Huaier Polysaccharide Attenuates Doxorubicin-Induced Acute Cardiotoxicity by Regulating Ferroptosis
X. Ma, H. Gao, B. Yang, H. Zhao, and Z. Zhu

We studied the effects of Huaier polysaccharide (HP) in doxorubicin-induced myocardial injury in mice. The content of HP in *Trametes robiniophila Murr* medicinal fungus determined by the phenol-sulfuric acid method was 85.25%. In the *in vitro* model, the viability of H9c2 cells was significantly increased after HP treatment compared to the control, while doxorubicin (DOX) decreased this parameter. The inhibitory effect of DOX on cell viability was attenuated after HP treatment. In the *in vivo* model, the body weight of mice in DOX and DOX+HP groups was significantly decreased compared to the control group. ECG showed significantly elevated ST segment in the DOX group, while in the DOX+HP group, ECG was close to normal. The levels of cardiotoxicity markers cTnI and lactate dehydrogenase in the DOX+HP group were significantly lower than in the DOX group. In the DOX group, the myocardial tissue had obvious structural disorder and interfibrillar vacuoles. In the DOX+HP group, the cardiomyocytes were neatly arranged without interfibrillar vacuoles. The expression of the ferroptosis marker glutathione peroxidase 4 was increased in the DOX+HP group compared to the DOX group. Thus, our study reveals that HP attenuated DOX-induced myocardial injury in mice probably by regulating ferroptosis.

**Key Words:** Huaier polysaccharide; doxorubicin; myocardial injury; ferroptosis; glutathione peroxidase 4 (GPX4)

Doxorubicin (DOX) is an anthracycline antineoplastic drug with a broad anticancer spectrum. However, it is often used with caution in clinical practice due to severe cardiotoxicity [1]. After entering cardiomyocytes, DOX is decomposed into semiquinone metabolites that damage cells by disrupting the redox balance of cells [2]. At the same time, DOX can also increase the concentration of myocardial Ca²⁺, thereby aggravating myocardial damage and inducing heart failure. These effects of DOX increase significantly with increasing its doses [3]. Therefore, it is very important to find drugs or measures to attenuate the cardiotoxicity of DOX, which has become a research hotspot all over the world.

It had been shown that the cardiotoxicity of DOX is mainly due to calcium overload and the effects of oxygen free radicals generated through the Fenton reaction and promoting LPO in cell membranes or various organelle membranes to induce ferroptosis, an important mechanism for DOX-induced cardiotoxicity [4]. Ferroptosis inhibitors have been shown to reduce DOX cardiotoxicity and protect the myocardium [5]. This means that toxicity of DOX to the myocardium can be attenuated by inhibition of DOX-induced ferroptosis in cardiomyocytes.

Polysaccharides are important bioactive ingredients that have good pharmacological effects in many fields. So, more and more attention was paid by researchers to find more and more new polysaccharide drugs [6]. It has been reported that fucoidan, a polysaccharide from *Fuco cesiculosus*, can attenuate DOX-induced acute cardiotoxicity [7]. This prompted us to study the cardioprotective effects of polysaccharide components. *Trametes robiniophila Murr* (Huaier) is a medicinal fungus used for the treatment...
of hemorrhoids, blood in the stool, rectal prolapse, and other diseases. Huaier polysaccharide (HP) is the main ingredient in Huaier, consisting of 6 monosaccharides and 18 amino acids [8]. Previous studies have demonstrated that HP has antitumor, antiviral, and immune-enhancing effects in vitro and in vivo [9,10]. However, the field of myocardial protection remains to be explored.

Our aim was to study the role of HP in DOX-induced myocardial injury from multiple perspectives, and preliminarily confirmed the potential targets of HP.

MATERIALS AND METHODS

HP characteristics. The content of HP was determined by the phenol-sulfuric acid method as previously described [9]. Briefly, glucose solutions of different concentrations were used as a standard, while HP solutions were the test solutions. To all samples, 6% phenol solution and concentrated sulfuric acid were added in an appropriate proportion, mixed quickly, and cooled with cold water. Absorbance was measured at 490 nm. A standard curve was drawn and the absorbance was regarded as the abscissa, while the concentration of glucose standard solution was regarded as the ordinate, then the content of polysaccharide was calculated.

Experiments in vitro. Rat cardiomyocyte cell line H9c2 was obtained from Cell Bank of Chinese Academy of Sciences and cultured in a DMEM medium (Gibco) containing 10% fetal bovine serum (Gibco), 100 U/ml penicillin G, 2.5 μg/ml amphotericin B, and 100 μg/ml streptomycin at 37°C and 5% CO2 in a humidified atmosphere. The cells were seeded in 96-well cell culture plates at a density of 2×104 cells/ml. After the cells were fully attached, HP or/and DOX were added. After 48 h, the cells were incubated with 10 μl Cell Counting Kit-8 (CCK-8; Beyotime Biotechnology) for another 4 h. Cell viability was measured using a Cell Imaging Multi-Mode Reader (BioTek) at 450 nm.

Experiments in vivo. BALB/c male mice (n=18; 7-8-weeks-old, body weight 25-28 g) were purchased from Shanghai Experimental Animal Center. The mice were randomly divided into 3 groups (6 mice per group) according to their body weight: control, DOX, and DOX+HP. The animal studies were approved by the Institutional Animal Care and Use Committee of Zhejiang Chinese Medical University (No. ZSLL-2013-108) and performed according to the Guidelines proposed by the Laboratory Animal Research Center of Zhejiang Chinese Medical University.

DOX (15 mg/kg; Shanghai Selleck Chemicals Co.) was injected intraperitoneally in 100 μl of 0.9% NaCl, HP (200 mg/kg) was gavaged with 200 μl bidistilled water. Control group received only 0.9% NaCl and bidistilled water in the same volume according to the same scheme. The mice were treated and weighed daily for 12 days. After euthanasia, the blood was quickly collected and centrifuged (3000g for 10 min at 4°C) to collect serum, part of the heart was fixed in formalin, the rest was stored at -80°C.

ELISA. The serum levels of cardiac troponin-I (cTnI) (cTnI ELISA kit; MMBIO) and lactate dehydrogenase (LDH) (LDH ELISA kit, Jiancheng Bioengineering Institute) were measured according to the manufacturers’ instructions.

Histology. Formalin-fixed myocardial tissue was dehydrated with graded ethanol, treated with xylene for 1 h, embedded in paraffin, and sliced into 3-μm sections. Then, the sections were stained with hematoxylin and eosin (Beyotime Biotechnology) and examined under a DMS500 light microscope (Leica).

For immunohistochemical analysis, the tissue sections were dewaxed and rehydrated, and then the slides were incubated with the primary anti-GPX4 antibody (1:200, ab125066; Abcam) at 4°C overnight. After that, the slides were incubated with horseradish peroxidase (HRP)-conjugated secondary antibodies (cat.# PV-8000; OriGene Technologies, Inc.) at room temperature. After being stained with DAB and counterstained with hematoxylin, the slides were examined under a microscope.

The data were analyzed using GraphPad Prism 5.0 (GraphPad Software) software and expressed as M±SD. Significance between the experimental and control groups was evaluated by Student’s t test for independent samples (Microsoft Excel 2021). The differences were significant at p<0.05.

RESULTS

Standard curve to determine HP concentration. Glucose solutions of different concentrations were used to draw a standard curve. The curve showed that the HP concentration and absorbance in the range of 0-0.07 mg/ml had a strong linear relationship (Fig. 1, b). Equal amounts of 0.05 mg/ml HP solutions (n=3) were used for absorbance determination, the content of the HP was determined to be 85.25% (Table 1) according to the standard curve.

The effect of HP in in vitro experiment. The CCK-8 assay showed that after HP treatment, the viability of H9c2 cells significantly increased (p<0.001, Fig. 1, c), suggesting that HP had a protective effect on normal cardiomyocytes. Treatment with different concentrations of DOX significantly decreased the viability of H9c2 cells (p<0.001, Fig. 1, d). HP reversed the inhibitory effect of DOX (1 μM) on H9c2 cells and showed a concentration dependence (p<0.001, Fig. 1, e).
Fig. 1. The role of HP on DOX-induced cardiotoxicity (a), the content of HP in the extract (b), viability of H9c2 cells treated with HP (c), DOX (d), and HP+DOX (e) according to CCK-8 assay data. *p<0.05, **p<0.01, ***p<0.001 in comparison with the control; ‘p<0.05, ‘’p<0.01, ‘’’p<0.001 in comparison with DOX group.

TABLE 1. Standard Curve of Glucose Concentration

| Parameter                  | Sample No. 1 | Sample No. 2 | Sample No. 3 |
|----------------------------|--------------|--------------|--------------|
| Absorbance                 | 0.229        | 0.252        | 0.251        |
| Mean value                 |              | 0.244        |              |
| HP content, %              |              | 85.25        |              |
| Standard deviation         |              | 0.01061      |              |
| Relative standard deviation, %|              | 4.35         |              |
The results showed that HP could significantly attenuate DOX-induced cardiomyocyte injury in vitro and could be used as a potential drug for attenuating DOX cardiotoxicity (Fig. 1, a).

**The effect of HP in in vivo experiment.** Compared to the control group, the body weight of mice in the DOX group and DOX+HP group decreased (p<0.05, Fig. 2, b). In addition, ECG were examined. ST segment elevation often occurs in acute myocardial ischemia or even myocardial infarction and other diseases [11]. The ST segment in the DOX group was significantly elevated in comparison with the control group, indicating that DOX has a strong damaging effect on the myocardium. In the mice treated with DOX and HP, the ST-segment almost returned to normal (Fig. 2, a). Our results demonstrate that the toxic effects of DOX on the myocardium were severely pathogenic, but could be attenuated by HP (Fig. 1, a).

**The effect of HP on cardiotoxicity markers cTnl and LDH.** When myocardial cells are damaged, cTnl can be released into the serum through the damaged myocardial cell membrane. The content of LDH will also increase. So, these proteins are often used as indicators of myocardial damage in clinical practice [12]. In our experiments, serum contents of cTnl and LDH in the DOX group were significantly increased (p<0.05, Fig. 2, a).
c, d) in comparison with the control, which confirmed DOX toxicity for the myocardium. In the DOX+HP group, serum contents cTnI and LDH were significantly lower than in the DOX group (p<0.05, Fig. 2, c, d). The results demonstrated that the toxic effect of DOX on the myocardium could be attenuated by HP.

**Results of histological study.** Hematoxylin and eosin staining of mouse myocardial tissue sections showed that the cardiomyocytes in the control group were regularly arranged and morphologically normal. In the DOX group, structural changes in the myocardium, hemolysis, and interfibrillar vacuoles were seen. Thus, DOX had caused great damage to the myocardial tissue. In the DOX+HP group, the myocardial tissue was regularly arranged, and no hemolysis and interfibrillar vacuoles were observed (Fig. 3, a). These results demonstrate that HP could attenuate myocardial morphological damage induced by DOX.

Ferroptosis is a recently discovered iron-dependent mechanism of cell death caused by inactivation of glutathione peroxidase 4 (GPX4), accumulation of lipid peroxides, and the imbalance of the GSH/GSSG redox system [13]. DOX degrades heme in cardiomyocytes by up-regulating heme oxygenase-1, then releases free iron, induces ferroptosis, and damages cardiomyocytes [14].

Accordingly, this study attempted to explore the relationship between the cardioprotective effect of HP and ferroptosis. Surprisingly, the expression of GPX4 in the DOX group was downregulated in comparison with the control group suggesting that DOX cardiotoxicity was related to induction of ferroptosis. In the DOX+HP group, the expression of GPX4 was increased in comparison with the DOX group (Fig. 3, b), demonstrating that HP could attenuate DOX-induced cardiotoxicity by inhibiting ferroptosis.

The cardiotoxicity of DOX often induces severe cardiomyopathy, such as myocardial infarction and heart failure, which greatly reduces the antitumor efficacy of DOX [15]. Therefore, there is an urgent need for a drug that can reduce the cardiotoxicity of DOX and protect the myocardium without weakening the antitumor effect of DOX. In summary, the results of our study demonstrated that HP had the effect of attenuating DOX-induced cardiotoxicity, possibly by inhibiting ferroptosis. Furthermore, the detailed molecular mechanisms of the cardioprotective effects of HP and the attenuating effect of HP on DOX cardiotoxicity are worthy to be studied in the future. There is no doubt of importance in studying the effect and mechanism of HP in DOX-induced myocardial injury in order to develop natural polysaccharide components for medicinal use and to provide theoretical and experimental basis. We expected to make a modest contribution to follow-up studies and attenuation of DOX-induced cardiotoxicity in the clinic.

**REFERENCES**

1. Monahan DS, Flaherty E, Hameed A, Duffy GP. Resveratrol significantly improves cell survival in comparison to dexrazoxane and carvedilol in a h9c2 model of doxorubicin induced cardiotoxicity. Biomed. Pharmacother. 2021;140:111702. doi: 10.1016/j.biopha.2021.111702

2. Carvalho FS, Burgeiro A, Garcia R, Moreno AJ, Carvalho RA, Oliveira PJ. Doxorubicin-induced cardiotoxicity:
from bioenergetic failure and cell death to cardiomyopathy. Med. Res. Rev. 2014;34(1):106-135. doi: 10.1002/med.21280

3. Vaidya TR, Mody H, Franco YL, Brown A, Ait-Oudhia S. Multiscale and Translational Quantitative Systems Toxicology, Pharmacokinetic-Toxicodynamic Modeling Analysis for Assessment of Doxorubicin-Induced Cardiotoxicity. AAPS J. 2021;23(1):18. doi: 10.1208/s12248-020-00542-0

4. Qin Y, Guo T, Wang Z, Zhao Y. The role of iron in doxorubicin-induced cardiotoxicity: recent advances and implication for drug delivery. J. Mater. Chem. B. 2021;9(24):4793-4803. doi: 10.1039/d1tb00551k

5. Zhang H, Wang Z, Liu Z, Du K, Lu X. Protective Effects of Dexazoxane on Rat Ferroptosis in Doxorubicin-Induced Cardiomyopathy Through Regulating HMGB1. Front. Cardiovasc. Med. 2021;8:685434. doi: 10.3389/fcvm.2021.685434

6. Chen X, Han W, Wang G, Zhao X. Application prospect of polysaccharides in the development of anti-novel coronavirus drugs and vaccines. Int. J. Biol. Macromol. 2020;164:331-343. doi: 10.1016/j.ijbiomac.2020.07.106

7. Zhang J, Sun Z, Lin N, Lu W, Huang X, Weng J, Sun S, Zhang C, Yang Q, Zhou G, Guo H, Chi J. Fucoidan from Fucus vesiculosus attenuates doxorubicin-induced acute cardiotoxicity by regulating JAK2/STAT3-mediated apoptosis and autophagy. Biomed. Pharmacother. 2020;130:101534. doi: 10.1016/j.biopha.2020.110534

8. Fang L, Zhang Y, Zang Y, Chai R, Zhong G, Li Z, Duan Z, Ren J, Xu Z. HP-1 inhibits the progression of ccrCC and enhances sunitinib therapeutic effects by suppressing EMT. Carbohydr. Polym. 2019;223:115109. doi: 10.1016/j.carbpol.2019.115109

9. Hu B, Yan W, Wang M, Cui X, Hu Y, Chen Q, Zhang Y, Qi X, Jiang J. Huaier polysaccharide inhibits the stem-like characteristics of ERα-36high triple negative breast cancer cells via inactivation of the ERα-36 signaling pathway. Int. J. Biol. Sci. 2019;15(7):1358-1367. doi: 10.7150/ijbs.27360

10. Yang A, Fan H, Zhao Y, Chen X, Zhu Z, Zha X, Zhao Y, Chai X, Li J, Tu P, Hu Z. An immune-stimulating proteoglycan from the medicinal mushroom Huaier up-regulates NF-kB and MAPK signaling via Toll-like receptor 4. J. Biol. Chem. 2019;294(8):2628-2641. doi: 10.1074/jbc.RA118.005477

11. Oleynikov VE, Matskeplishvili S, Shigotarova E, Kulytsin A, Burko N. Diagnosis of coronary artery rethrombosis after effective systemic thrombolytic therapy in patients with ST-segment elevation myocardial infarction. J. Investig. Med. 2022;70(4):892-898. doi: 10.1136/jim-2021-001945

12. Yu YW, Que JQ, Liu S, Huang KY, Qian L, Weng YB, Rong FN, Wang L, Zhou YY, Xue YJ, Ji KT. Sodium-Glucose Co-transporter-2 Inhibitor of Dapagliflozin Attenuates Myocardial Ischemia/Reperfusion Injury by Limiting NLRP3 Inflammasome Activation and Modulating Autophagy. Front. Cardiovasc. Med. 2022;8:768214. doi: 10.3389/fcvm.2021.768214

13. Lin JH, Yang KT, Lee WS, Ting PC, Luo YP, Lin DJ, Wang YS, Chang JC. Xanthohumol Protects the Rat Myocardium against Ischemia/Reperfusion Injury by Limiting Ferroptosis. Oxid. Med. Cell. Longev. 2022;2022:9523491. doi: 10.1155/2022/9523491

14. Fang X, Wang H, Han D, Xie E, Yang X, Wei J, Gu S, Gao F, Zhu N, Yin X, Cheng Q, Zhang P, Dai W, Chen J, Yang F, Yang HT, Linkermann A, Gu W, Min J, Wang F. Ferroptosis as a target for protection against cardiomyopathy. Proc. Natl Acad. Sci. USA. 2019;116(7):2672-2680. doi: 10.1073/pnas.1821022116

15. Sheibani M, Azizi Y, Shayan M, Nezamoleslami S, Eslami F, Farjoo MH, Dehpour AR. Doxorubicin-Induced Cardiotoxicity: An Overview on Pre-clinical Therapeutic Approaches. Cardiovasc. Toxicol. 2022;22(4):292-310. doi: 10.1007/s12012-022-00721-1