A herbal formula, SYKT, reverses doxorubicin-induced myelosuppression and cardiotoxicity by inhibiting ROS-mediated apoptosis

TING CHEN[1,2*], HONG-MEI SHEN[3*], ZHI-YONG DENG[1*], ZUO-ZHANG YANG[4], RUI-LIAN ZHAO[3], LI WANG[2,5], ZHI-PING FENG[1], CHAO LIU[1], WEN-HUI LI[5] and ZHI-JIE LIU[2]

[1]Department of Nuclear Medicine, Tumor Hospital of Yunnan Province, The Third Affiliated Hospital of Kunming Medical College, Kunming, Yunnan 650118; [2]The Institute of Molecular and Clinical Medicine of Kunming Medical College, Kunming, Yunnan 650500; Departments of [3]Combination of Chinese Traditional and Western Medicine; [4]Orthopaedics and [5]Radiotherapy, Tumor Hospital of Yunnan Province, The Third Affiliated Hospital of Kunming Medical College, Kunming, Yunnan 650118, P.R. China

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Abstract. Doxorubicin (DOX) is an antineoplastic drug widely used for the treatment of various types of cancer; however, it can induce severe side effects, such as myelosuppression and cardiotoxicity. Sanyang Xuedai (SYKT) is a natural medicine originating from an ancient prescription of the Dai nationality in Southwest China. With eight Chinese herbal medicines, including sanguis draconis, radix et rhizoma notoginseng, radix et rhizoma glycyrrhizae and radix angelicae sinensis as the primary ingredients, SYKT has been reported to possess numerous biological functions. The present study investigated whether SYKT can confer protection against DOX-induced myelosuppression and cardiotoxicity, and explored the potential mechanism involved. Mice were treated with DOX, SYKT or a combination of the two; hematopoietic functions were assessed by measuring the number of peripheral blood cells, cluster of differentiation $CD34^+/CD44^+$ bone marrow cells and apoptotic cells. Myocardial enzymes, including aspartate aminotransferase, lactate dehydrogenase, creatine kinase (CK) and its isoform CK-MB, were assessed using a biochemical analyzer. The apoptotic rate of cardiomyocytes was assessed using flow cytometry. Histopathological analysis was conducted using hematoxylin-eosin staining. Intracellular reactive oxygen species (ROS) production was evaluated using a dichlorofluorescein intensity assay. The mice treated with DOX exhibited a reduced survival rate, reduced peripheral blood and $CD34^+/CD44^+$ cell counts, elevated myocardial enzymes and apoptotic indices in bone marrow cells and cardiomyocytes, all of which were effectively prevented by SYKT co-administration. Furthermore, bone marrow cells and myocytes from mice treated with DOX demonstrated increased dichlorofluorescein intensity, which was attenuated by SYKT. Notably, SYKT did not interfere with the effects of DOX on tumor volume or the induction of tumor cell apoptosis in tumor-bearing mice. The present study indicated that SYKT may counteract DOX-induced myelosuppression and cardiotoxicity through inhibiting ROS-mediated apoptosis. These findings suggested that SYKT may have potential as a means to counteract the potentially fatal hematopoietic and cardiac complications associated with DOX treatment.

Correspondence to: Professor Zhi-Jie Liu, The Institute of Molecular and Clinical Medicine of Kunming Medical College, 1168 West Chunrong Road, Kunming, Yunnan 650500, P.R. China
E-mail: liuzhj@shanghaitech.edu.cn

Dr Wen-Hui Li, Department of Radiotherapy, Tumor Hospital of Yunnan Province, The Third Affiliated Hospital of Kunming Medical College, 519 Kunzhou Road, Kunming, Yunnan 650118, P.R. China
E-mail: wenhuili64@aliyun.com

*Contributed equally

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Introduction

Cancer is the second leading cause of mortality in developing countries (1). Despite the advances in cancer biology and cancer therapeutics, most of the currently available anticancer drugs are characterized by immunosuppressive and cytotoxic adverse effects (2). Doxorubicin (DOX) is widely used in the treatment of solid tumors and hematological malignancies. Despite its extensive and longstanding clinical applications, its use is associated with serious adverse effects, which include cardiotoxicity and hematopoietic suppression (3,4). However, the mechanism of DOX-induced cytotoxicity remains to be elucidated. Reactive oxygen species (ROS) are involved in various processes of DOX metabolism, including the redox cycling of its quinone moiety, the disturbance of

iron metabolism and the production of DOX metabolites in myocardial tissue (5). DOX can directly induce platelet cytotoxicity by stimulating the generation of ROS, decreasing glutathione levels, and depleting protein thiol groups (6). The potentiation of ROS production and the depletion of endogenous antioxidants can trigger the intrinsic apoptotic pathway in hematopoietic cells and cardiomyocytes (7). Therefore, oxidative stress appears to hold a key role in DOX-induced myelosuppression and cardiotoxicity.

The deleterious effects associated with ROS production could be counteracted with antioxidants (7). Antioxidants and free radical scavengers can prevent oxidative tissue damage by directly neutralizing reactive hydroxyl and peroxy radicals, or through regulating ROS signaling involved in gene expression and enzymatic cascades (8). Various antioxidants have been identified, including preventative antioxidants, such as superoxide dismutase and catalase, and lipid peroxidation blockers, such as vitamins C and E (9). In addition, some herbs, such as ginseng, astragalus, poria and notoginseng, have exhibited significant antioxidant potential (10). Nevertheless, to the best of our knowledge, the use of antioxidants in counteracting the deleterious actions of DOX has yet to be investigated. Therefore, the identification of an alternative, safe and widely available treatment is required to mitigate the pathophysiological effects of DOX.

Previous studies have investigated the potential of natural products in minimizing the toxic effects of chemotherapeutic agents on healthy cells, without compromising their antineoplastic activity (11). SYKT is a natural medicine originating from an ancient prescription of the Dai nationality in Southwest China. SYKT is composed of eight primary ingredients, including sanguis draconis, radix et rhizoma notoginseng, radix et rhizoma glycyrrhizae, radix angelicae sinensis, ginger, Rhizoma Dioscoreae, Poria cocos, and Fructus Amomi. SYKT has been demonstrated to significantly improve the condition of anemic patients (12). Previous pharmacological and toxicological studies, as well as clinical trials, have revealed that SYKT has no toxicity or negative adverse effects (13). It has previously been demonstrated that SYKT can potentiate hematopoiesis and the function of the immune system; in addition, it can decrease chemotherapy- and radiotherapy-induced toxicity (13). Therefore, it may be hypothesized that SYKT can prevent chemotherapy-induced toxicity due to its antioxidant and cytoprotective properties. The present study evaluated the potential of SYKT administration as a protective strategy against DOX-induced myelosuppression and cardiotoxicity, and investigated the underlying mechanism using mouse models.

Materials and methods

Materials. DOX was purchased from Shenzhen Main Luck Pharmaceuticals Inc. (Shenzhen, China) and dissolved in PBS. SYKT (1.02 g/ml) containing sanguis draconis, radix et rhizoma notoginseng, radix et rhizoma glycyrrhizae, radix angelicae sinensis, ginger, Rhizoma Dioscoreae, Poria cocos and Fructus Amomi., was purchased from Great Tao Pharmaceutical Co., Ltd (Yunnan, China). A mixture of Cremophor RH40 (BASF SE, Ludwigshafen, Germany) and dehydrated alcohol (1:1, w/w) was used to dissolve vitamin E (d, l-a-tocopherol; BASF SE) to a stock concentration. Further dilutions were made with water prior to injection at a concentration corresponding to 100 IU/kg for each animal. All drugs were prepared immediately prior to use, with sterile solvents and under sterile conditions. Mouse lymphoma (EL4) cells were obtained from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China). Anti-CD34 (catalogue no. 551387) and CD44 antibodies (catalogue no. 561859) were purchased from BD Biosciences (Franklin Lakes, NJ, USA). A terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL) apoptosis assay kit was purchased from Merck KGaA (Darmstadt, Germany). ROS detection reagents were purchased from Thermo Fisher Scientific, Inc. (Waltham, MA, USA).

Animals. Inbred female C57BL/6J mice (age, 8-9 weeks; weight 20-25 g) were purchased from Dashuo Laboratory Animal Technology (Chengdu, China). The mice were housed in well-ventilated cages (5 mice per cage) under standard room temperature, pressure and humidity conditions. The animals were provided with free access to normal mouse chow and water. All experiments and procedures involving animals and their care were conducted in conformity with NIH guidelines (NIH Pub. No. 85-23, revised 1996) and were approved by Animal Care and Use Committee of Kunming Medical College (Kunming, China).

Mortality analysis. DOX was dissolved in normal saline prior to injection in mice. A total of 24 mice were randomly assigned into four groups (n=6/group). Mice in the SYKT group received SYKT (1.2 ml/kg/d) for 5 days, in a cumulative dosing scheme resembling its clinical use. Mice in the DOX group received a single dose of DOX (20 mg/kg, subclinical lethal dose) on day 5. Mice in the SYKT/DOX group received both SYKT and DOX as aforementioned. Control mice received normal saline. Mice in the control group were gavaged with normal saline at an equal volume to the SYKT solution during day 1 to day 5, and treated with normal saline at an equal volume to the DOX solution on day 5 via peritoneal perfusion. Mortality was monitored daily.

Subgrouping and drug administration. A total of 24 mice were randomly assigned into four groups (n=6/group) and were treated as follows. The control group received normal saline at an equal volume to the SYKT solution on day 1, 3 and 5; and treated with normal saline at an equal volume to DOX solution on day 2, 4 and 6 via peritoneal perfusion. The SYKT group received SYKT via gavage at a dose of 1.2 ml/kg/d on days 1, 3 and 5. In the DOX group, the mice were injected intraperitoneally with DOX at a dose of 3 mg/kg (14) on days 2, 4 and 6. This cumulative dose (9 mg/kg) was equivalent to 630 mg for a 70-kg human male, just above the threshold at which DOX cardiomyopathy is expected to occur in a clinical setting (15). The SYKT/DOX group received SYKT via gavage at a dose of 1.2 ml/kg/d on days 1, 3 and 5, and DOX at a dose of 3 mg/kg/d (i.p.) on days 2, 4 and 6. The mice were sacrificed at different times according to the experimental plan. Whole blood samples were collected, and the femur bone was isolated to obtain

bone marrow cells. The cardiac tissue was fixed in 10% formalin for histopathological examination or stored at -80˚C for further analysis.

Blood cell test. A 10 μl blood sample was drawn from the tail vein of each mouse into an EDTA-coated capillary tube prior to administration (day 0), and day 5, 10, 15, 20 and 30 following administration. The samples were mixed with 10 ml PBS. Peripheral white blood cells (WBCs), red blood cells (RBCs) and platelets (PLTs) were counted using a hematometer with an optical microscope.

Bone marrow cells analysis. Bone marrow cells were flushed from the bilateral femurs after mice were sacrificed. Following two washes with PBS, bone marrow cells were collected. After erythrocytes were lysed with RBC lysis buffer (BioLegend, Inc., San Diego, CA, USA), total bone marrow cell viability was assessed using trypan blue. Bone marrow cells were labeled with anti-CD34 and anti-CD44 antibodies (1:1,000), which are markers of hematopoietic stem cells, mesenchymal stem cells and other cell types in the bone marrow, by incubating the mixture at 4˚C for 30 min. Results were analyzed using a BD FACSCalibur (BD Biosciences). All antibodies and dyes were purchased from BD Biosciences.

Myocardial enzymes. Biochemical tests were used to detect aspartate aminotransferase (AST), creatine kinase (CK), CK-MB and lactate dehydrogenase (LDH) levels. Blood sampling was performed via orbital sinus puncture while the animals were under light diethylether anesthesia (common concentration of diethylether: 2-4%) on day 15 following drug delivery, then placed in safe-lock centrifuge tubes (1.5 ml) and maintained for 1 h at room temperature to allow spontaneous coagulation. The segregated sera were extracted via collection in a 1.5 ml test tube, separated by centrifugation at 1,006 x *g* for 15 min, stored as 50 μl aliquots at -20˚C and assayed within 72 h. The serum parameters were analyzed using an Aeroset Clinical Chemistry Analyzer (Abbott Pharmaceutical Co., Ltd, Lake Bluff, IL, USA). The activity measurements were expressed in international units of enzyme activity per liter (IU/L) of serum. The reference change limits were obtained from the sera of 30 untreated mice.

Myocardial histological analysis. The mice were sacrificed with an overdose of sodium pentobarbital (45 mg/kg) and hearts were removed. The right and left ventricles were transected into sections parallel to the atrioventricular sulcus according to the method of heart preparation (16) and placed in 10% buffered formalin for 24 h at room temperature. Further fixation of the tissue and paraffin embedding were carried out according to standard procedures (17). The obtained specimens were stained with hematoxylin-eosin and observed with an optimal microscope. The degree of myocardial damage was scored according to rules described by Billingham *et al* (18) as follows: Grade 0, cells show no anthracycline (DOX) damage; grade 0.5, the myocardium is not completely normal but no anthracycline-specific changes are evident; grade 1.0, a few cells (<5%) have myofibrillar loss or distended sarcoplasmic reticulum, or both; grade 1.5, small groups of cells (5-15%) exhibit anthracycline effects consisting of marked myofibrillar loss or cytoplasmic vacuolization, or both; grade 2.0, 16-25% of cells demonstrate the aforementioned alterations; grade 2.5, 26-35% of cells demonstrate the aforementioned alterations; grade 3.0, specimens exhibit diffuse cell damage with >35% of cells exhibiting pathologic changes, the loss of contractile elements and organelles, and mitochondrial and nuclear degeneration. The tissue specimens were analyzed by three pathologists in a blind manner. The results were recorded as the median of the three independent scores for each animal. A total of six animals per group were included. A statistical evaluation was performed using the Kruskal-Wallis test.

Measurement of intracellular ROS. A different group of mice (6 mice in each group) were treated with DOX combined with d, l-a-Tocopherol (vitamin E). Vitamin E was used as a ROS scavenger positive control (19). Vitamin E was orally administered at a 0.2 ml bolus dose on days 1, 3 and 5, and DOX was administered at a dose of 3 mg/kg (i.p.) on days 2, 4 and 6. Day 15 post-treatment was selected as the most appropriate time point for further investigation due to myelotoxicity and cardiotoxicity being the most significant at this time following treatment with DOX. The heart and bilateral femur bones were removed after sacrificing the animals; a single-cell suspension of bone marrow and myocardial cells was obtained. The mice were immersed in 75% ethanol for 3-5 min following sacrifice. The four limbs were isolated while keeping the humerus and femurs intact. The two ends of the epiphysis were broken to expose the bone marrow cavity. The bone marrow cavity was rinsed with DMEM culture medium to suspend the bone marrow. The myocardial cell suspension was then prepared with neonatal mice, with procedures briefly described as follows. The isolated heart was placed on a petri dish containing serum-free DMEM culture medium, to clean the stained blood and remove the base of the heart and epicardial connective tissue. The ventricular muscle was cut into 0.5-1 mm^3 sized pieces and digested with PBS containing 0.125% Trypsin. The medium was mixed and the culture flask was placed in a CO_2-enriched incubator at 37˚C for 5-7 min, followed by mixing with suction tubes. The supernatant was moved into a centrifugation tube and an equal volume of 15% NBC-DMEM culture medium was added to prevent digestion. Undigested tissue blocks were further treated by addition of 0.06% Trypsin. Tissue blocks were generally digested following a repeated digestion of 3-4 times. The digested solution and cell suspension were filtered using stainless steel 100-mesh and then collected, followed by centrifugation at 335 x *g* for 5-7 min. The supernatant was removed and the precipitated section was added to a DMEM culture medium containing 15% BSA. The myocardial cell suspension was ready following gentle mixing. The cells were incubated with 2',7'-dichlorofluorescin diacetate (DCFDA; 10 mM) for 1 h at 37˚C in the dark, followed by an immediate wash in PBS. Intracellular ROS production was detected using the intensity of fluorescence of the oxidant sensitive probe DCFDA. The intensity of emitted fluorescence was measured using flow cytometry at a wavelength of 525 nm.

Assessment of bone marrow and myocardial cell apoptosis. Cells (1-2x10^6) were harvested and fixed in 1% paraformaldehyde at room temperature for 30 min. The cells were washed

in PBS containing 0.1 M glycine, resuspended in ice-cold 70% ethanol, and stored overnight at -20˚C. To detect apoptosis, the cells were washed in PBS and resuspended in a 50 μl reaction mixture containing 0.1 mM dithiothreitol, 0.05 mg/ml bovine serum albumin (BSA) (Roche Diagnostics GmbH, Mannheim, Germany), 2.5 mM $CoCl_2$, 0.4 mM biotin-16-dUTP, and 0.1 U/ml terminal deoxynucleotidyl transferase in 0.1 M sodium cacodylate buffer (pH 7.0). The mixture was incubated at 37˚C for 30 min. The cells were then washed in PBS and resuspended in 100 μl staining buffer containing 2.5 mg/ml fluoresceinated avidin, 4X concentrated saline-sodium citrate buffer, 0.1% Triton X-100 and 5% (w/v) low-fat dried milk. The cells were incubated for 30 min at room temperature in the dark and then rinsed twice in PBS prior to flow cytometry (FACScan; BD Biosciences). An excitation wavelength of 488 nm was obtained using a 15-mW air-cooled argon ion laser. Fluorescence emission was collected through a 530/30 band-pass filter for fluorescein isothiocyanate (FITC) and a 585/42 band-pass filter for phycoerythrin (PE). Both FITC and PE fluorescence data were collected on a linear scale (FlowCytomixPro Software, Version 2.1, Bender MedSystems GmbH, Vienna, Austria).

Measurement of tumor volume and apoptosis in mouse solid tumor xenograft model. The cell suspension was cultured in 1640 medium containing 10% BSA and 10% penicillin and streptomycin. Mouse lymphoma (EL4) cells ($1x10^6$) were then subcutaneously injected into the right hind limb of each animal. Mice bearing tumors of similar sizes were randomly divided into four groups (n=6/group). Mice in the SYKT group received SYKT (1.2 ml/kg/d on days 1, 3 and 5, p.o.). Mice in the DOX group received DOX (3 mg/kg/d on days 2, 4 and 6, i.p.). Mice in the SYKT/DOX group received both SYKT and DOX as aforementioned. Control mice were gavaged with normal saline at an equal volume to SYKT solution on day 1, 3, and 5; and normal saline at an equal volume to DOX solution on day 2, 4 and 6 via peritoneal perfusion. The radii of the developing tumors were measured using Vernier calipers on days 3, 5, 7, 10 and 15, and the tumor volume was calculated using the formula $V=4/3\pi r_1^2 r_2$, where r_1 and r_2 denote the radii of the tumor in two different planes. For the tumor cell apoptosis assay, the animals were sacrificed and xenografts were isolated on days 7, 10 and 15. The tumors were homogenated after thoroughly rinsing with ice-cold PBS. The tumor stroma was then fully degraded with collagenase (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) at 37˚C in an incubator for 1.5 h. After the tumor tissue was dispersed with ice-cold PBS containing 1% BSA, a single-cell suspension was acquired via filtration through a 400 mm filter. The cells were stained with Fluorescein-12-dUTP and propidium iodide (PI)-PE for TUNEL apoptosis assay and analyzed using flow cytometry. Procedures for preparing the sample ready for flow cytometry detection were briefly described as follows: Formaldehyde-fixed cells (15 min, 4˚C) were centrifuged at 755 x *g* for 5 min, followed by rinsing twice with PBS. The supernatant was then discarded and 70% pre-cold ethanol was added to fix the cells for 15 min at 4˚C, followed by centrifugation at 755 x *g* for 5 min, and a rinse twice with PBS. Double distilled water (30 μl), bio-dNTP (1 μl), TdT (terminal deoxynucleotidyl transferase) (5 μl) and TdT buffer solution (10 μl) were added in proper sequence, mixed and cultured at 37˚C for 30 min. Then, 100 μl of FITC-avidin was added and the mixture was kept away from light, at room temperature for 30 min, followed by centrifugation at 1500 rpm at 4˚C for 5 min. The product was washed twice with PBS containing 0.1% tritonX-100 and then 50 μl of PI was added. The mixture was kept away from light, at room temperature for 20 min prior to testing with flow cytometry to evaluate the apoptotic rate. All samples were analyzed in triplicate using FlowCytomixPro Software (Version 2.1).

Statistical analysis. Statistical analysis was performed using SPSS software version 10.0 (SPSS, Inc., Chicago, IL, USA). Data were expressed as the mean ± standard deviation (n=6). Statistical significance was assessed using one-way analysis of variance, and the group means were compared using Duncan's Multiple Range Test. $P<0.05$ was considered to indicate a statistically significant difference.

Results

SYKT reduces DOX-induced mortality in mice. In order to explore the potential of SYKT in counteracting DOX toxicity, mice survival was monitored daily. Under the experimental conditions, 100% of the mice succumbed within 15 days following treatment with DOX alone, whereas only 33% of the animals treated with a combination of SYKT and DOX succumbed within 15 days following treatment (Fig. 1). The surviving animals were monitored for 20 more days and did not exhibit any adverse effects. There were no cases of mortality in the control or SYKT groups. These results suggested that SYKT may reduce DOX-associated mortality.

SYKT mitigates the DOX-induced reduction in peripheral blood cell counts. To determine the effects of SYKT on DOX-induced myelosuppression, WBC, RBC and PLT counts were performed in peripheral blood samples. Mice treated with DOX alone significantly decreased the WBC count to minimum on day 10 of treatment ($4.067\pm0.677x10^9$/l), which persisted until day 30. In mice treated with DOX plus SYKT, WBC count appeared to be initially reduced ($4.4\pm0.42x10^9$/l on day 10); however, after day 15 WBC count gradually increased and reached a count similar to that of the control group by day 30 (Fig. 2A). The control and SYKT groups exhibited no significant alterations in WBC count. Similarly, the number of RBCs was markedly decreased following treatment with DOX, reaching a minimum ($7.383\pm0.788x10^{12}$/l) on day 15, and remained at $8.7\pm0.901x10^{12}$/l on day 30. SYKT significantly attenuated the effects of DOX on RBC count, particularly at later time points (Fig. 2B). In addition, PLT counts in mice receiving chemotherapy were markedly decreased, reaching a minimum ($206.667\pm30.722x10^9$/l) on day 15; PLT count remained at $388\pm83.465x10^9$/l on day 30. SYKT significantly mitigated the effects of DOX on PLT count throughout the experiment (Fig. 2C). The present results indicated that SYKT may significantly attenuate the DOX-induced reduction in peripheral blood cell counts.

SYKT improves DOX-induced myelotoxicity. CD34 and CD44 are transmembrane phosphoglycoproteins expressed in bone

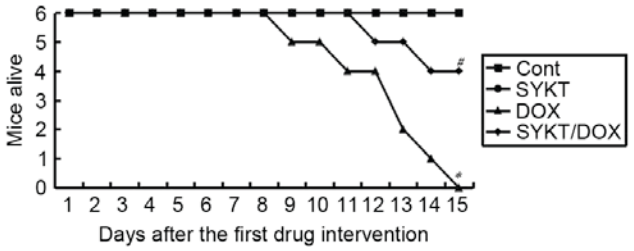

Figure 1. SYKT reduces DOX-induced mortality in mice. Mice in the SYKT group received SYKT (1.2 ml/kg/d) for 5 days. Mice in the DOX group received a single dose of DOX (20 mg/kg) on day 5. Mice in the SYKT/DOX group received both SYKT and DOX as aforementioned. Control mice received normal saline. Mortality was monitored daily. Mortality among mice treated with DOX alone was significantly higher compared with in mice receiving a combination of DOX and SYKT (P<0.05). DOX, doxorubicin.

marrow cells. To evaluate the myelotoxic effects of DOX, $CD34^+$ and $CD44^+$ cells were assessed using flow cytometry on post-treatment day 15 (the optimal time points for bone marrow sampling were established in a pilot experiment). The results indicated that DOX reduced the expression of CD34 and CD44, whereas SYKT co-administration counteracted this effect (Fig. 3A). Although CD34 and CD44 are not specific markers, both are usually expressed in hematopoietic and mesenchymal stem cells, and other types of bone marrow cells. The improved CD34 and CD44 expression following SYKT co-administration may reflect a recovery in bone marrow function. To further assess the effects of SYKT on DOX-induced myelotoxicity in mice, the apoptotic rate of bone marrow cells was evaluated on post-treatment day 15 via TUNEL staining coupled with flow cytometry. The apoptotic rate in the control group was 5.8% compared with 28.4% in the DOX group. However, the apoptotic rate in the SYKT/DOX group was reduced to 17.7%, suggesting that SYKT may prevent DOX-induced apoptosis of bone marrow cells (Fig. 3B).

SYKT ameliorates DOX-induced cardiotoxicity. To assess the cardiotoxic effect of DOX and to investigate whether SYKT can prevent this toxicity, myocardial enzyme levels were determined by analyzing the activity of AST, LDH, CK and CK-MB in serum samples. The optimal time points for blood and heart tissue sampling were established in a pilot experiment. Blood samples were collected from mice on days 7, 10, 15, 22 and 30 post-treatment with DOX (9 mg/kg, i.p.). The elevation of myocardial enzymes was the greatest on day 15 post-treatment, and this was selected as the most appropriate time point for further investigation. DOX induced a significant elevation in all measured serum parameters compared with in the control group. The observed changes were similar to those following acute myocardial infarction (20), which may indicate that DOX induced extensive myocardial injury. However, in mice in the SYKT/DOX group, all biochemical parameters appeared significantly decreased compared with in the DOX alone group (Fig. 4A).

In order to further investigate the effects of DOX and SYKT on cardiomyocytes, cell apoptosis was assessed via flow cytometry on day 15 post-treatment. Compared with the control (3.8%) and SYKT (3.4%) groups, cardiomyocytes from

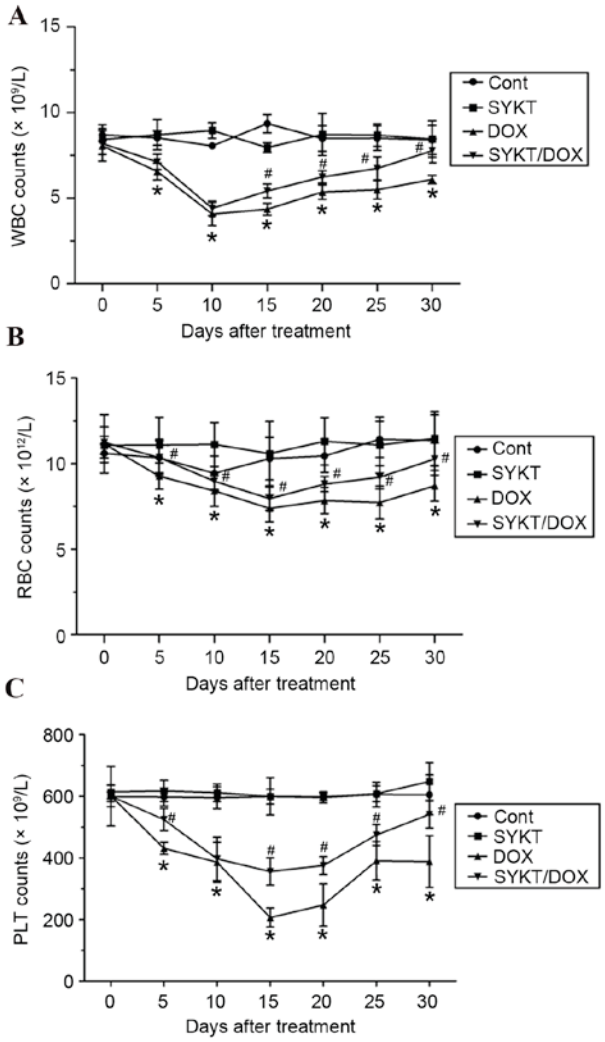

Figure 2. SYKT mitigates the DOX-induced reduction in peripheral blood cell counts. Mice were treated with DOX (3 mg/kg/d, on days 2, 4 and 6) and SYKT (1.2 ml/kg/d, on days 1, 3 and 5) alone or combined. Peripheral blood cell counts were performed at different time points. (A) WBC counts. (B) RBC counts. (C) PLT counts. Data are expressed as the mean ± standard deviation. *P<0.05 vs. control group; #P<0.05 vs. DOX group. DOX, doxorubicin; WBC, white blood cell; RBC, red blood cell; PLT, platelet; Cont, control.

mice in the DOX group exhibited maximum dUTP-FITC binding (8.5%) but very little PI staining (0.9%), indicating that the majority of cells were apoptotic but not necrotic. In mice receiving DOX and SYKT, the percentage of apoptotic cardiomyocytes was low (4.8%), indicating that SYKT protected cardiomyocytes from DOX-induced apoptosis (Fig. 4B).

Histological analysis revealed that DOX administration disturbed the normal radiating pattern of cell plates in the heart; however, SYKT co-administration mitigated the DOX-induced alterations, so that the observed organ pattern remained similar to in the control group (Fig. 4C). Furthermore, the extent of typical histopathological changes on day 15 post-treatment with DOX was ~20%, scored as grade 2 according to Billingham *et al* (18). Evaluation of Billingham scores in mice receiving a combination of DOX and SYKT revealed a median score of 1.5, which was substantially improved compared with in the DOX group (Table I). The strong correlation between histopathology changes

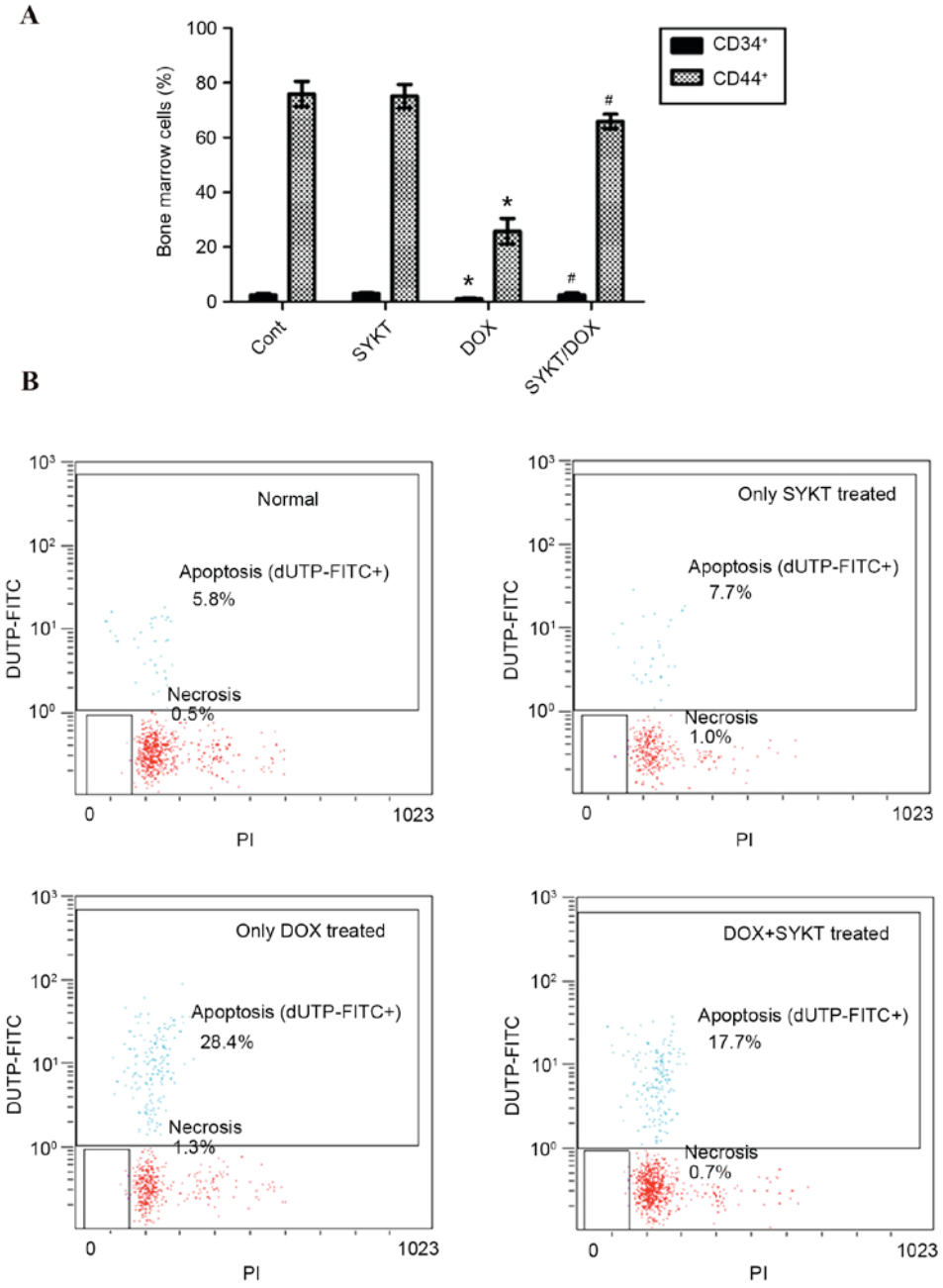

Figure 3. SYKT reduces DOX-induced myelotoxicity. Mice were treated with DOX (3 mg/kg/d on days 2, 4 and 6) and SYKT (1.2 ml/kg/d on days 1, 3 and 5) alone or combined, and the number and apoptotic rate of bone marrow cells were determined. (A) Flow cytometric analysis of $CD34^+$ and $CD44^+$ cells in bone marrow on day 15. (B) Apoptotic rate of bone marrow cells. The cell distribution was analyzed using dUTP binding and PI uptake. Representative dot plots are shown for one of the six independent experiments. Data are expressed as the mean ± standard deviation. $^*P<0.05$ vs. control group; $^\#P<0.05$ vs. DOX group. DOX, doxorubicin; CD, cluster of differentiation; dUTP, 2'-deoxyuridine, 5'-triphosphate; PI, propidium iodide; Cont, control group; FITC, fluorescein isothiocyanate.

scored≥2 and presence of congestive heart failure has been established (21). The present results suggested that SYKT may mitigate DOX-induced cardiotoxicity in mice.

SYKT reverses DOX-induced myelosuppression and cardiotoxicity by inhibiting ROS-mediated apoptosis. Mounting evidence indicates the involvement of ROS in DOX-induced pathophysiology (22,23). The redox cycling of DOX is generally attributed to the production of ROS (24). In order to investigate the potential of SYKT in inhibiting ROS production, bone marrow and myocardial cells were treated with CM-H_2DCFDA on day 15 post-treatment. Flow cytometric analysis revealed that treatment with DOX caused a rightward shift in the intensity of fluorescence of DCFDA, representing the ROS content in bone marrow and myocardial cells. This shift was markedly decreased when vitamin E (positive control) or SYKT were co-administered with DOX (Fig. 5A and B), indicating that SYKT co-administration reduced ROS production. These results suggested that ROS production participates in DOX-induced toxicity in bone marrow and myocardial cells. In addition, DOX induced apoptosis in bone marrow (Fig. 5C) and myocardial cells (Fig. 5D),

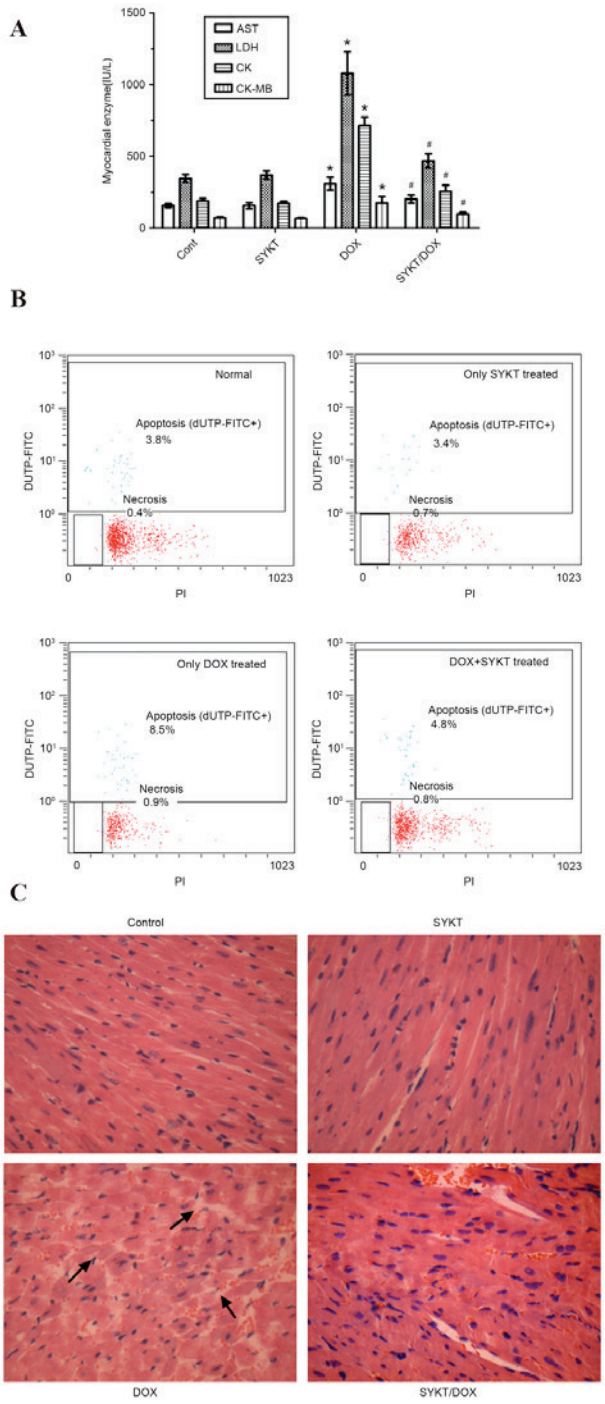

Figure 4. SYKT reduces DOX-induced cardiotoxicity. Mice were treated with DOX (3 mg/kg/d on days 2, 4 and 6) and SYKT (1.2 ml/kg/d on days 1, 3 and 5) alone or combined. (A) Serum activity of myocardial enzymes was determined using an automatic biochemical analyzer on day 15. (B) Apoptotic rate of cardiomyocytes. Cell distribution was analyzed using dUTP binding and PI uptake. The results are expressed as dot plots, as represented in one of the six independent experiments. (C) Hematoxylin-eosin staining pattern in cardiac sections. Arrows indicate abnormal ultrastructural changes (as indicated by the loss of the normal radiating pattern of the cell plates) (magnification, x200). Data are expressed as the mean ± standard deviation. *P<0.05 vs. control group; $^{\#}$P<0.05 vs. DOX group. DOX, doxorubicin; AST, aspartate aminotransferase; LDH, lactate dehydrogenase; CK, creatine kinase; dUTP, 2'-deoxyuridine, 5'-triphosphate; PI, propidium iodide; Cont, control group.

which was significantly inhibited by SYKT co-administration. The present results suggested that SYKT may reverse DOX-induced myelosuppression and cardiotoxicity by inhibiting ROS-mediated apoptosis.

SYKT does not interfere with the antitumor efficacy of DOX. In order to investigate whether SYKT interferes with the antitumor effects of DOX, a mouse tumor xenograft was developed via subcutaneous injection of EL4 cells. Mice were treated with DOX and SYKT alone or in combination when xenograft tumors reached a volume of ~5.25±0.876 cm^3 (range 3.9-6.3 cm^3). Treatment with DOX reduced the tumor volume to ~1.817±0.975 cm^3 (range, 0.6-3.2 cm^3) on day 15 post-treatment. No significant differences in tumor volume were observed between mice treated with DOX alone and mice receiving a combination of DOX and SYKT (Fig. 6A). Tumor volumes in control mice were significantly larger compared with mice treated with DOX alone or combined with SYKT. Furthermore, tumor cell apoptosis was evaluated via TUNEL staining coupled with flow cytometry. Compared with the control and SYKT alone groups, on days 7, 10 and 15, treatment with DOX markedly increased the apoptotic rate. No differences were observed between groups receiving DOX alone and DOX combined with SYKT (Fig. 6B). The present results demonstrated that SYKT does not interfere with the antitumor efficacy of DOX.

Discussion

DOX is an anthracycline compound that is widely used in the treatment of several types of solid tumor and leukemia in humans. Despite its effectiveness, the clinical application of DOX has been limited due to its dose-dependent and cumulative myelosuppressive and cardiotoxic effects (25,26). However, the mechanism of DOX-induced acute and chronic toxicity remains to be elucidated. DOX induces the generation of ROS during the redox cycling of its quinone moiety; it also disturbs iron metabolism, whereas toxic DOX metabolites are produced in the heart and hematopoietic tissue (6,27). Oxidative stress triggers the intrinsic mitochondria-dependent apoptotic pathway in cardiomyocytes and hematopoietic cells (7). Molecules with antioxidant properties can prevent ROS-mediated cytotoxicity. Numerous traditional Chinese medicines, such as ginseng, astragalus membranaceus, poria cocos and notoginseng, possess significant antioxidant activity (28). The present study demonstrated that the traditional herbal formula SYKT may reverse DOX-induced myelosuppression and cardiotoxicity by inhibiting ROS-mediated apoptosis.

DOX damages bone marrow cells and cardiomyocytes via ROS production, which can be inhibited by SYKT. To determine the mechanism underlying DOX-induced myelosuppression and cardiotoxicity, as well as to investigate the potential of SYKT in preventing these actions, myocardial function was assessed via myocardial enzyme spectrum and histological analysis, whereas hematopoietic function was assessed via peripheral blood cell and bone marrow cell counts. The present results revealed that DOX induced cardiotoxicity and myelosuppression, whereas SYKT effectively protected the myocardial and hematopoietic tissues. Numerous lines of evidence converge to suggest a role for

Table I. Billingham scores of cardiomyopathy in mice from different intervention groups.

	Billingham scores							
Group	0	0.5	1	1.5	2	2.5	3	Median score
Control	6							0
SYKT	6							0
DOX					4	2		2[a]
DOX + SYKT			2	3	1			1.5[b]

[a]P<0.05 vs. control group; [b]P<0.05 vs. DOX group. Data are expressed as the median of three independent scores for each animal. Statistical analysis was performed using Kruskal-Wallis test.

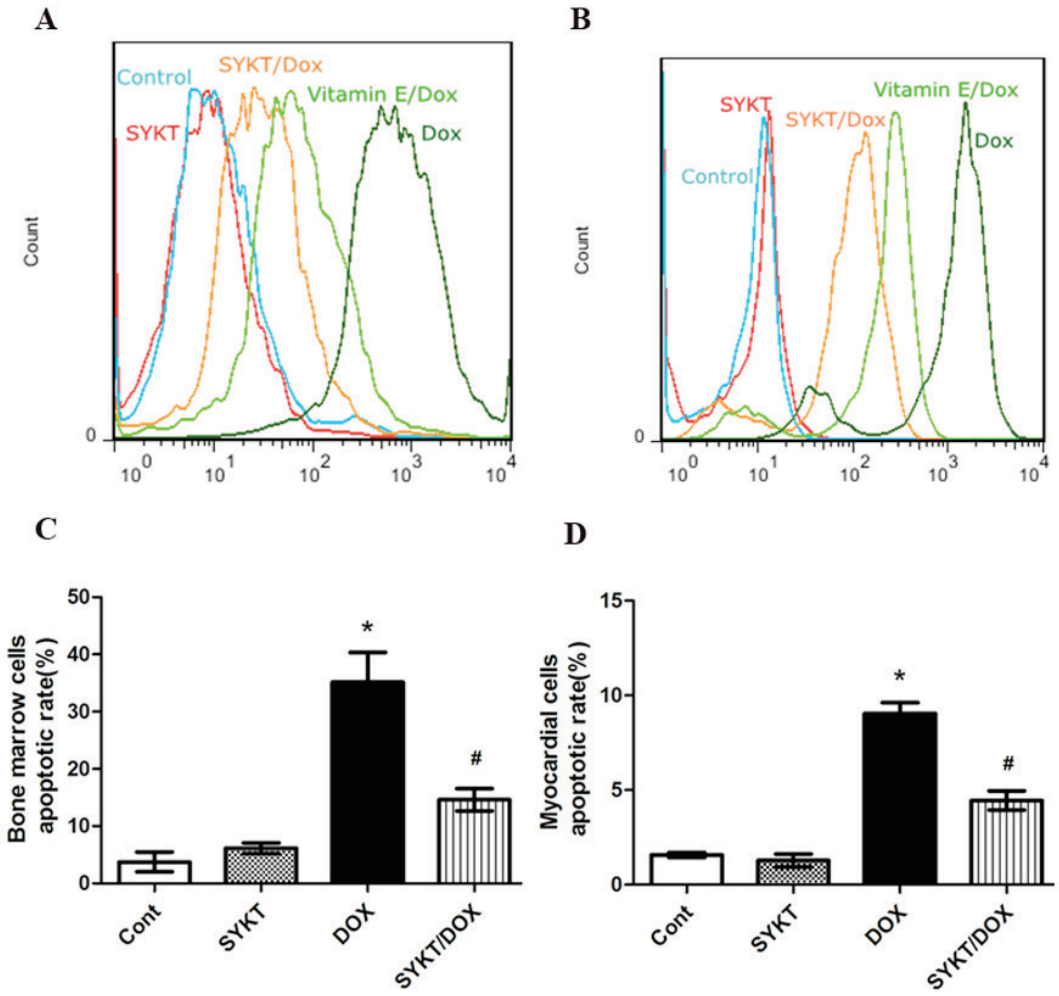

Figure 5. SYKT reduces DOX-induced myelosuppression and cardiotoxicity by inhibiting ROS-mediated apoptosis. Mice were treated with DOX (3 mg/kg/d on days 2, 4 and 6) and SYKT (1.2 ml/kg/d on days 1, 3 and 5) in the presence or absence of vitamin E (0.2 ml bolus dose on days 1, 3 and 5). ROS production and apoptotic rate of bone marrow cells and cardiomyocytes were measured on day 15. DCFDA fluorescence of (A) bone marrow cells and (B) cardiomyocytes was evaluated using flow cytometry. (C) Bone marrow cells and (D) cardiomyocytes apoptosis was assessed using terminal deoxynucleotidyl transferase dUTP nick end labeling staining and flow cytometry. Data are expressed as the mean ± standard deviation. *P<0.05 vs. control group; #P<0.05 vs. DOX group. DOX, doxorubicin; DCFDA, 2',7'-dichlorofluorescin diacetate; Cont, control.

ROS in DOX-induced toxicity (22-24). The present study demonstrated that treatment with DOX induced an increase in ROS production, which was attenuated with the addition of SYKT to the therapeutic scheme. These results suggested that ROS may serve an important role in the mechanism of DOX-induced toxicity in cardiomyocytes and bone marrow cells. The protective effect of SYKT may be attributed to the antioxidant potential of some of its components, which are able to scavenge superoxide, hydroxyl, hydrogen peroxide and nitric oxide free radicals. Therefore, it is possible

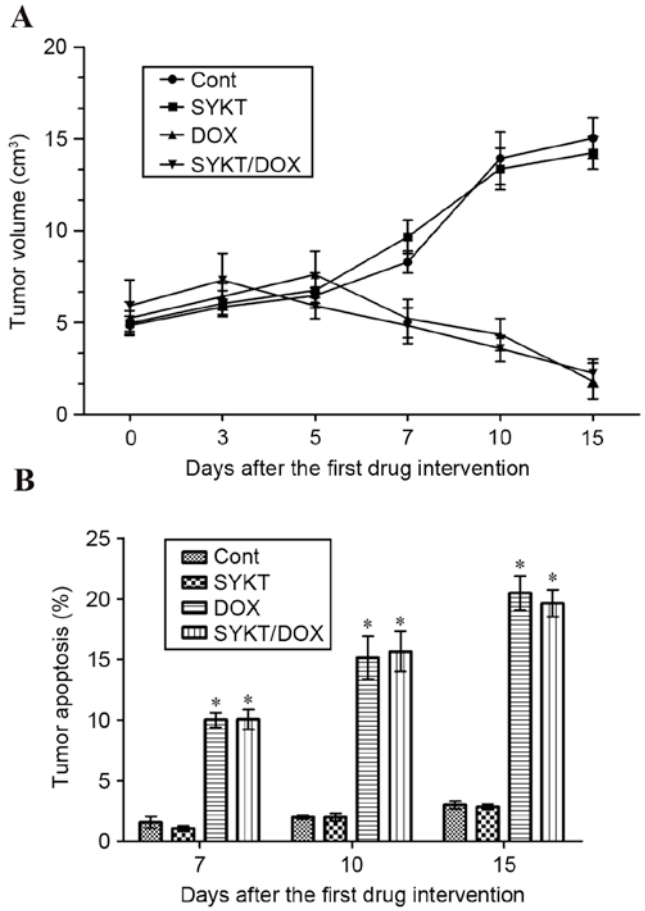

Figure 6. SYKT does not interfere with the antitumor efficacy of DOX. Mice bearing tumors were treated with DOX (3 mg/kg/d on days 2, 4 and 6) and SYKT (1.2 ml/kg/d on day 1, 3 and 5) alone or combined. Tumor volume and apoptosis were assessed. (A) Tumor volume was measured using Vernier calipers. (B) Tumor cell apoptotic rate was assessed using terminal deoxynucleotidyl transferase dUTP nick end labeling staining and flow cytometry. Data are expressed as the mean ± standard deviation. *P<0.05 vs. control group. DOX, doxorubicin; Cont, control.

that some of the beneficial effects associated with SYKT co-administration may result from its neutralizing action on ROS produced by DOX.

SYKT reverses DOX-induced myelosuppression and cardiotoxicity by inhibiting ROS-mediated apoptosis. Under physiological conditions, low levels of ROS function as 'redox messengers' in intracellular signaling; however, aberrant ROS production results in oxidative damage of cellular macromolecules and promotes cell death. Apoptosis can be initiated by extracellular and intracellular signals via death receptor- and mitochondria-mediated pathways (29). Disequilibrium in intracellular redox homeostasis, due to an increase in ROS production or a dysfunction in antioxidant mechanisms, can cause irreversible oxidative modifications to lipids, proteins and DNA, which can result in oxidative stress-induced apoptotic signaling (30). The present study demonstrated that cardiomyocytes and bone marrow cells from mice treated with DOX exhibited a significantly increased apoptotic rate. SYKT co-administration significantly reduced the number of apoptotic cells, indicating that SYKT can protect cardiomyocytes and bone marrow cells from DOX-induced apoptosis.

The intrinsic mitochondrial apoptotic pathway, initiated by ROS and mitochondrial DNA damage, triggers permeabilization of the outer mitochondrial membrane and the translocation of cytochrome *c*, apoptosis-inducing factor and the mitochondrial protein Smac/DIABLO from the mitochondria to the cytosol, where they activate caspase-dependent or -independent cytosolic signaling events (31). Mitogen-activated protein kinases (MAPKs) serve an important role in apoptotic signaling (32). Oxidative stress can activate members of the MAPK protein family (33). Among these, p38 and c-Jun N-terminal kinases (JNK) serve critical roles in the mechanism of DOX-induced cell death (34). Activated p38 and JNK MAPKs phosphorylate the apoptosis regulator B-cell lymphoma 2-associated X protein (Bax) and promote its translocation to the mitochondria (33,35). Once there, Bax triggers the opening of mitochondrial permeability transition pores and subsequent release of cytochrome *c*. The maintenance of the mitochondrial membrane potential ($\Delta\psi_m$) is fundamental to cell survival, and the loss of $\Delta\psi_m$ activates a cascade leading to cellular apoptosis (36,37). Following its release into the cytosol, cytochrome *c* initiates the formation of apoptosomes and subsequently activates caspases (37). Further studies are required to investigate whether SYKT inhibits DOX-induced apoptosis of myocardial and bone marrow cells via preventing the MAPK-mediated Bax mitochondrial translocation, thus promoting the preservation of $\Delta\psi_m$ and inhibiting cytochrome *c* release into the cytosol.

SYKT does not affect the antitumor efficacy of DOX. Chemoprotectants have relatively limited clinical application, due to concerns regarding the potential for a negative interaction with antineoplastic agents, resulting in reduced chemotherapeutic efficacy. The present study demonstrated that SYKT did not impair the antitumor efficacy of DOX in tumor-bearing mice. DOX can induce apoptosis in healthy and tumor cells via various mechanisms (38). In endothelial cells and cardiomyocytes, DOX has been demonstrated to induce apoptosis through a ROS-mediated mechanism independent of p53 activation. Conversely, the tumor suppressor p53 has a crucial role in the mechanism of DOX-induced apoptosis in cancer cells (38). Therefore, it may be hypothesized that SYKT reduces the damage induced by DOX to myocardial and bone marrow cells by inhibiting ROS-mediated apoptosis, while having no effect on the p53-mediated apoptotic pathway.

In conclusion, the present results demonstrated that SYKT may prevent DOX-induced myelosuppression and cardiotoxicity without impairing its antitumor efficacy, through a mechanism involving the inhibition of ROS-mediated apoptosis. Therefore, the present study suggested that SYKT co-administration may be considered a potential solution to counteract the myelosuppressive and cardiotoxic action of DOX.

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