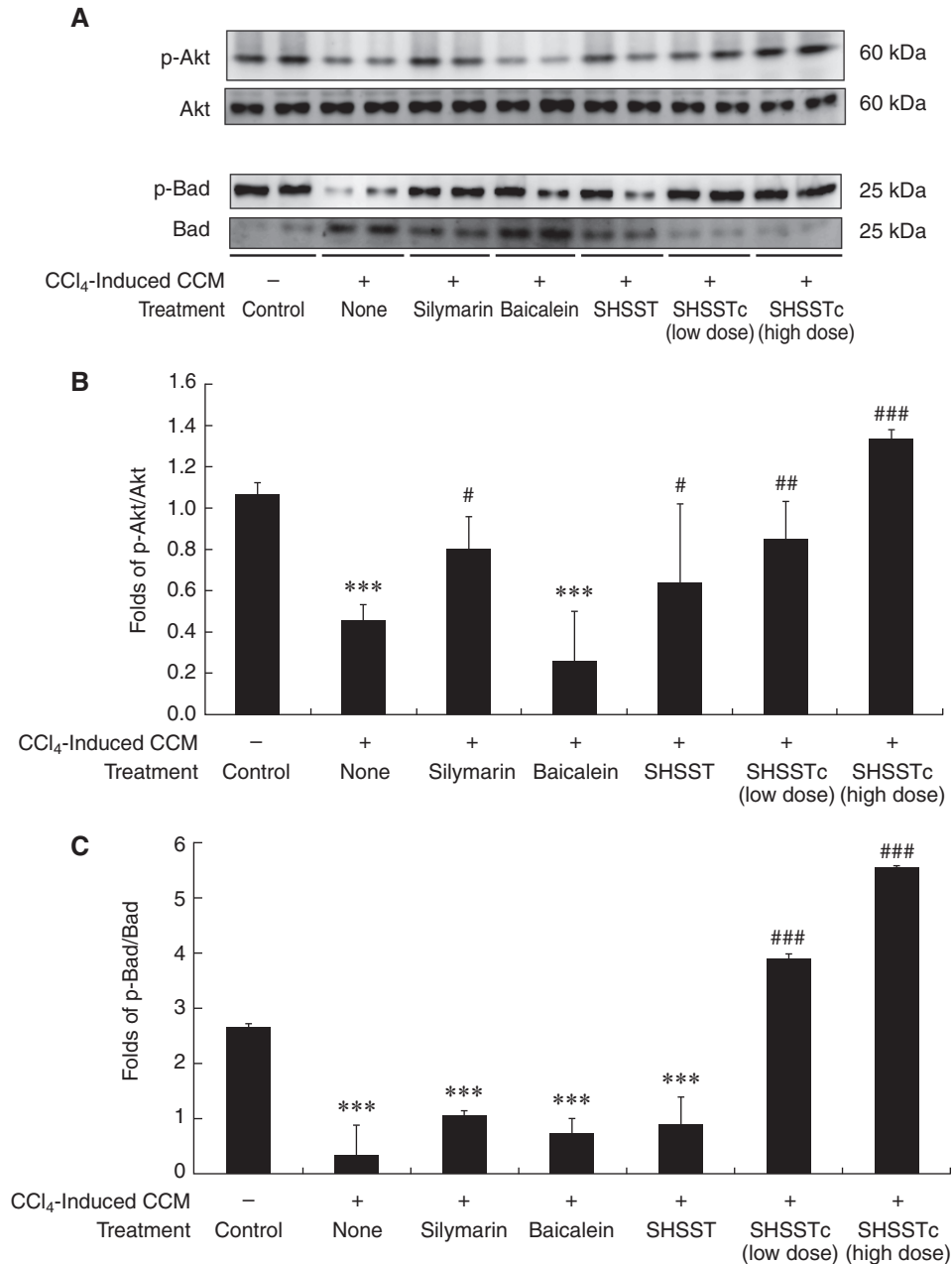


Fig. 2. (A) Analysis of expression of Akt and Bad phosphorylation. (B-C) Normalized protein expression levels of p-Akt/Akt (B) and p-Bad/Bad (C). *P* values are as defined in the legend of Fig. 1.

acrylamide gel electrophoresis (SDS-PAGE) with a constant voltage of 75 V for 120 min after the concentration was determined using the Lowry protein assay. Protein samples in the gels were transferred to Hybond-C PVDF membranes (GE healthcare UK limited, Buckinghamshire, UK) using 50 volt for 3 h. PVDF membranes were then incubated in 3% bovine serum albumin (BSA) in TBS buffer for blocking. Except for p-Akt (#9271), which was obtained from Cell Signaling, Maryland, USA, all primary antibodies for subsequent analysis were purchased

from Santa Cruz Biotechnology, CA, USA. The primary antibodies included Fas (SC-7886), FADD (SC-6035), caspase-8 (SC-6134), α -tubulin (SC-5286), PI3K (SC-423), p-PI3K (SC-12929), Akt (SC-5298), Bad (SC-8044), p-Bad (SC-7999) and Caspase 3 (SC-7148). The primary antibodies were added into the TBS solution with the membranes for targeting specific proteins. Pictures were then taken with Fujifilm LAS-4000 (GE healthcare UK limited, Buckinghamshire, UK) after horseradish peroxidase-labeled antibodies were used.

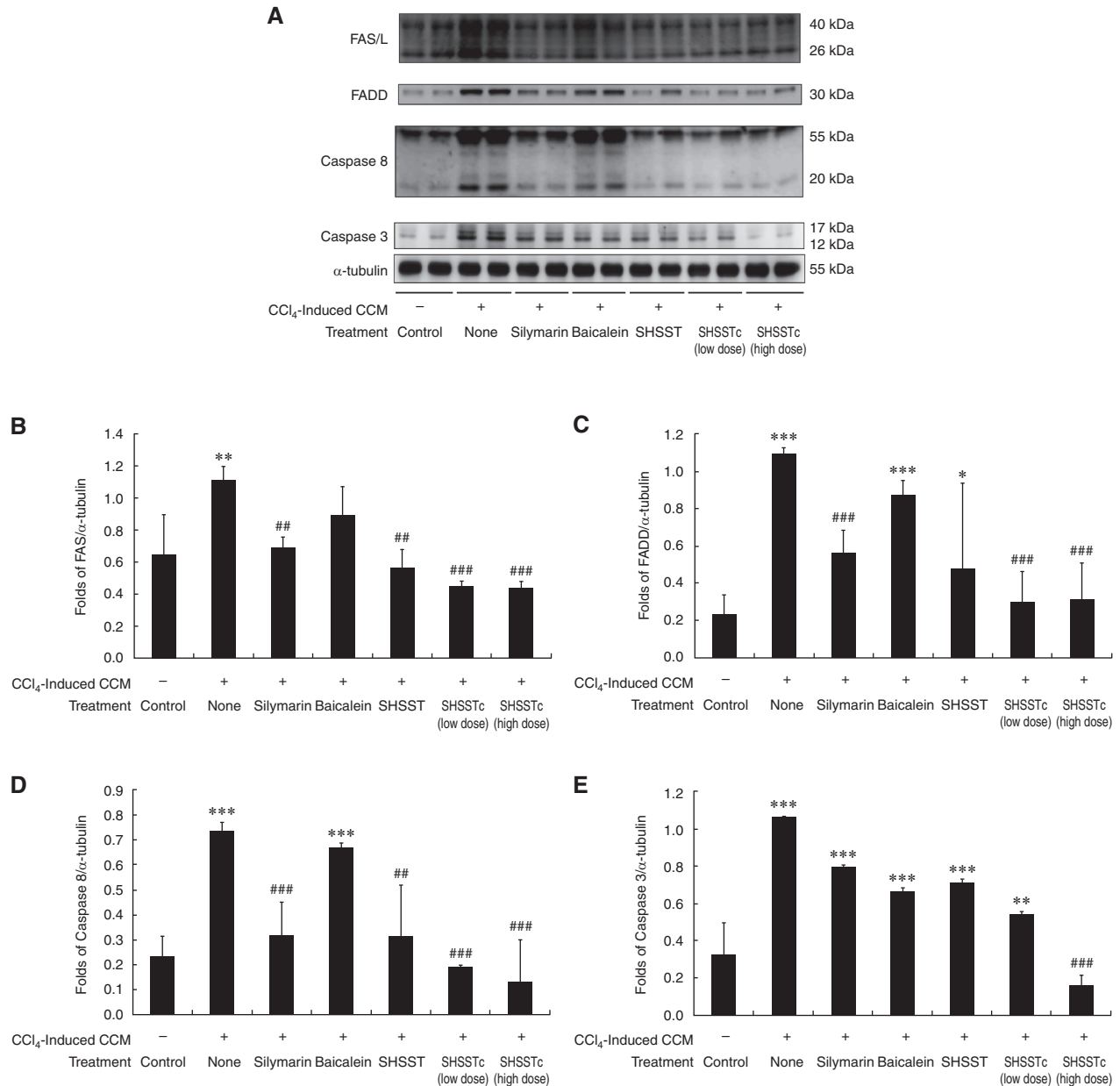


Fig. 3. (A) Analysis of expression of the FAS-FADD signaling pathway. (B-D) Normalized protein expression levels of FAS/ α -tubulin (B), FADD/ α -tubulin (C), caspase 8/ α -tubulin (D) and caspase 3/ α -tubulin (E). *P* values are as defined in the legend of Fig. 1.

Statistical Analysis

The results shown are means \pm SD of three independent experiments. Statistical analysis was performed using one-way analysis of variants. Student's *t*-test was applied for paired samples.

Results

After 4 weeks of treatments with twice CCl₄-induced challenges, the IGF1R and its downstream PI3K phosphorylation were inhibited in the heart of

CCM rat compared to that of the control rat (Fig. 1). In the silymarin (100 mg/kg/day) treatment group, phosphorylated IGF1R and PI3K expression levels were both reversed back to a level equal to that of the control group. Treatments with baicalein (30 mg/kg/day), SHSST (30 mg/kg/day), SHSSTc at low dose (30 mg/kg/day) and high dose (300 mg/kg/day) increased the phosphorylation of IGF1R and PI3K.

The phosphorylated Akt is dominated by Bad phosphorylation (25). p-Bad plays a role in anti-apoptosis (Fig. 2). After 4 weeks of treatments with twice CCl₄-induced challenges, Akt and its down-

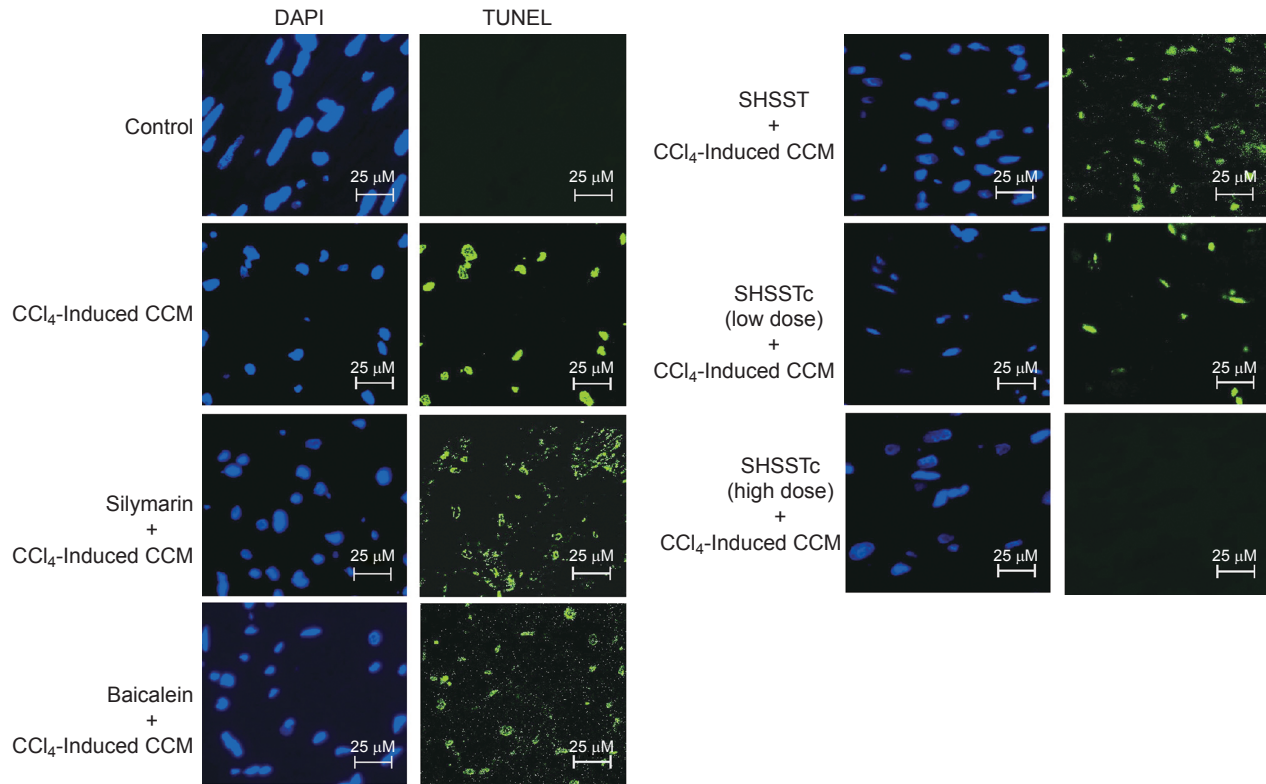


Fig. 4. DAPI and TUNEL staining of rat hearts of each treatment group. Nuclei of the cells were stained with DAPI in blue, and the nuclei of apoptosis cells were indicated by TUNEL stain in green. The bar represents 25 μ m.

stream Bad phosphorylation were inhibited by more than 50% in the hearts of the CCM rats. The baicalein treatments did not increase Akt and Bad phosphorylation. In the silymarin and SHSST treatment groups, the ratios of the p-Akt/Akt expression levels were increased by 0.81- and 0.70-fold, but downstream p-Bad expression levels were not significantly increased. However, in the SHSSTc treatment group, p-Akt and p-Bad phosphorylation increased in a dose-dependent manner.

Expression levels of Fas and FADD and the downstream caspase 8 and 3 were increased (Fig. 3). After silymarin and SHSST treatments, the FAS/FADD-induced apoptotic protein expression levels were reduced. However, baicalein treatment did not provide a protective effect better than silymarin. The SHSSTc treatments, at both low and high doses, provided significant protective effects through FAS/FADD-induced down-regulation of caspase 8 and 3.

Apoptotic cells were clearly detected by the TUNEL assay (16). The hearts of the CCl₄-induced CCM rats presented more apoptotic cardiomyocyte cells than the hearts of the control rats (Fig. 4). The apoptotic cells in hearts were reduced after 4 weeks of treatment with silymarin, baicalein, SHSST and low-dose SHSSTc. The high-dose SHSSTc treatment almost totally inhibited cell apoptosis in hearts of

the CCl₄-induced CCM rats.

Discussion

Cardiac dysfunction is usually attributed to concomitant diseases in cirrhosis patients, such as excess alcohol consumption (2, 24). We previously used a common dose of CCl₄-induced hepatic fibrosis and found that within 24 h the heart exhibited increased BNP expression (20). This result shows that CCl₄ may also directly damage the heart. Therefore, in this study, the CCl₄ dose was reduced to 0.2 ml/kg, and the injections were done twice daily in order to avoid directly affecting the heart of the injected rat.

Based on our previous reports, cardiac functional defects are an obvious phenomenon that can be found in the decline of expression of the IGF1R/PI3K/Akt survival pathway (13, 14, 15, 39). After treatment, we found that IGF1R could be activated in promoting downstream PI3K phosphorylation. PI3K and Akt phosphorylation requires the Akt downstream anti-apoptotic protein BAD to maintain AKT phosphorylation to stabilize 14-3-3 on BAD to avoid apoptosis (25). Although this study could not directly prove that CCM depression was the main cause of PI3K/Akt expression, SHSSTc or silymarin treatment could indeed reply to PI3K/Akt activation by reducing the

CMM scale of the incidence.

FAS-ligand is one of the soluble cytokines released into the blood circulation system in the early stages of liver fibrosis (9). As reported, Fas over-expression is highly related to cardiac dysfunction, because FAS on heart cells can be activated by the FAS-ligand by direct activation of the FADD death domain, initiating cell apoptosis (8). Caspase 8 and 3 are activated downstream inducing apoptotic signals and resulting in cardiomyocyte death (10, 26).

Silymarin has been shown to resist hepatocyte and cardiomyocyte apoptosis (4, 7, 18). The results from this study also confirmed that CCl₄-induced liver fibrosis in rat hearts indeed utilized the FAS-L/FAS/FADD-induced death pathway. Silymarin did provide protective functions for the heart. Baicalein, the main bioactive constituents of SHSST, provided less protection. However, β -CD-modified SHSSTc exerted an outstanding heart protection effect in a dose-dependent manner.

According to the experimental evidences in our research, hepatoprotective drugs such as silymarin exerted anti-apoptosis effects and PI3K/Akt survival pathway activation, proving silymarin to be a good therapeutic candidate for CCM treatment. However, SHSSTc provides a particularly stronger amelioration effect than silymarin in treating CCM.

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