Effects of fermented *Cordyceps sinensis* on oxidative stress in doxorubicin treated rats

Rong Wu, Jian-Ping Gao, Hui-Lin Wang, Yan Gao, Qian Wu, Xiao-Hua Cui

Department of Pharmacology, School of Pharmacy, Shanghai University of Traditional Chinese Medicine, NO. 1200 Cailun Road, Shanghai, 201203, China

ABSTRACT

Background: *Cordyceps sinensis* (CS) is one of the rare traditional Chinese herbs, only a very limited amount of natural CS is produced. Fermented CS, as a substitute for natural CS, is widely used in the field of supplementary medical treatment and health products. Its antagonistic effect on oxidative stress (OS) *in vivo* has not been investigated. **Objective:** Our aim was to investigate the antagonistic effect of fermented CS on OS in doxorubicin (DOX) treated rats and to compare the anti-OS effects in heart and liver tissues. **Materials and Methods:** OS rats were induced by tail-intravenous injection of DOX (total of 7.5 mg/kg), and then administered intragastrically with fermented CS (1.5 g/kg) for 4 weeks. At the end of the experiment, heart, liver and serum samples were taken for and biochemical analyses. **Results:** Fermented CS significantly increased the activities of glutathione peroxidase and catalase and the scavenging activity of O_2^- in serum, and the total superoxide dismutase activity in cardiac tissue; reduced the malondialdehyde content in liver and cardiac tissues. **Conclusion:** Fermented CS can inhibit DOX-induced OS reactions, and the anti-OS effects have high selectivity to heart and liver, especially to heart. Thus, fermented CS may be a candidate used for the prevention against various cardiac diseases induced by OS.

Key words: Antioxidant enzyme system, doxorubicin, fermented *Cordyceps sinensis*, oxidative stress, supplementary medical treatment

INTRODUCTION

Reactive oxygen species (ROS) mainly consists of superoxide anion (O_2^-), hydroxyl radical (OH^-), hydrogen peroxide (H_2O_2), and nitric oxide (NO^-). Under normal circumstances, ROS possess an important role in maintaining an aerobic life.[1] The body's antioxidant system, which is composed of enzymatic and nonenzymatic systems, can effectively remove free radicals. The enzymatic system contains superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GSH-Px), and other enzymes. Oxidative stress (OS) refers to the cellular damage and pathologic change that occur when an imbalance occurs between oxidants and antioxidants within a living organism.[2] With an active chemical property, ROS can react with other substances within a short period. When the balance between ROS generation and clearance is broken, ROS will quickly accumulate, resulting in OS. Accumulated ROS react with macromolecules in the body, such as lipids,

proteins, polypeptides, and nucleic acids, and then give rise to lipid membrane peroxidation, protein cross-linking, and nucleic acid damage, which will thus cause oxidative damages of cells, tissues, and organs. In recent years, researches have focused on OS and its relevance of many diseases, such as cardiovascular disease,[3] inflammation,[4] and cataract.[5] The intake of exogenous antioxidants is an effective approach to preventing oxidative damage and maintaining the balance between generation and clearance of free radicals in the body.[6] Studies show that some of the exogenous antioxidants can increase the activities of antioxidant enzymes.[7,8]

Doxorubicin (DOX), a broad-spectrum anticancer drug, has considerable side effects in body, especially in heart and liver. DOX can promote the generation of ROS and malondialdehyde (MDA),[9] and can inhibit the activities of SOD, CAT, GSH-Px, and other anti-oxidative enzymes, resulting in OS injury. *Cordyceps sinensis* (CS), a widely used traditional Chinese medicine, is a composite consisting of CS (Berk.) Sacc. Parasitized on the larva of some species of insects of family *Hypocreaceae* and the dead caterpillar. CS has long been used to improve quality of life and promote longevity. It is now commonly used as a health product,[10] with a wide range of pharmacological effects,

Address for correspondence:
Prof. Jian-Ping Gao, Department of Pharmacology, School of Pharmacy, Shanghai University of Traditional Chinese Medicine, NO. 1200 Cailun Road, Shanghai, 201203, China.
E-mail: zydgjp@aliyun.com

such as immune regulation, antitumor, anti-senescence, and hypoglycemic and hypolipidemic actions.[11,12] Owing to the strict natural conditions, only an extremely limited amount of natural CS is produced. Fermented CS (Cs-C-Q80), as a substitute for natural CS, is widely used in the field of supplementary medical treatment and health products. Fermented CS is produced by purifying and artificially fermenting the fungus isolated from fresh Qinghai CS. Its pharmacological effects are similar to natural CS.[13,14] Some studies demonstrate its antioxidant action.[15,16] However, most studies mainly focus on *in vitro* experiments, and no research has assessed the effects of CS on inhibiting OS injury of different organs, especially the liver and heart. In the present study, we selected two different formulations and sources of fermented CS to explore their effects on OS treated with DOX and to compare their anti-OS effects in heart and liver.

MATERIALS AND METHODS

Herbal materials and reagents
Cordyceps sinensis mycelia are isolated from natural CS to produce fermented CS (Cs-C-Q80) by artificial fermentation. Its chemical components are similar to natural CS and contain amino acids and mannitol, the products meet the standard of the Pharmacopoeia of the people's Republic of China (2010 Edition). A comparison of the chemical compositions and bioactive ingredients between natural CS and fermented CS was reported elsewhere.[17] The natural CS contains 29.1% protein, 8.62% fat, 24.2% carbohydrate, 2.85% ash, 8.93% moisture, 18.1% amino acid and 5.4% cordycepin; the fermented CS contains 14.8% protein, 6.63% fat, 39.4% carbohydrate, 2.95% ash, 6.4% moisture, 9.23% amino acid and 1.4% cordycepin. CS capsules were supplied by Hangzhou Zhongmei Huadong Pharmaceutical Co., Ltd., (Hangzhou, China). CS tablets were supplied by Qinghai Everest Aweto Pharmaceutical Co., Ltd., (Qinghai, China). The two formulations of fermented CS are listed in the Pharmacopoeia of the people's Republic of China (2010 Edition). DOX Hydrochloride for injection was obtained from Zhejiang Hisun Pharmaceutical Co., Ltd., (Taizhou, China).

Animals
Male Sprague-Dawley rats (190 g^{-2}10 g) were supplied by Shanghai Laboratory Animal Co., Ltd., (Shanghai, China). All animals were maintained on a 12 h light/12 h dark cycle room with a temperature of 22–24°C and humidity of 40% ± 5%. These rats received humane care and had free access to a standard diet and drinking water. Animal experiments were conducted in accordance with the guide for the care and use of laboratory animals published by the U.S. National Institutes of Health (NIH Publication No. 85–23, revised 1996) and approved by the Animal Care and Use Committee of the Shanghai University of Traditional Chinese Medicine.

Experimental design
Rats received a tail-intravenous injection of 0.2% DOX (total of 7.5 mg/kg, twice, interval of 1-week) to induce OS. The normal control group consisting of 12 rats received a tail-intravenous injection of 0.9% NaCl (the solvent for DOX, the same volume with DOX) to eliminate the influence of solvent and injection on rats. One week after modeling, OS rats were randomly divided into three groups: DOX control, DOX + CS tablet, and DOX + CS capsule. Rats in the drug treatment groups were administered intragastrically with 1.5 g/kg/day CS for 4 weeks, while rats in the normal control and DOX control groups were administered intragastrically with the same volume drinking water. At the end of the experiment, the rats were sacrificed under anesthesia (urethane, 1 g/kg). Blood samples were collected and centrifuged (4°C, 2325 × g, 10 min) to recover serum, and heart and liver tissues were excised and stored at −80°C.

Endogenous antioxidant enzymes in the heart, liver, and serum
Liver and heart tissues were homogenized with cold 0.9% NaCl and centrifuged (4°C, 1780 × g, 15 min) to recover the supernatants. The supernatants and serum were used for enzyme activity assays. Total SOD (T-SOD), GSH-Px, and CAT activities in the heart, liver, and serum were assayed by the xanthinoxidase method, DTNB colorimetric method, and ammonium molybdate spectrophotometric method using kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, China).

Malondialdehyde and the scavenging activity of O_2^- assay
Malondialdehyde contents in the heart, liver, and serum were determined by a thiobarbituric acid method using a kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, China). The scavenging activity of O_2^- in the heart, liver, and serum were assayed spectrophotometrically by the pyrogallol autoxidation method at 325 nm. The scavenging activity of O_2^- was calculated using the following formula:

$$\text{Scavenging effect } (\%) = (1 - A_1/A_0) \times 100$$

Where A_0 is the absorbance of control without the tested samples and A_1 is the absorbance in the presence of the tested sample.

Measurement of cardiac and liver marker enzymes
Serum creatine kinase (CK), lactate dehydrogenase (LDH), alanine aminotransferase (ALT), and aspartate aminotransferase (AST) activities were analyzed with the Hitachi 7080 biochemical analyzer (Tokyo, Japan). CK,

LDH, ALT, and AST kits were obtained from Shino-Test Co., Ltd., (Tokyo, Japan).

Measurement of transforming growth factor-β1 and interleukin-8 in serum

Serum transforming growth factor-β1 (TGF-β1) and interleukin-8 (IL-8) levels were measured using enzyme-linked immunosorbent assay kits (Shanghai Yu Sen Biotechnology Co., Ltd., Shanghai, China).

Statistical analysis

All values were expressed as mean ± standard deviation and analyzed using SPSS 18.0 software (Statistical Product and Service Solutions, IBM Co., Ltd., New York, America). One-way analysis of variance (ANOVA) was performed for multiple comparisons, followed by Dunnett's test to evaluate the difference between two groups. Rank-sum test was used as an alternate test for variance heterogeneity. When several measurements were taken on the same experimental unit, statistical analysis was evaluated by repeated-measures ANOVA. (P < 0.05) was considered statistically significant.

RESULTS

Total superoxide dismutase activity and malondialdehyde content

Six weeks after injection of DOX, the content of serum MDA was significantly higher (P < 0.01) and the serum T-SOD activity was significantly lower (P < 0.01). Treatment with CS capsule for 4 weeks significantly reduced the MDA contents in liver and cardiac tissues (P < 0.05), but no significant change occurred in the content of serum MDA [Figure 1]. In addition, CS capsule and CS tablet significantly increased the T-SOD activities in cardiac tissues, but the improvement of T-SOD activities in serum and liver tissues was not evident (P > 0.05) [Figure 2].

Catalase and glutathione peroxidase activities

Six weeks after injection, DOX significantly reduced the GSH-Px and CAT activities in serum and liver and cardiac tissues (P < 0.05). After treatment for 4 weeks, CS capsule and CS tablet significantly increased the CAT activities in cardiac (P < 0.01) and liver tissues (P < 0.05), and the GSH-Px activities of serum and liver and cardiac tissues (P < 0.01) [Figure 3]; CS tablet significantly increased the serum CAT activity (P < 0.05) [Figure 4].

Scavenging activity of O_2^-

Six weeks after injection, DOX significantly reduced the serum O_2^- radical scavenging activity (P < 0.05) [Figure 5]. Compared with DOX control group, CS capsule increased the scavenging activity of O_2^- in liver tissues (P < 0.05) [Figure 6] and serum (P = 0.05028); CS tablet increased the scavenging activity of O_2^- in cardiac tissues (P < 0.05) [Figure 7] and trended to increase the scavenging activity of O_2^- in liver tissues (P = 0.07844).

Creatine kinase, lactate dehydrogenase, alanine aminotransferase, and aspartate aminotransferase activities in serum

Six weeks after injection of DOX, no significant change occurred in the serum CK, LDH, ALT, and AST activities, suggesting that the influences on liver and myocardial enzyme systems induced by DOX for 6 weeks were not evident in this experiment. After treatment for 4 weeks, CS tablets significantly decreased the CK activity (P < 0.05); CS capsule and CS tablet significantly decreased the LDH activity (P < 0.05); and no significant change occurred in the ALT and activities [Figure 8].
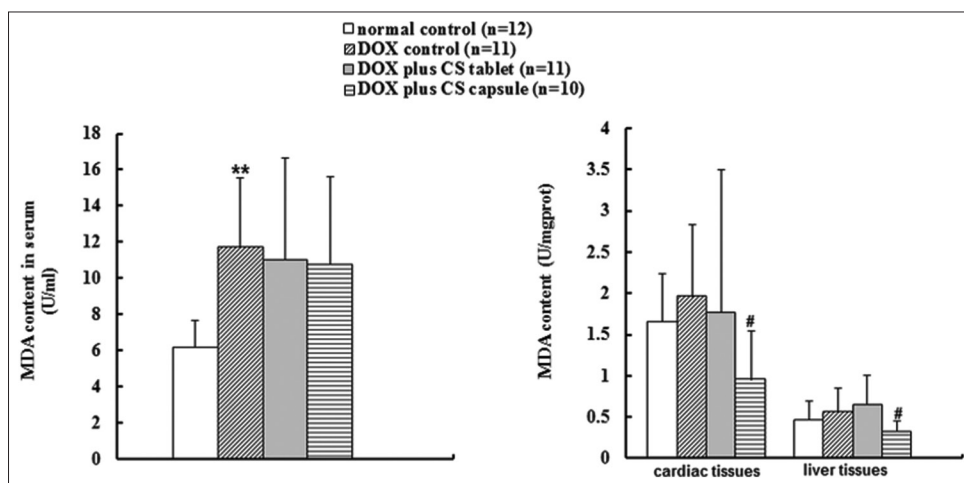

Figure 1: Effects of fermented *Cordyceps sinensis* on malondialdehyde content in doxorubicin (DOX) treated rats. Data are presented as mean ± standard deviation. MDA, malondialdehyde. *P < 0.05, **P < 0.01, compared with the normal control group. #P < 0.05, ##P < 0.01, compared with the DOX control group
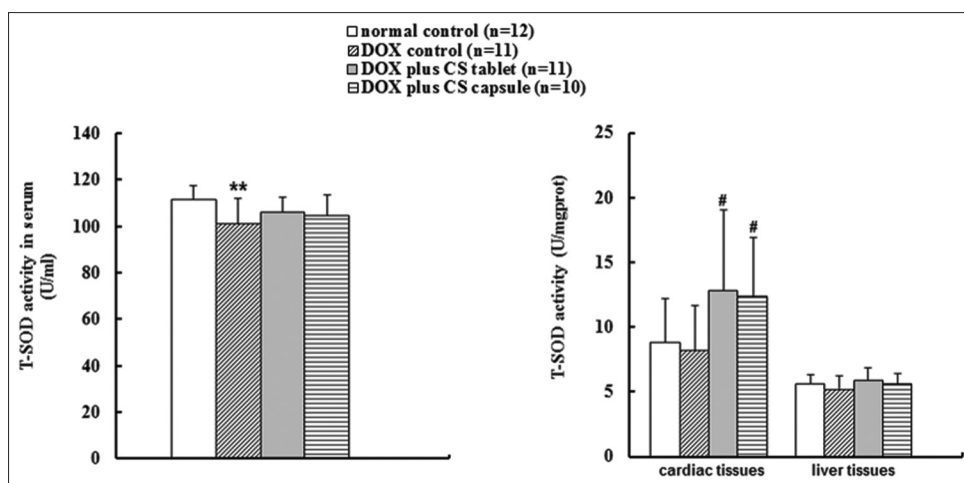
Figure 2: Effects of fermented *Cordyceps sinensis* on total superoxide dismutase (T-SOD) activity in doxorubicin (DOX) treated rats. Data are presented as mean ± standard deviation. T-SOD, total superoxide dismutase. *$P < 0.05$, **$P < 0.01$, compared with the normal control group. #$P < 0.05$, ##$P < 0.01$, compared with the DOX control group
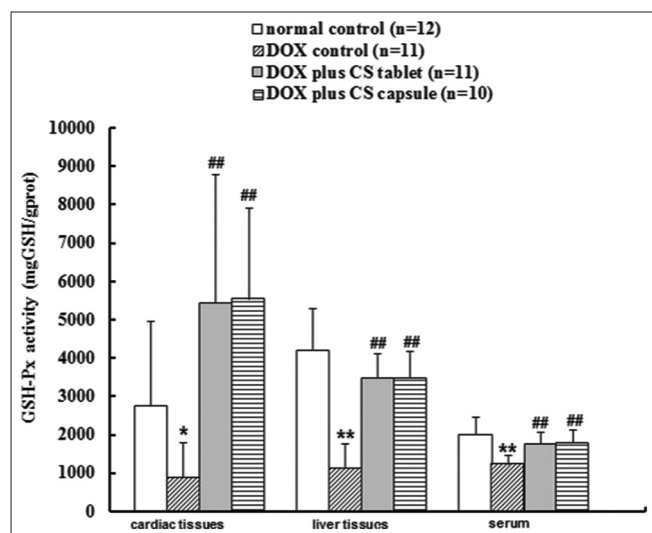

Figure 3: Effects of fermented *Cordyceps sinensis* on GSH-Px activity in doxorubicin (DOX) treated rats. Data are presented as mean ± standard deviation, glutathione peroxidase. *$P < 0.05$, **$P < 0.01$, compared with the normal control group. #$P < 0.05$, ##$P < 0.01$, compared with the DOX control group

Transforming growth factor-β1 and interleukin-8 concentrations in serum

Six weeks after injection of DOX, no significant change occurred in the levels of TGF-β1 and IL-8, suggesting that the effect of DOX on inflammation was not evident in this experiment. Treatment with CS tablet for 4 weeks significantly decreased the TGF-β1 and IL-8 concentrations ($P < 0.05$) [Figure 9].

DISCUSSION

Doxorubicin is an anthracycline anticancer antibiotic which is extensively used as a potent and broad spectrum anti-cancer agent. The anticancer activity of DOX comes from its inhibition effect in the process of DNA replication,[18] it can interact with DNA by reducing of macromolecular biosynthesis.[19] It is used to treat several solid tumors and hematological malignancies such as lymphomas, breast cancer and leukemia,[20] but the clinical use of DOX is limited by its serious adverse effect including cardiotoxicity and hepatotoxicity. DOX has a high affinity of binding to phospholipid in the inner mitochondrial membrane.[21] The heart and liver are susceptible to DOX injury, as they contain more mitochondria. Mechanism of DOX damage in the body is associated with the free radicals it produces,[22] which lead to OS.

Oxidative stress is the basis of many diseases and pathological processes. DOX-induced OS can lead to the increase in OS products and the decrease in the activities of antioxidant enzymes and thus cause the damage of membrane lipids and other cell components, and eventually give rise to multiple organ injury. For this reason, we chose to measure the relevant biomarkers in serum and liver and cardiac tissues of rat to evaluate whether fermented CS inhibits the DOX-induced OS response, and then assess the difference of its antioxidant capacity in liver and heart. The present study is designed to provide an experimental basis for the application of fermented CS to treat OS injury in heart and liver.

As a final product of lipid peroxidation, MDA can generate covalent adducts that probably lead to protein damage.[23] It is a relevant biomarker for assessing the degree of oxidative damage. Thus, we can evaluate the level of OS by measuring the MDA content and reveal the damage degree of the body.[24,25] T-SOD, CAT, and GSH-Px belong to the antioxidant enzyme system of the body and are able to remove free radicals and protect cells and tissues. Among them, SOD is the essential enzyme in removing
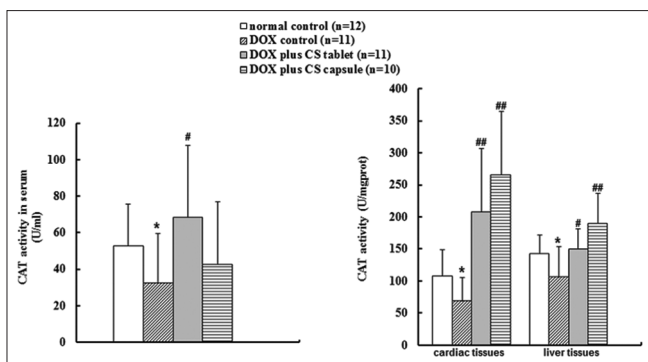
Figure 4: Effects of fermented *Cordyceps sinensis* on catalase (CAT) activity in doxorubicin (DOX) treated rats. Data are presented as mean ± standard deviation. CAT, CAT. *$P < 0.05$, **$P < 0.01$, compared with the normal control group. #$P < 0.05$, ##$P < 0.01$, compared with the DOX control group
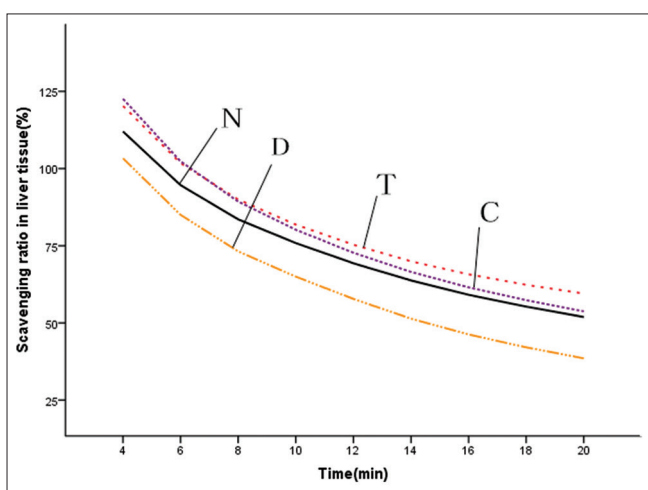

Figure 5: Effects of fermented *Cordyceps sinensis* (CS) on superoxide radical scavenging ability in serum. N, normal control; D, doxorubicin (DOX) control; T, DOX plus CS tablet (1.5 g/kg); C, DOX plus CS capsule (1.5 g/kg)

Figure 6: Effects of fermented *Cordyceps sinensis* (CS) on superoxide radical scavenging ability in liver tissue. N, normal control; D, doxorubicin (DOX) control; T, DOX plus CS tablet (1.5 g/kg); C, DOX plus CS capsule (1.5 g/kg)

Figure 7: Effects of fermented *Cordyceps sinensis* (CS) on superoxide radical scavenging ability in cardiac tissue. N, normal control; D, doxorubicin (DOX) control; T, DOX plus CS tablet (1.5 g/kg); C, DOX plus CS capsule (1.5 g/kg)

free radicals, it can catalyze the disproportionation reaction and thus remove O_2^-, constituting a critical defense mechanism of cells against the harmful effects of ROS.[26,27] CAT and GSH-Px are two vital enzymes that inhibit oxidative damage, they play important roles in anti-oxidant enzyme system.[28-30] The main role of CAT is to catalyze the conversion of H_2O_2 to H_2O and O_2, and the main biological function of GSH-Px is to turn lipid peroxide into the corresponding alcohol and reduce free H_2O_2 into H_2O, thus protecting the body from oxidative damage. Therefore, the activities of T-SOD, CAT, and GSH-Px can reflect the capacity of the body in removing free radicals. Some studies,[31,32] show that O_2^- clearance is multifactorial, and the related compounds include endogenous SOD, vitamin C, vitamin E, and exogenous active ingredients. In the present study, we adopted pyrogallol autoxidation method,[33] to measure superoxide radical scavenging ability of the biological samples of experimental rats to reveal
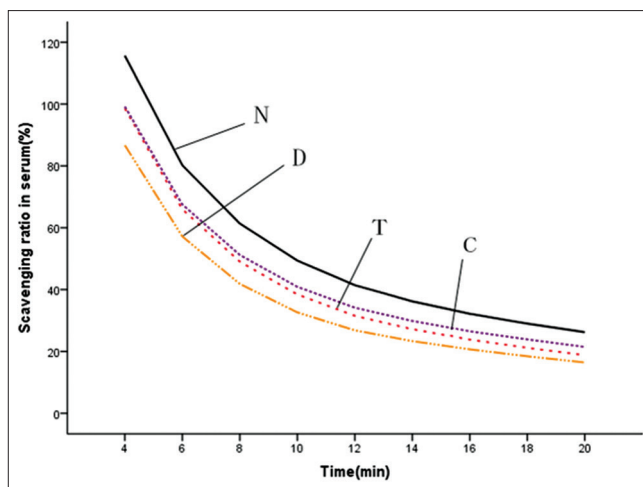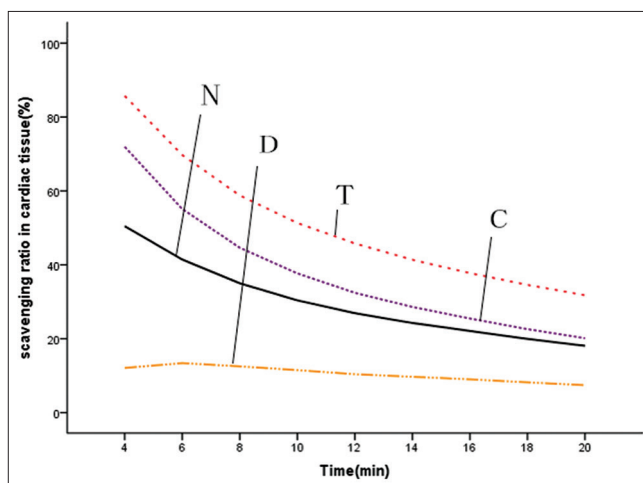
more comprehensively the effect of drugs on improving the body's ability in clearing O_2^- free radicals. Six weeks after injection of DOX, an evident decrease in both the superoxide radical scavenging ability and the activities of T-SOD, GSH-Px, and CAT in the serum of DOX control group rats and an obvious increase in the amount of MDA were observed. Those results indicate that DOX significantly reduces the activities of the anti-oxidant enzymes of rats and significantly increases the lipid peroxidation products. However, after rats were fed with fermented CS by oral gavage for 4 weeks, it significantly improved the activities of GSH-Px and CAT. In addition, fermented CS had a certain effect in improving the activity of SOD and raising the O_2^- clearance rate as well, and some effect in reducing the amount of MDA. The results
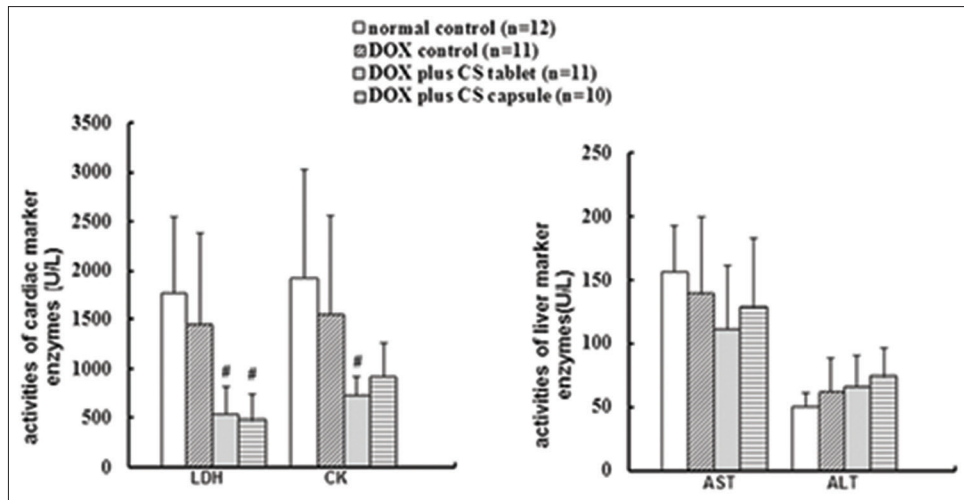
Figure 8: Effects of fermented *Cordyceps sinensis* on creatine kinase, lactate dehydrogenase, alanine aminotransferase and aspartate aminotransferase activities in doxorubicin (DOX-)-induced rats. Data are presented as mean ± standard deviation. CK, *P* < 0.05, **P* < 0.01, compared with the normal control group. #*P* < 0.05, ##*P* < 0.01, compared with the DOX control group
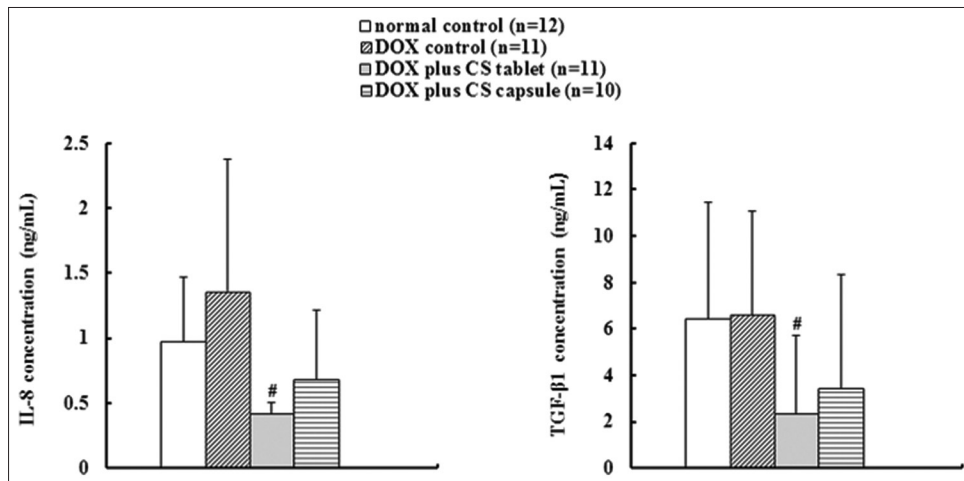

Figure 9: Effects of fermented *Cordyceps sinensis* on transforming growth factor-β1and interleukin-8concentration in doxorubicin (DOX) treated rats. Data are presented as mean ± standard deviation. *P* < 0.05, **P* < 0.01, compared with the normal control group. #*P* < 0.05, ##*P* < 0.01, compared with the DOX control group

show that fermented CS has a significant effect in inhibiting DOX-induced OS, especially in increasing the activities of the anti-oxidant enzymes.

Doxorubicin causes damage to the body and especially leads to heart diseases,[34] and liver damage,[35] by inducing OS. LDH and CK are both cardiac enzymes. In the case of myocardial injury or necrosis, these enzymes will be released into blood, and then their activities in serum will be increased. Based on this reason, testing the activities of these enzymes in serum is essential in diagnosing myocardial injury. ALT and AST mainly exist in the liver and other tissues. In the case of liver damage or cell necrosis, the release of ALT and AST from liver cells will induce the increase of their activities in serum. Therefore, liver injury can be assessed by detecting the activities of ALT and AST in serum. Six weeks after injection, DOX did not

significantly influence the serum activities of CK, LDH, ALT, and AST. This result indicates that DOX significantly increases the level of OS in the heart and liver 6 weeks after injection in this experiment but the damage of hepatocytes and cardiomyocytes may be not so obvious. However, treatment with fermented CS for 4 weeks significantly reduced the activity of LDH and CK but did not influence the activities of AST and ALT. This result indicates that fermented CS may potentially have protective effect against myocardial tissue damage.

Oxidative stress is closely related to inflammation. The increase of ROS can activate IL-8 and other cytokines,[36,37] which will cause a certain degree of neutrophil infiltration, intensify inflammatory response, and lead to cell damage. As a major inflammatory cytokine, IL-8 is an effective diagnostic biomarker for inflammatory diseases.[38] The

mechanism of IL-8 in the immune system is complex, including chemotactic effects on target cells (neutrophils and other granulocytes) and pro-inflammatory effects by enhancing degranulation and activation of basophils, neutrophils and macrophages.[39] When inflammatory reaction occurs, IL-8 concentration significantly increases in inflammatory tissues, serum, and body fluids. ROS are also associated with the generation of TGF-β1 and at the same time takes part in a series of TGF-β1-induced pathological response.[40] TGF-β1, as one kind of immunosuppressive factors, participates in the immune responses mainly by suppressing natural killer cells and macrophages, inhibiting the production of regulatory T cells and the differentiation of helper T cells, and suppressing the proliferation of B and T cells.[41] TGF-β1 is also a key fibrosis factor. Under pathological conditions, such as inflammation, increasing TGF-β1 will lead to extracellular matrix overproduction and thus cause liver fibrosis and cardiac fibrosis.[42,43] Six weeks after injection of DOX, the level of IL-8 slightly increased, but no significant difference was observed between normal control and DOX control rats. After treatment for 4 weeks, fermented CS showed a certain effect in reducing IL-8 and TGF-β1, indicating that it may play a potential role in protecting the body against inflammation.

Mitochondrial respiratory chain complexes produce adenosine triphosphate through electron transfer. As the main source of ROS in the body, mitochondria are abundant in hepatocytes and cardiomyocytes. Thus, heart and liver are easily attacked by ROS. Closely associated with cardiovascular diseases, OS can lead to atherosclerosis, myocardial hypertrophy, and cardiac myocyte apoptosis and other diseases.[44] OS can also be a precipitating factor in certain liver diseases, closely related to liver cancer, liver fibrosis, and other liver injuries.[45,46] Therefore, by comparing the effect of fermented CS on inhibiting the OS in heart and liver, we can make a clearer assessment on its selective role on inhibiting OS in different organs. By comparing the antioxidant indicators in serum and liver and cardiac tissues, fermented CS showed a significant effect in increasing the activity of SOD in cardiac tissues, but had insignificant effect in serum and liver tissues. It also had significant effects in increasing the activities of CAT and GSH-Px in serum and liver and cardiac tissues. The rates of increase in the activities of CAT and GSH-Px in liver and heart were obviously higher than those in the serum, and most notably in cardiac tissue. In addition, in the groups of fermented CS, no significant decrease was observed in the MDA content in serum. The significant decrease in MDA occurred in heart and liver tissues, with a higher rate of decrease in heart tissues. Therefore, fermented CS has more obvious effect of anti-OS in heart and liver, especially in heart.

CONCLUSION

Fermented CS can ameliorate the OS in DOX treated rats. It is mainly manifested in the obvious improvement of the antioxidant enzyme system. Its protective effects are more obviously in heart and liver, especially in heart. Therefore, fermented CS can be recommended for a promising adjuvant of DOX in clinical application. It can also be a candidate used for the prevention against various cardiac diseases induced by OS. The additional studies are necessary to evaluate the efficacy of fermented CS in treating DOX-induced cardiotoxicity and hepatotoxicity.

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