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Original Article

Polysaccharides from *Phellinus linteus* (Phps) Protect against Doxorubicin-Induced Myocardial Inflammatory Injury in Mice through PI3K/Akt/mTOR Signaling Pathway

Xiaoyu Ren[1,#], Jinfeng Wang[2,#], Yuan Zhou[1], Lili Jiang[1], Fei Liu[1], Qingxian Mei[3,*]

[1]Institute of Molecular Medicine, Medical College of Liaodong University, Dandong, Liaoning, CHINA.
[2]The Second People's Hospital of Yichang, China Three Gorges University, Yichang, CHINA.
[3]Three Gorges University Hospital of Traditional Chinese Medicine & Yichang Hospital of Traditional Chinese, Yichang, Hubei, CHINA.
[#]*These authors contributed equally to this work.*

ABSTRACT

Aim/Background: To study the improvement effect of *Phellinus linteus* polysaccharides (Phps) on doxorubicin-induced myocarditis injury in mice. **Materials and Methods:** The mice were randomly divided into 5 groups, namely blank control group, model group, and Phps low-, medium-, high-dose groups. The model group and Phps groups were induced by doxorubicin to establish a myocardial injury model of mice. After 7 days, the physiological status of the mice was observed and the cardiac function indicators of each group (+dp/dtmax,-dp/dtmax,-dp/dtmax, BP, EF, FS, HR) were measured. The activities of myocardial enzymes (AST, CK, LDH) were detected using a fully automated biochemical analyzer. The levels of inflammatory factors (IL-6, IL-1 β, TNF-α) in myocardial tissue were detected with ELISA kits. The pathological changes in myocardial tissue were detected using HE staining method. The mRNA expression levels of PI3K, Akt, and mTOR in myocardial tissue were detected using Real-time PCR. **Results and Conclusion:** Compared with the blank control group, the model group mice showed poorer states, with significant reductions in blood pressure, ejection fraction, and heart rate ($p<0.05$), the serum myocardial enzyme activity and inflammatory factor levels were significantly increased ($p<0.05$), and myocardial cells were thickened and swollen and deformed. Compared with the model group, the above indicators in treatment groups were effectively improved ($p<0.05$). Phps may mainly inhibit myocarditis through the PI3K/Akt/mTOR signaling pathway, thus effectively improving doxorubicin-induced myocardial injury in mice.

Keywords: *Phellinus linteus* polysaccharides, Doxorubicin, Myocardial injury, Inflammatory reaction.

Correspondence:
Ms. Qingxian Mei
Attending Physician, Three Gorges University Hospital of Traditional Chinese Medicine & Yichang Hospital of Traditional Chinese, Yichang, Hubei, CHINA.
Email: 13217174427@163.com

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INTRODUCTION

Doxorubicin (Dox) is an anthracycline antineoplastic drug commonly used in the treatment of various solid tumors and malignant tumors. However, Dox exhibits dose-dependent cardiotoxicity, with a dose above 550 mg/m^2 increasing the incidence of heart failure.[1] Long-term use of Dox not only leads to adverse reactions such as myocardial damage and heart dysfunction in cancer patients,[2] but it can also cause irreversible congestive heart failure and significantly increase patient mortality, severely affecting patient prognosis and limiting its clinical application.[3,4] Currently, the only clinically approved .drug for mitigating doxorubicin-induced myocardial injury is dexrazoxane. Although it has some cardioprotective effects, its use can cause severe adverse reactions such as decreased appetite, nausea and vomiting, and abnormalities in liver and kidney function, and it is also expensive.[5] In search of more drugs to alleviate or treat doxorubicin-induced myocardial injury, researchers have conducted in-depth studies on its pathogenesis. Studies have found that inflammatory responses are one of the main factors in doxorubicin-induced myocardial injury.[6] Therefore, effectively controlling myocardial inflammatory responses is one of the important ways to prevent and treat doxorubicin-induced myocardial injury.

The *Phellinus linteus*, commonly known as "mulberry fungus," "tree chicken," "mulberry minister," or "monkey's eye," belongs to the *Polyporaceae* family.[7] It often parasitizes on broadleaf trees such as mulberry, poplar, willow, and birch. *Phellinus linteus* is a rare medicinal and edible fungus with a history of more than 2000 years, first recorded in "Shennong's Herbal Classic," and is known for its effects of promoting blood circulation to stop

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bleeding, nourishing yin and kidney, and softening hard masses.[8] *Phellinus linteus* Polysaccharides (Phps) constitute the primary active component, primarily in the form of polysaccharides, glycoproteins, and glycosides.[9] Research has demonstrated that Phps possess a multitude of pharmacological effects, including immune modulation, anti-tumor properties, anti-inflammatory, and antioxidant effects, among others.[10-12]

Many studies have reported the anti-inflammatory effects of PHPs, such as the significant inhibition of DSS induced pathological changes in mouse colon tissue by Phps, and the reduction of the expression of inflammatory factors IL-6, IL-1 β, TNF-α, and iNOS.[13] Phps (0-100 μg/mL) could also regulate the phosphorylation of PPARα and PPARγ, inhibit the MAPK pathway, and suppress the translocation of NF-κB and AP-1, thereby reducing the levels of inflammatory factor in LPS-induced RAW 264.7 macrophages.[14] Additionally, it was proved that Phps could upregulate the production of anti-inflammatory cytokines in LPS-stimulated RAW264.7 macrophages, while downregulating the production of pro-inflammatory cytokines.[15] These anti-inflammatory effects suggested that Phps may become a promising treatment for inflammatory diseases. Nevertheless, there is still a lack of research on the effect and mechanism of Phps on myocardial inflammatory injury. Therefore, this study attempted to use doxorubicin to establish a mouse model of myocardial injury, and evaluates the improving effects of Phps on myocardial inflammatory injury by detecting indicators such as cardiac function, myocardial enzyme activity, and inflammatory factor levels. Molecular biology methods were further used to verify its molecular mechanisms, providing a reference for the clinical prevention and treatment of doxorubicin-induced myocardial injury, and offering a scientific basis for the further development and utilization of Phps.

MATERIALS AND METHODS

Chemicals and Reagents

Phps was purchased from Shaanxi Baoyifeng Biotechnology (Shaanxi, China). Doxorubicin (D1515) was purchased from Sigma-Aldrich Co., Ltd., (Michigan, USA). Aspartate aminotransferase (WS-T2001) assay kit, Creatine (WS-T3005) kinase assay kit, and Lactate dehydrogenase (WS-T4002) assay kit were purchased from Beijing Wantai Drd Co., Ltd., Mouse IL-6 (mI098430) Elisa kit, Mouse IL-1β (ml106733) Elisa kit, and Mouse TNF-α (ml002095) Elisa kit were purchased from Shanghai Enzyme-linked Biotechnology Co., Ltd., Physiological saline was purchased from Shijiazhuang No.4 Pharmaceutical Co., Ltd., Polyformaldehyde (P1110) was purchased from Solarbio Technology Co., Ltd (Beijing, China). Isopropanol chloroform, Anhydrous ethanol (10009218), Trizol reagent were purchased from Sinopharm Chemical Reagent Co., Ltd., (Shanghai, China). SYBR Green PCR Master Mix detection kit (FP209) and FastReal qPCR PreMix (FP316) (SYBR Green) was purchased from Tiangen Biotech Co., Ltd., (Beijing, China). TransScript R First-Strand cDNA Synthesis SuperMix (AT311) was purchased from TransGen Biotech Co., Ltd., (Beijing, China).

Animal experiments

The SPF-grade male C57BL/6 mice were randomly divided into five groups: a blank control group, a model group, and Phps treatment (low, medium, high) groups, with 8 mice per group. The treatment groups were intubated with different concentrations of Phps (50, 100, 200 mg/kg), while the blank control group and the model group were intubated with the same dose of physiological saline, and this was continued for 14 days. On the 15th day, the model group and the treatment groups were intraperitoneally injected with 15 mg/kg of doxorubicin for model establishment.[16] On the 21st day, the mice were euthanized, and serum and heart tissues were collected and preserved for further use. All our operations adhered to animal welfare standards and received approval from the administration Committee of Experimental Animals, Liaoning Province, China.

Assessment of cardiac function parameters

Mice were anesthetized with isoflurane, and their positions were fixed. The cardiac function-related parameters of the mice in each group were then detected using a high-resolution small animal ultrasound system. These parameters included: the maximum rate of rise of left ventricular pressure during isovolumic contraction (+dp/dtmax), the maximum rate of decline of left ventricular pressure during isovolumic relaxation (-dp/dtmax), Blood Pressure (BP), Ejection Fraction (EF), Fractional Shortening (FS), and changes in Heart Rate (HR).

Determination of serum cardiac enzyme activity

After mice were anesthetized, blood is drawn from the heart. The plasma samples were isolated via centrifuging whole blood at 4^{o}C and 3500 r/min for 10 min, and the supernatant is collected. The activities of Aspartate Transaminase (AST), Creatine Kinase (CK), and Lactic Dehydrogenase (LDH) were then measured using a fully automated biochemical analyzer.

Histopathological examination of myocardial tissue

After euthanasia of mice, the hearts were carefully collected and rinsed several times with Phosphate-Buffered Saline (PBS). The hearts were then fixed in 4% paraformaldehyde. Following this, they underwent a gradient dehydration in alcohol, were embedded in paraffin, and sectioned. The sections were stained with Hematoxylin-Eosin (HE), and then examined under a microscope for changes in myocardial tissue such as degeneration, hypertrophy, and the extent of inflammatory infiltration. Photos were taken for documentation.

Quantitative real-time PCR analysis

Trizol was used to extract total RNA from cardiac tissue. After the concentration and integrity of the RNA were qualified, reverse transcription reaction was carried out (37°C for 15 min; 85°C for 5 sec) to synthesize cDNA. The cDNA served as a template for amplification (95°C for 1 min, 1 cycle; 95°C for 20 sec, 40 cycles; 60°C for 1 min, 40 cycles). The mRNA expression levels of the target genes in each group were analyzed using the $2^{-\Delta\Delta Ct}$ method to obtain the ratio between different groups. The primer sequence is in Table S1.

Data analysis

The experimental data were statistically analyzed using the SPSS (21.0) software. Quantitative data that conform to a normal distribution were expressed as the mean±standard deviation. Inter-group comparisons were made using one-way ANOVA, and a *p*-value less than 0.05 was considered to indicate statistically significant differences.

RESULTS

The effect of Phps on physiological condition of mice

Compared with the blank control group, the mice in the model group showed poor mental state, sluggish movement, reduced food intake, weight loss, and dull and lifeless fur. Compared with the model group, the symptoms of mice in the Phps group were significantly improved, not only did their body weight increase, but their mental state also showed a clear recovery.

The effect of Phps on cardiac function of mice

The impact of Phps on cardiac function in mice was investigated by examining various cardiac function indicators. Compared with the blank control group, the model group mice showed a significant decrease in +dp/dtmax, -dp/dtmax, EF, and FS levels ($p<0.001$, Figure 1a, d, c, f). BP and HR levels were also significantly reduced ($p<0.01$, Figure 1b, e), indicating that doxorubicin could significantly suppress cardiac function indicators in mice, and the model was successfully established. Compared with the model group, the Phps groups showed a significant increase in +dp/dtmax, -dp/dtmax, HR, and EF levels ($p<0.05$; Figure 1a, b, c, d), while BP and FS did not show significant changes (Figure 1e, f), indicating that Phps could significantly improve cardiac function indicators in mice, and showed a certain dose-dependent effect (Figure 1).

The effect of Phps on activity of myocardial enzymes (AST, CK, LDH) in mice

The activity of myocardial enzymes (AST, CK, LDH) in mice was investigated by detecting the changes in the activity of myocardial enzymes in the serum of mice in each group. Compared with the blank control group, the activity of myocardial enzymes (AST, CK, LDH) in the model group mice was significantly increased ($p<0.01$). Compared with the model group, the activity of AST in the medium and high dose groups were significantly reduced ($p<0.001$) (Figure 2), the activity of LDH in the low and high dose groups were significantly reduced ($p<0.001$), the activity of CK in the high dose group was significantly reduced ($p<0.05$) (Figure 2). The results indicated that doxorubicin could increase the activity of myocardial enzymes, causing myocardial damage and acute inflammatory responses. Phps could significantly reduce the

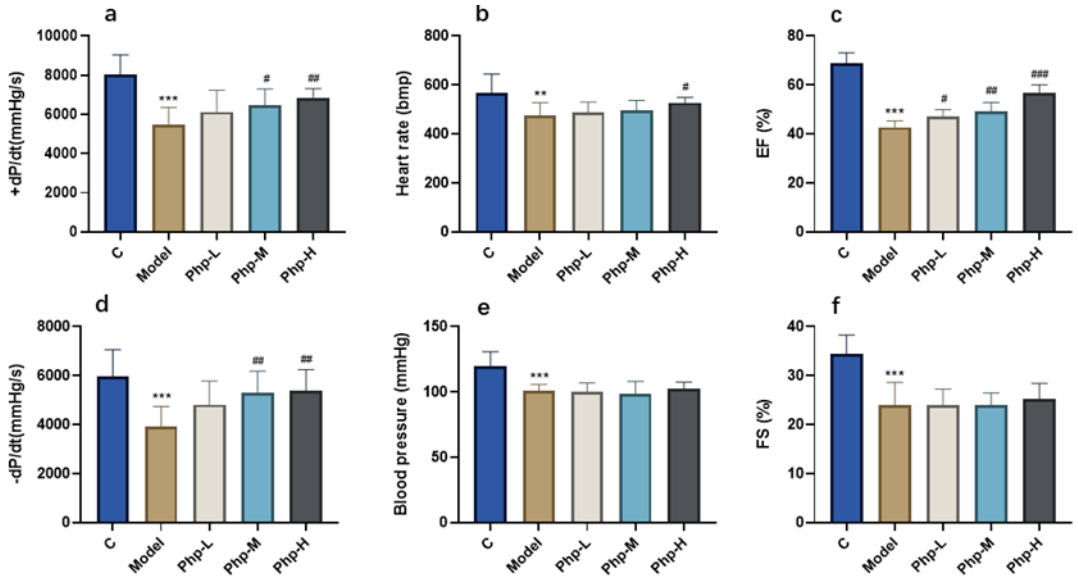

Figure 1: The effect of Phps on cardiac function of mice. (a) +dp/dtmax. (b) Heart rate (HR). (c) Ejection fraction (EF). (d) -dp/dtmax. (e) Blood Pressure (BP). (f) Fractional Shortening (FS). The values are expressed as the Mean±SD (n=8 per group). **$p<0.01$, ***$p<0.001$ vs. control group. #$p<0.05$, ##$p<0.01$, ###$p<0.001$ vs. model group.

activity of myocardial enzymes, restoring them to normal levels, and improve the myocardial damage caused by doxorubicin.

The effect of Phps on the levels of inflammatory factors (IL-1 β, IL-6, TNF - α) in mice

The levels of inflammatory factors (IL-1β, IL-6, TNF-α) in myocardial tissue was investigated by measuring the activity changes of mice in each group. Compared with the blank control group, the levels of IL-1β, IL-6, and TNF-α in the myocardial tissue of the model group mice were significantly increased (p<0.05). Compared with the model group, the levels of IL-1β in the low dose group was significantly reduced (p<0.05), the levels of IL-6 in the medium and high dose group were significantly reduced (p<0.01), and TNF-α in all treatment groups were significantly reduced (p<0.001) (Figure 3).

The effect of Phps on the pathological changes in mice

The pathological changes in myocardial tissue were examined through pathological staining results. The blank control group showed intact cardiac structure, with orderly arrangement of myocardial cells and clear myocardial texture, without any inflammatory cell infiltration or hyperplasia (Figure 4a). In contrast, the model group exhibited pathological changes such as degeneration of cytoplasmic granules in myocardial cells, inflammatory cell infiltration, vacuolation, and interstitial edema (Figure 4b). In the treatment group, the arrangement of myocardial tissue cells gradually became more orderly, the degree of damage improved, swelling and deformation were reduced, and inflammatory infiltration was less pronounced. The results indicated that Phps could significantly ameliorate myocardial tissue damage induced by doxorubicin (Figure 4).

The effect of Phps on the mRNA expression levels of PI3K, Akt, and mTOR in mice

As shown in Figure 5. Compared with the normal control group, the mRNA expression levels of *PI3K*, *Akt*, and *mTOR* in the model group were significantly increased (p<0.001). Compared with the model group, both medium and high doses groups significantly reduced the mRNA expression levels of *PI3K*, *Akt*, and *mTOR* (p<0.05 or p<0.01). There were no statistical differences in the low dose group, but there was a trend of downregulation. The results suggest that Phps may improve doxorubicin-induced myocardial injury in mice through the PI3K/Akt/mTOR signaling pathway.

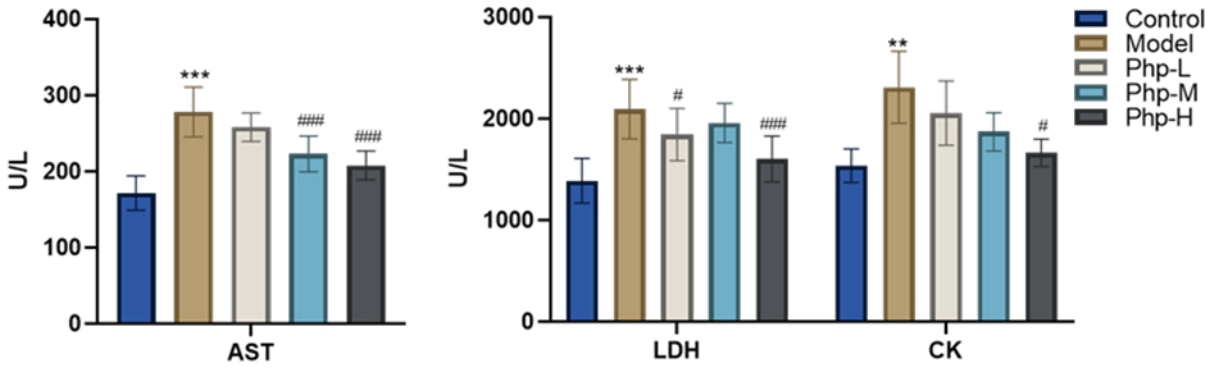

Figure 2: The effect of Phps on activity of myocardial enzymes (AST, CK, LDH) in mice. (a) AST. (b) LDH. (c) CK. The values are expressed as the Mean±SD (n=8 per group). **p<0.01, ***p<0.001 vs. control group. #p<0.05, ##p<0.01, ###p<0.001 vs. model group.

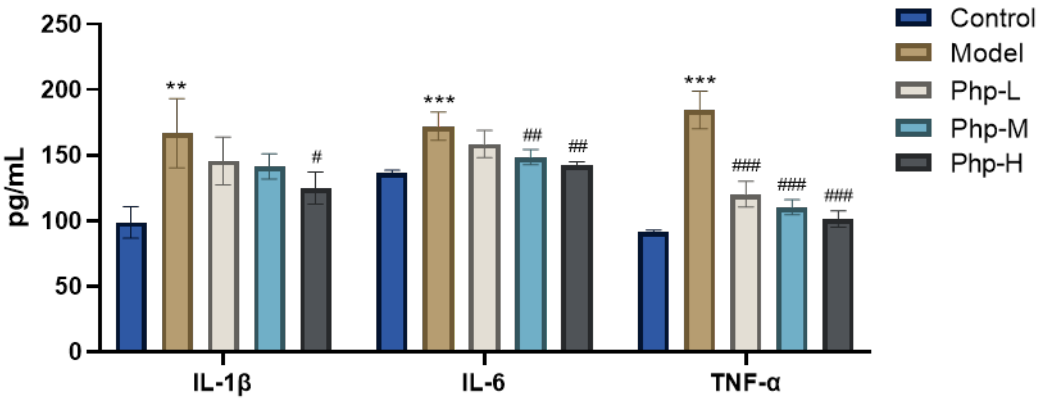

Figure 3: The effect of Phps on the levels of inflammatory factors in mice. (a) IL-1β. (b) IL-6. (c) TNF-α. The values are expressed as the Mean±SD (n=8 per group). **p<0.01, ***p<0.001 vs. control group. #p<0.05, ##p<0.01, ###p<0.001 vs. model group.

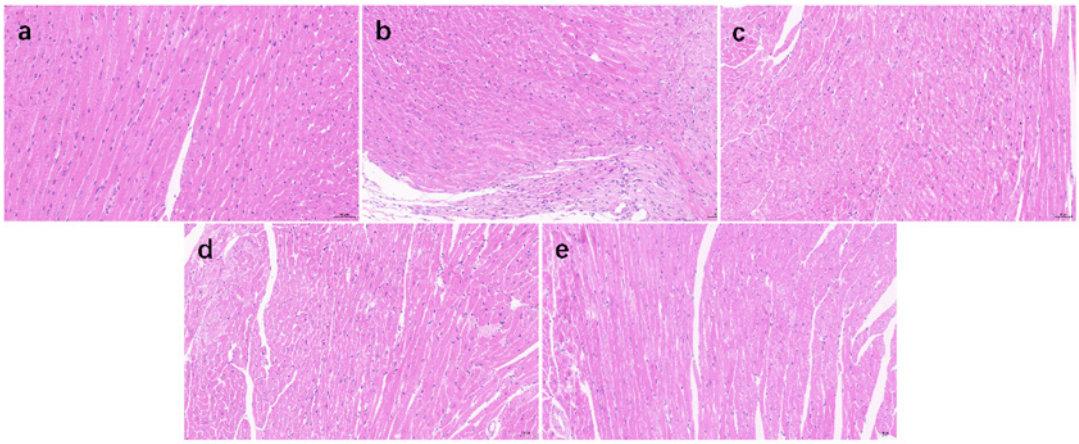

Figure 4: Pathological staining of myocardial tissue in each group of mice (× 200). (a) Blank control group, (b) Model group, (c) Low-dose group, (d) Medium dose group, (e) High-dose group.

DISCUSSION

Myocardial injury is relatively common among patients with cardiovascular diseases, meaning that the structure and function of the heart are compromised, and in severe cases, it can further progress to heart failure, even leading to patient death.[17] To delve into the pathological mechanisms of myocardial injury and seek its prevention and treatment methods, various myocardial injury animal models are applied in the basic and clinical research of cardiovascular diseases, such as: isoproterenol-induced myocardial injury, doxorubicin-induced myocardial injury, and myocardial ischemia-reperfusion injury.[18-20] Among them, the doxorubicin-induced myocardial injury method typically involves administering doxorubicin via intraperitoneal injection in rats and mice, with a usual dose of 2-8 mg/kg for rats and 4-20 mg/kg for mice.[21] Since myocardial injury induced by doxorubicin in rats and mice is highly similar to drug-induced heart failure in clinical settings, an increasing number of researchers are choosing to use doxorubicin to establish myocardial injury models.[22]

In this study, based on the successful establishment of a mouse model of myocardial injury, different concentrations of Phps were used to treat mice with myocardial injury to evaluate the impact of Phps on cardiac function in the myocardial injury model mice. Clinically, Heart Rate (HR), Left Ventricular Systolic Pressure (LVSP), Left Ventricular End-Diastolic Pressure (LVEDP), the maximum rate of pressure development in the left ventricular cavity (+dp/dtmax), and the maximum rate of pressure decline in the left ventricular cavity (-dp/dtmax) are important parameters for assessing cardiac function and cardiac health.[23] AST, CK, LDH, and other myocardial enzymes are enzymatic substances released into the bloodstream when myocardial cells are damaged or die, and are commonly used to diagnose heart diseases and monitor the extent of myocardial injury. AST is found in the myocardium, liver, kidneys, and other tissues, and its levels may increase when myocardial injury occurs.[24] CK is found in the myocardium, skeletal muscle, and other tissues, and the level of its CK-MB isoenzyme increases when myocardial injury occurs.[25] LDH is found in the myocardium and various other tissues, and its levels may increase when myocardial injury occurs.[26,27] By observing the physiological condition of mice and measuring cardiac function indicators and myocardial enzyme activities in each group of mice, it was found that Phps could significantly improve doxorubicin-induced myocardial cell injury, which was conducive to the recovery of cardiac function and myocardial enzyme activity in mice with myocardial injury. The results of HE histopathological staining further illustrated that doxorubicin severely damaged the structure of myocardial tissue in mice, while Phps could significantly improve the structural and functional damage to myocardial tissue caused by doxorubicin.

Doxorubicin-induced inflammation is a high-risk factor for the occurrence of heart failure, and drugs with anti-inflammatory properties may improve heart failure.[28,29] Previous studies have proven that under the stimulation of doxorubicin, the expression of inflammatory factors such as IL-8 and TNF-α in myocardial cells significantly increased, and NF-κB was overactivated.[30,31] Wang X *et al.*,[32] found that dihydrotanshinone I, which had a variety of anti-tumor and anti-inflammatory properties, improved doxorubicin-induced cardiotoxicity by inhibiting the activation of M1 macrophages and the over-release of pro-inflammatory cytokines both *in vitro* and *in vivo*. This study found that a large amount of inflammatory factors IL-6, IL-1β, and TNF-α were produced in the myocardial tissue of mice in the doxorubicin-induced model group, and the levels of inflammatory factors in the myocardial tissue of mice treated with Phps significantly decreased. These results were consistent with previous studies, indicating that Phps could improve doxorubicin-induced myocardial injury by inhibiting inflammatory responses and regulating the levels of inflammatory factors. However, the specific mechanism of action is still unclear.

Zhang X *et al.* found that Akt, as a downstream effector of PI3K, could play a crucial role in protecting myocardial tissue from injury by mediating downstream signaling pathways through

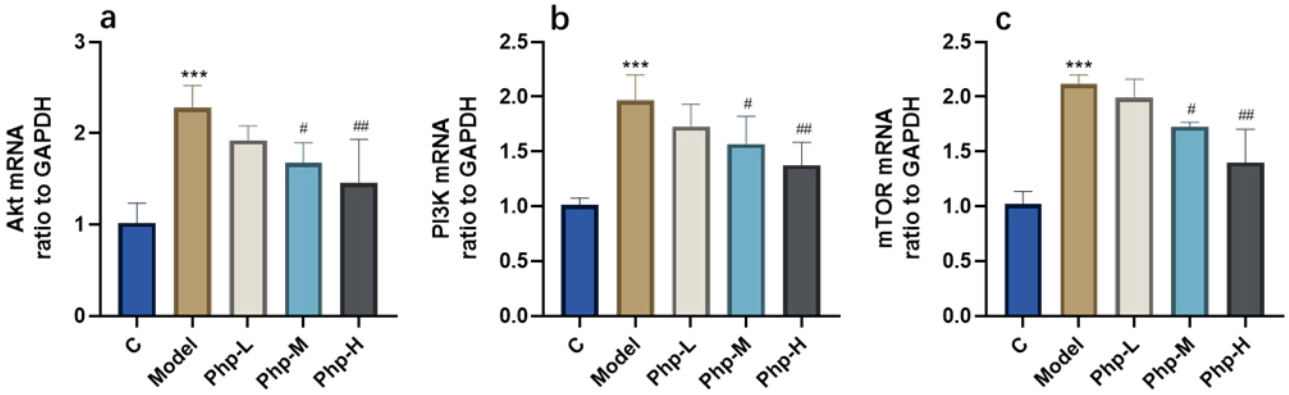

Figure 5: The effect of Phps on the mRNA expression levels of Akt, PI3K, and mTOR in mice. (a) Akt. (b) PI3K. (c) mTOR. The values are expressed as the Mean±SD (n=8 per group). ***p<0.001 vs. control group. #p<0.05, ##p<0.01 vs. model group.

phosphorylation.[33] Sun L *et al.* discovered that the activation of the PI3K/Akt signaling pathway could reverse the detrimental effects of the anticancer drugs lapatinib and doxorubicin on myocardial cells H9c2, further indicating that the activation of the myocardial inflammatory signaling pathway PI3K/Akt was closely related to doxorubicin-induced cardiotoxicity.[34] mTOR is a serine-threonine kinase that plays an important role in cell growth, proliferation, and regulation.[35] A substantial amount of research has proven that after the activation of PI3K/Akt, it could promote the phosphorylation of mTOR and play a significant protective role in various inflammatory diseases by regulating immune cells and the NF-κB pathway.[36] The activation of the PI3K/Akt/mTOR pathway could also alleviate myocardial ischemia-reperfusion injury and pathological myocardial hypertrophy by negatively regulating autophagy activity.[37,38] Combined with the experimental results of Real-time PCR, it further proved that the PI3K/Akt/mTOR signaling pathway might be a key pathway for Phps to improve myocardial inflammatory injury and protect myocardial cells. However, the specific mechanism of action is still unclear.

CONCLUSION

Phps may mainly inhibit myocarditis through the PI3K/Akt/mTOR signaling pathway, thus effectively improving doxorubicin-induced myocardial injury in mice.

ABBREVIATIONS

Phps: *Phellinus linteus* polysaccharides; **Dox:** Doxorubicin; **+dp/dtmax:** Maximum rate of rise of left ventricular pressure; **-dp/dtmax:** Maximum rate of decline of left ventricular pressure; **BP:** Blood pressure; **EF:** Ejection fraction; **FS:** Fractional shortening; **HR:** Heart rate; **AST:** Aspartate transaminase; **CK:** Creatine kinase; **LDH:** Lactate dehydrogenase; **PBS:** Phosphate-Buffered Saline; **HE:** Hematoxylin-Eosin; **PI3K:** Phosphoinositide 3-kinase; **AKT:** Protein kinase B; **mTOR:** Mammalian Target of Rapamycin; **IL-1β:** Interleukin-1 beta; **IL-6:** Interleukin-6; **TNF-α:** Tumor Necrosis Factor-alpha.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

FUNDING

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AUTHOR CONTRIBUTIONS

Conceptualization, X.R. and Q.M.; methodology, X.R.; software, Y.Z.; validation, Y.Z., L.J. and F.L.; formal analysis, Q. M.; investigation, Q. M.; resources, X.R.; data curation, X.R.; writing-original draft preparation, Q. M.; writing-review and editing, Q. M.; visualization, Y.Z.; supervision, J.W.; project administration, J. W.; funding acquisition, X.R. All authors have read and agreed to the published version of the manuscript.

SUMMARY

In summary, Phps could significantly improve doxorubicin-induced myocardial cell injury, which was conducive to the recovery of cardiac function and myocardial enzyme activity in mice with myocardial injury. And the PI3K/Akt/mTOR signaling pathway might be a key pathway for Phps to improve myocardial inflammatory injury and protect myocardial cells.

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Table S1: The primer sequence.

Gene	Primer Direction	Sequence (5′→3′)
PI3K	Forward	ATGGCGCTGAAGATCGTGAC
	Reverse	TCACGGTCCAGCTCCTTGAT
Akt	Forward	CCTGGACTACCTGCACTCGG
	Reverse	GGTGTCGATGCCATCAAAGG
mTOR	Forward	GAGCATCCGCACCAACTTCT
	Reverse	TCCAGCCACAGCCAAATGTA
GAPDH	Forward	GAAGGTGAAGGTCGGAGTCA
	Reverse	CATGGGTGGAATCATATTGGAA