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Sheng-Mai-San Reduces Adriamycin-Induced Cardiomyopathy in Rats

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ABSTRACT

The traditional Chinese medicine prescription "Sheng-Mai-San (SMS)" has been used for treating patients with coronary heart disease for a long time and was found to have antioxidative effect. Here, we applied adriamycin (doxorubicin, ADR), a highly effective anticancer agent, as an inducer to establish the animal model of dose-related cardiomyopathy due to inhibition of nucleic acid as well as protein synthesis, formation of free radicals and lipid peroxidation. The objective of this study was to investigate the protective effects of SMS on adriamycin-induced cardiomyopathy. Wistar rats were divided into four groups: CONT (control), ADR, SMS and ADR+SMS. ADR (cumulative dose, 15 mg/kg) was administered to rats in six equal intraperitoneal injections over a period of 2 weeks and SMS was administered via a feeding tube throughout the mouth once a day for 30 days (cumulative dose, 150g/kg). At the end of the 5-week posttreatment period, hearts of the rats were surgically removed for the study of synthesis rates of DNA, RNA and protein. Besides, myocardial antioxidants, lipid peroxidation and morphological ultrastructure were also evaluated. Three weeks of the end of treatment, cardiomyopathy and congestive heart failure were characterized according to assessment in ascites, congested liver, depressed cardiac function and myocardial cell damage. The results demonstrated that nucleic acid as well as protein synthesis was inhibited, while lipid peroxidation was increased. Myocardial glutathione peroxidase (GSHPx) activity was decreased and electron microscopic examination revealed myocardial lesion indicative of ADR-induced cardiomyopathy. In contrast, administration of SMS before and concurrent with ADR significantly attenuated the myocardial effects. It also lowered mortality as well as the amount of ascites. In addition, indexes in myocardial GSHPx, macromolecular biosynthesis and superoxide dismutase activities were increasing, with a concomitant decrease in lipid peroxidation and preserved myocardial ultrastructure. These results indicated that SMS may be partially protective against ADR-induced cardiomyopathy.

Keywords: Traditional Chinese Medicine; Adriamycin; Sheng-Mai-San; Cardiomyopathy; Antioxidation

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INTRODUCTION

Traditional Chinese medicine (TCM) has become an increasingly popular alternative for the treatment of the pathophysiological conditions relating to life style, aging and cancer (Cheng, 2000; You *et al.,* 2003). The TCM prescription "Sheng-Mai-San (SMS)" has long been used for more than 700 years for patients with coronary heart disease (Wang *et al.,* 2003) and was found to have antioxidative effect from the report of China (Lu *et al.,* 1994). It was also found to protect against isoproterenol-induced myocardial injury in rats, and in ischemia-reperfusion injury in isolated perfused hearts prepared from pretreated animals (Li *et al.,* 1996). Furthermore, it was reported that SMS protect against cerebral oxidative damage induced by ischemia-reperfusion in rats (Wang *et al.,* 1999; Ichikawa *et al.,* 2003). Our preliminary results also suggested that SMS might improve the exercise tolerance in patients with congestive heart failure and have no side effects (You *et al.,* 2001).

Adriamycin (doxorubicin, ADR) is a broad spectrum anti-tumor antibiotic used to treat cancer patients. However, the potential usefulness of this drug is limited by the development of life-threatening cardiomyopathy (Singal and Siveski-Iliskovic, 1998) and congestive heart failure (Gewitz, 1999). Adriamycin-induced myocardial dysfunction has been suggested to involve inhibition of nucleic acid as well as protein synthesis (Monti *et al.,* 1995), release of vasoactive amines, changes in adrenergic function (Singal *et al.,* 1998), abnormalities in the mitochondria, lysosomal alterations, alterations in sarcolemmal $Ca2^{+}$ transport, membrane-bound enzymes, imbalance of myocardial electrolytes (Siveski-Iliskovic *et al.,* 1994), free radical formation (Rabelo *et al.,* 2001) and lipid peroxidation (Kumar *et al.,* 2001). Although adriamycin-induced injury appears to be multifactorial and complex, however, oxygen radical-induced injury of membrane lipids is considered to be the most important factor responsible for the development of adriamycin-induced cardiomyopathy (Singal *et al.,* 1987; Morishima *et al.,* 1998).

Since inhibition of nucleic acid as well as protein synthesis, formation of free radicals and lipid peroxidation are suggested to be involved in ADR cardiomyopathy, we therefore utilized a rat model of ADR-induced cardiomyopathy, to determine whether SMS has cardioprotective activity.

MATERIALS AND METHODS

Shengmai San Preparation

Sheng-mai-san used in the current study was supplied by Sun Ten Laboratories, Taipei. Its identification was authenticated by experts in pharmacognosy. The three component herbs of SMS, Panax ginseng (600g), Radix Ophiopogonis (900g) and Fructus Schisandra (360g) were suspended in 24180g of distilled water and then boiled for one hour. The decoction was filtered through a mesh, and the filtrate (19723g) was then concentrated to 1860g by a vacuum pump and stored at -80˚C until use.

Animal Model

Male Wistar rats, body weight 250~300g, were maintained on a normal rat chow diet. Rats were divided into four groups: CONT (control), ADR (adriamycin treated), SMS (sheng-mai-san treated), and ADR+SMS. ADR was administered intraperitoneally in six equal injections (each containing 2.5mg/kg ADR) over a period of 2 weeks for a total cumulative dose of 15mk/kg ADR. SMS was administered via a feeding tube throughout the mouth once a day for 30 days (5g/kg/day, cumulative dose, 150g/kg/body weight). ADR+SMS were administered in the same manner as SMS, alternating with adriamycin injections. CONT rats were injected with a same volume of normal saline. Treated as well as CONT rats were observed for up to 5 weeks after the last treatment for their body weight, general appearance, behavior, ascites, limbs edema and mortality. At the end of the 5-week posttreatment period, hearts of the rats were used for the study of synthesis rates of DNA, RNA and protein, myocardial antioxidants, lipid peroxidation and ultrastructure.

Hemodynamic Measurements

Immediately prior to sacrifice, rats were anesthetized with sodium pentobarbital (30mg/kg IP). A catheter was inserted into the right common carotid artery, and arterial pressure was measured with a pressure transducer and recorded on a Beckman Dynograph.

Synthesis Rates of DNA, RNA and Protein

The hearts were washed with normal saline three times and 70% alcohol once respectively, then washed with PBS at 40C, fragmented, and weighed. The resulting fragments after being washed with PBS were disaggregated by gentle agitation for 30 minutes with a 5 ml enzyme cocktail of trypsin (0.2%) and collagenase (0.05%). The resulting cell suspension was filtered through polyester mesh (50-µm pore size) and centrifuged at 2000 rpm for five minutes, then the cell pellet was resuspended in 5 ml RPMI for sorting. Cell suspensions were routinely counted on a hemocytometer with trypan blue, enabling heart cells yield to be ascertained. In order to measure the protein, RNA and DNA synthesis rates, the aliquots of the aforementioned cell pellet suspensions (3×10^{6} cells in 96 well microtiter plate) were labeled by adding 200 µl

(50 μCi/ml) ^{3}H-thymidine (20 Ci/m mole), ^{3}H-uridine (28.9 Ci/m mole) and ^{3}H-leucine (53 Ci/m mole). After 18 hours, the cells were collected in the filter paper disk. Each disk was placed in a counting vial, and 5 ml of scintillation mixture was added (0.47% PPO and 0.01% POPOP in 100% toluene). The sample was counted in a liquid scintillation counter (nuclear-Chicago Mark I).

Glutathione Peroxidase (GSHPx) Assay

GSHPx activity was expressed as nanomoles reduced nicotinamide adenine dinucleotid phosphate (NADPH) oxidized to nicotinamide adenine dinucleotide phosphate (NADP) per minute per milligram protein, with a molar extinction coefficient for NADPH at 340 nm of 6.22×10^{6}. Cytosolic GSHPx was assayed in a 3-mL cuvette containing 2.0 mL of 75 mmol/L phosphate buffer, pH 7.0. The following solutions were then added: 50 μL of 60 mmol/L glutathione, 100 μL glutathione reductase solution (30 U/mL), 50 μL of 0.12 mol/L NaN_3, 0.10 of 15 mmol/L Na_2EDTA, 100 μL of 3.0 mmol/L NADPH, and 100 μL of cytosolic fraction obtained after centrifugation at 20000g for 25 minuters. Water was added to make a total volume of 2.9 mL. The reaction was started by the addition of 100 μL of 7.5 mmol/L H_2O_2, and the conversion of NADPH to NADP was monitored by a continuous recording of the change of absorbance at 340 nm at 1-minute intervals for 5 minutes. Enzyme activity of GSHPx was expressed in terms of milligrams of protein.

Superoxide Dismutase Assay

Supernatant was assayed for superoxide dismutase (SOD) activity by following the inhibition of pyrogallol auto-oxidation. Pyrogallol (24 mmol/L) was prepared in 10 mmol/L HCl and kept at 4°C before use. Aliquots of supernatant (150 μg protein) were added to Tris HC1 buffer containing 25 μL pyrogallol and 10 μL catalase. The final volume of 3 mL was made up of the same buffer. Changes in absorbance at 420 nm were recorded at 1-minute intervals for 5 minutes. SOD activity was determined from a standard curve of percentage inhibition of pyrogallol auto-oxidation with a known SOD activity. This assay was highly reproducible, and the standard curve was linear up to 250 μg protein with a correlation coefficient of 0.998. Data are expressed as SOD units per milligram protein compared with the standard.

Malondialdehyde Assay

Measurement of lipid peroxidation by determination of myocardial malondialdehyde (MDA) content was performed by a modified thiobarbituric acid (TBA) method. Hearts were quickly excised and washed in buffered 0.9% KCl (pH7.4). After the atria, extraneous fat, and connective tissue were removed, the ventricles were homogenized in the same buffer (10% wt/vol). The homogenate was incubated for 1 hour at 37°C in a water bath. A 2-mL aliquot was withdrawn from the incubation mixture and pipetted into an 8-mL Pyrex tube. One milliliter of 40% trichloroacetic acid (TCA) and 1 mL of 0.2% TBA were promptly added. To minimize peroxidation during the subsequent assay procedure, 2% butylated hydroxytoluene was added to the TBA reagent mixture. Tube contents were vortexed briefly, boiled for 15 minutes, and cooled in a bucket of ice for 5 minutes. Two milliliters of 70% TCA was then added to all tubes, and contents were again vortexed briefly. The tubes were allowed to stand for 20 minutes. This was followed by a centrifugation of the tubes for 20 minutes at 3500 rpm. The color was read at 532 nm on a Zeiss spectrophotometer and compared with a known MDA standard.

Electron Microscopy

Three hearts from each group were washed in cold 0.067 M sodium phosphate buffer (pH 7.4). Samples were taken from four different areas of the free left ventricular wall between the mid region and the apex, and prefixed overnight at 4°C in 0.067 M sodium phosphate buffer (pH 7.4) containing 2.5% glutaraldehyde and 4.5% glucose. After washing in sodium phosphate buffer, the samples were postfixed in 2% OsO_4 for 2 hours at 4°C. The samples were dehydrated in a graded alcohol series, and embedded in epon, and ultrathin sections were sliced off with a diamond knife, stained with uranyl acetate and lead citrate and examined with a Hitachi S-5000 scanning electron microscope operated at 3 kV.

Statistical Analysis

Data were expressed as the mean ±SEM. For a statistical analysis of the data, group means were compared by one-way ANOVA followed by Scheffe's multiple range test.

RESULTS

General Observations and Mortality

The general appearance of all groups of animals was recorded during the time course of the study. After completion of adriamycin treatment, the animals' fur became scruffy and developed a light yellow tinge. There were red exudates around the eyes in ADR treated groups which appeared to be sicker, weaker, and lethargic compared with the SMS+ADR groups. The most predominant feature in the ADR group animals was the development of a grossly enlarged abdomen and ascites. This condition became apparent within a week after

the completion of treatment with adriamycin. When these rats were sacrificed, all ADR group animals had a significant amount of peritoneal fluid. In addition, the liver was enlarged and congested. In the SMS+ADR group, the amount of peritoneal fluid was about one forth compared with animals in the ADR group. During the posttreatment period, the mortality rate was significantly higher in the ADR group than the SMS+ADR group while there were no deaths in the CONT and SMS groups.

Data on heart weight and ratio of heart weight/body weight are listed in **Table 1.** Despite the ascites, the body weight gained in the ADR group was significantly less. Mere treatment with adriamycin resulted in obviously decrease in heart weight and ratio of heart to body weight. There was no significantly different in ratio of heart to body weight for SMS+ADR, the CONT and SMS groups. Heart weight in the SMS+ADR group was much heavier than that in the ADR group but were still lighter than that in the CONT and SMS groups.

Arterial Pressure

We observed that both systolic and diastolic arterial pressure were significantly lower in the ADR group than that in either the CONT or SMS group. In the SMS+ADR group, however, arterial pressure did not differ significantly from CONT. **(Table 2)**

Effects of the Drugs on DNA, RNA and Protein Synthesis Rates

Fig. 1. shows that ADR inhibited synthesis rates of DNA, RNA and protein nearly by 50% compared with the CONT group; while SMS increased the synthesis of DNA by 35%, RNA by 30%, and protein by 80%. In the SMS+ADR group,

Table 1. Effects of Sheng-mai-san on Adriamycin-Induced Changes in Heart Weight, Body Weight, Mortality Rate and Ascites

Animal group	Heart Weight, g	Heart Weight/Body Weight Ratio x10^3	Mortality, %	Ascites, mL
CONT	1.28± 0.06	4.00±0.23	0	0
ADR	0.80± 0.03*	2.24±0.01*	78	98.3±15.0*
SMS	1.26± 0.06	4.03±0.22	0	0
ADR+SMS	0.95± 0.04**	2.90±0.15**	35	23.8±7.4**

CONT indicates control; ADR, adriamycin; and SMS, sheng-mai-san.
Data are mean ± SEM of 9 to 14 rats in all studies. Mortality data are 30 rats each in the ADR and SMS+ADR groups and 15 rats each in the CONT and SMS groups.
*and **p<0.05 compared with all other groups.

Table 2. Effects of Sheng-mai-san on Adriamycin-Induced Changes in Arterial Pressure.

	CONT	ADR	SMS	SMS+ADR
SAP(mmHg)	127±3	108±5*	132±5	125±2
DAP(mmHg)	108±2	90±3*	116±5	108±2

Data are mean± SEM of 9 to 14 rats in each group. SAP indicates systolic arterial pressure; DAP, diastolic arterial pressure.
*p<0.05 compared with all other groups.

Table 3. Effects of Sheng-mai-san on Adriamycin-Induced Changes in GSHPx, SOD, and MDA.

Animal group	GSHPx, nmol/mg protein	SOD, U/mg protein	MDA, nmol/g heart
CONT	29.5±3.3	30.3±4.1	28.4±5.2
ADR	18.9±2.2*	16.2±3.3*	50.1±6.6*
SMS	31.9±3.3	32.1±5.3	17.6±3.6
ADR+SMS	23.6±3.0**	24.3±4.5**	34.6±4.7**

GSHPx indicates glutathione peroxidase; SOD, superoxide dismutase; MDA, malondialdehyde; CONT, control; ADR, adriamycin; and SMS, sheng-mai-san. Data are mean ± SEM from four to six experiments.
*p<0.001 as compared with the CONT group.
**p<0.001 as compared with the ADR group.

DNA synthesis was inhibited by 10%, RNA synthesis by 0%, and protein synthesis was promoted by 40%.

Antioxidant Enzyme and Lipid Peroxidation

Different antioxidant enzyme activities were examined in all groups; these data are shown in **Table 3.** GSHPx activity in the ADR group was reduced by about 40% compared with the CONT group. In the SMS+ADR group, GSHPx activity was almost the same as control levels. Total SOD activity in the SMS+ADR group was significantly higher than that in the ADR group. The amount of lipid peroxidation was determined by evaluating myocardial MDA content; these data are also shown in **Table 3.** MDA levels were lower in the SMS, and CONT groups, whereas the MDA content was significantly higher in the ADR group alone.

Morphological Microscopy

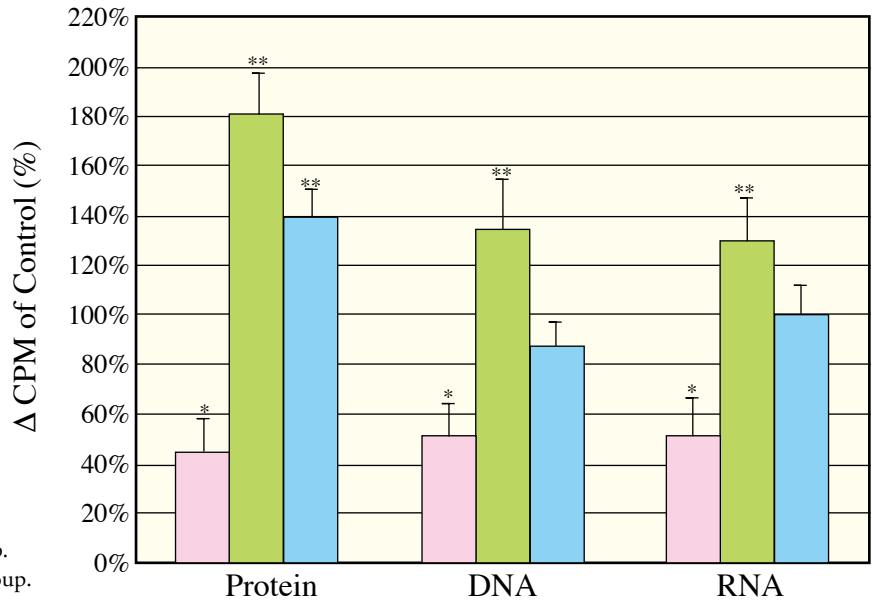
When we examined heart tissues by electron microscopy, we found that the myocardial subcellular structures in the Cont **(Fig 2A)** and SMS (data not shown) groups displayed normal morphological appearance. In contrast, rats in the ADR group exhibited myocardial lesions, mainly resulting from cytoplasmic vacuolization, myofibril loss or disarrangement, and mitochondrial degeneration **(Fig 2B)**. The myocardial ultrastructure of rats in the SMS+ADR group was almost well preserved **(Fig 2C)**. Although traces of perimitochondrial edema and some myofibril loss were observed in a few specimens, there alterations were not obvious.

DISCUSSION

Repeated administration of adriamycin beyond a certain dose had been shown to cause cardiopathic changes in patients (Steinhertz *et al.,* 1991) as well as in a variety to animal species (Van Acker *et al.,* 1996). In this study, we utilized a rat model of adriamycin-induced cardiomyopathy, and it has been shown that these animals have increased mortality and accumulation of ascites, as well as significantly decreased heart weight compared to CONT. The results demonstrated that a simultaneous treatment with SMS mitigates adriamycin – induced cardiomypathic changes as well as congestive heart failure, and indicated the improvement in cardiac function. Reduced mortality and preserved myocardial ultrastructure have also been shown in the SMS+ADR group.

The radioactive tracer method (the thymidine, uridine, and leucine incorporation assay) indicated that DNA, RNA, and protein synthesis rates of heart cells were inhibited by ADR, which reflects its cytotoxic properties, while SMS offered the cytoprotection against ADR cardiotoxic effect through promoting the macromolecular biosynthesis of heart cells.

SMS has been reported to protect the heart against anoxia/reoxygenation-induced injury in isolated perfused rat hearts *in vivo* and adriamycin-induced myocardial damage in rats (Rong *et al.,* 1988). It reduces myocardial infarct size which may be through activation of protein kinase C and opening of mitochondrial KATP channel (Wang *et al.,* 2001). We suggest myocardial protection afforded by SMS results from



Values are CPM of the control (%)
□—ADR ■—SMS ■—ADR+SMS
*p<0.001 compared with CONT group.
**p<0.001 compared with the ADR group.

Fig 1. Effects of sheng-mai-san on adriamycin-induced changes in protein, DNA and RNA synthesis rates of heart cells.

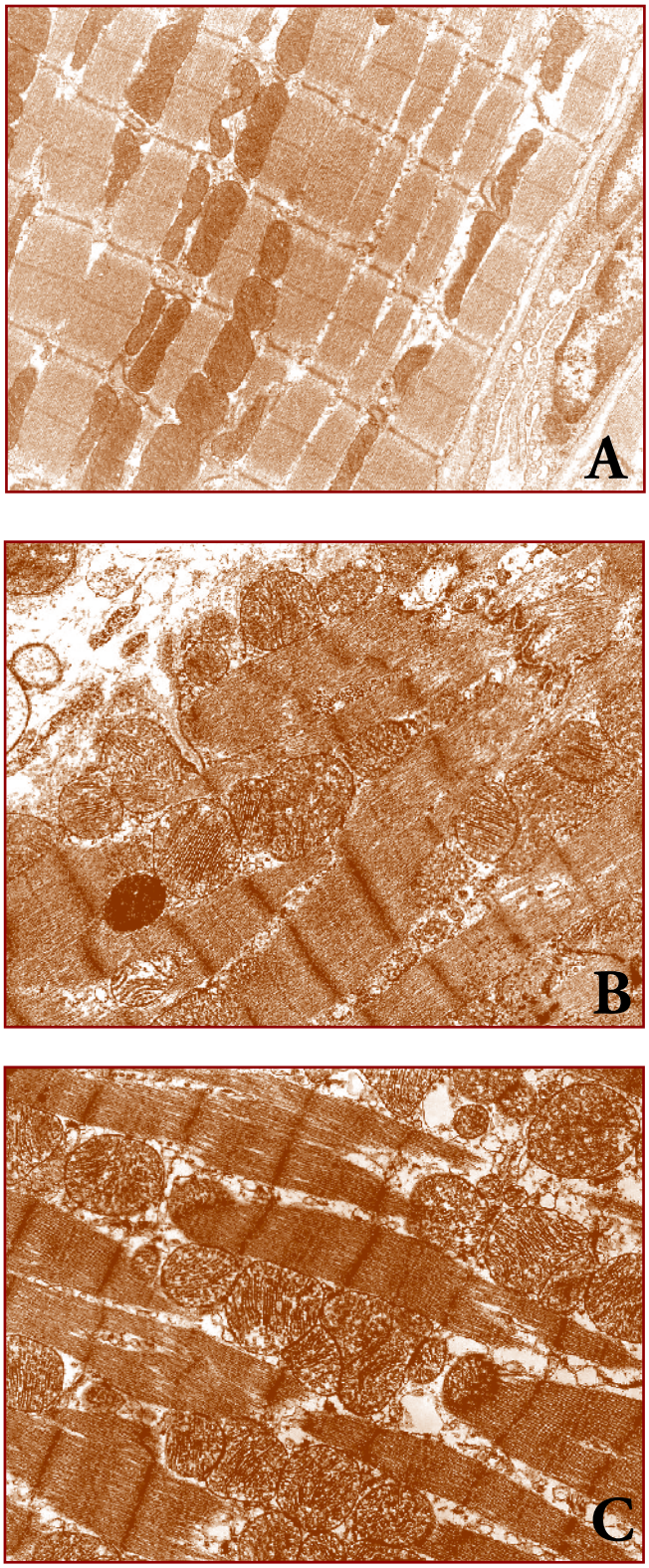


Fig 2. Myocardial ultrastructure of rat left ventricles. (A) Control. (B) Adriamycin (C) Sheng-mai-san + adriamycin. Three rats in each group were examined, and representative results are shown for each.

the presence of antioxidant activities which may contribute to some of its therapeutic effects (Ko *et al.,* 1995). In this regard, ADR has been shown to promote the production of free radicals, these toxic substances are known to cause myocardial dysfunctiom. Data from lipid peroxidation are also in concert with this hypothesis, inasmuch as SMS causes a significant attenuation in the ADR-induces increase in MDA levels. The beneficial effect of SMS against restenosis after percutaneous transluminal coronary angioplasty has also been suggested to be due to its antioxidant properties (Wang *et al.,* 2002).

In addition to antioxidant properties, SMS may also act as a stimulator for the activity of antioxidant enzymes. It has been reported that ADR reduces the activity of GSHPx in rat hearts (Li *et al.,* 2000). SMS has been shown to stimulate GSHPx activity in the brain (Ichikawa and Konishi, 2002). Accordingly, it might as well exert on the heart, thus providing indirect and direct protection against free radical attack. SMS not only prevented ADR-induced decreases by activating GSHPx but also increased SOD activity. The mechanisms for the ADR-induced decrease in GSHPx and the SMS-induced increase in antioxidants (GSHPx and SOD) are still not clear. Recently, some data provided evidences about both cardiomyocyte and endothelial cell apoptosis may play an important role in ADR-induced cardiomyopathy (Wu *et al.,* 2002). The related mechanisms need further studies.

In conclusion, it can be proposed that ADR cardiomyopathy is associated with the antioxidant deficit and SMS treatment changes the antioxidant status of the heart to improve cardiac function. The mechanism may be related to the maintenance of the antioxidant status and promote biosynthesis of the heart. It would also be of importance to elucidate the antitumor effects of the combination of SMS and ADR. The influence of SMS on tumor response to ADR should be evaluated in further studies.

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