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Qishen Yiqi Dropping Pills Ameliorates Doxorubicin-Induced Cardiotoxicity in Mice via Enhancement of Cardiac Angiogenesis

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Data Interpretation D
Manuscript Preparation E
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Background: Qishen Yiqi Dropping Pills (QYDP) is a Chinese traditional medicine that has been applied to treat coronary heart disease and ischemic heart failure in China. However, few studies have explored whether QYDP exerted an effect on doxorubicin (Doxo)-induced cardiotoxicity. Hence, in this study we investigated the effect of QYDP on cardiotoxicity induced by doxorubicin (Doxo) and its potential mechanism.





Material/Methods: Male C57BL/6 mice (20–25 g, 8–10 weeks old) were randomly assigned to 4 groups: Control group, QYDP group, Doxo group, and QYDP+Doxo group. The mice were intraperitoneal injected with Doxo weekly for 4 weeks to mimic the chronic toxicity. Four weeks after Doxo injection, echocardiography was applied to evaluate the left ventricular (LV) function, and the structure of the cardiac muscle fibers was analyzed with anti-actinin-2 antibody staining by immunofluorescence. Moreover, TUNEL staining and western blot analysis of Bax protein, Bcl-2 protein, and cleaved caspase-3 protein expression levels were conducted to explore whether QYDP exerted effect on cardiac apoptosis. In addition, Masson trichrome staining and western blot analysis of α -SMA protein expression levels were used to evaluate whether QYDP exerted an effect on cardiac fibrosis. Western blots and quantitative real-time polymerase chain reaction were applied to detect the vascular endothelial growth factor (VEGF) protein and mRNA levels in the myocardial tissue, and anti-CD31 antibody staining by immunohistochemistry was employed to explore whether QYDP exerted an effect on cardiac angiogenesis.

Results: QYDP effectively attenuated cardiac dysfunction and cardiac muscle fibers disruption in Doxo treated mice. Moreover, QYDP reduced myocardial apoptosis and myocardial fibrosis in Doxo treated mice, accompanied with elevated protein levels of VEGF and enhancement of myocardial microvessel density.

Conclusions: QYDP could protect against Doxo-induced cardiotoxicity, which may be closely associated with enhanced cardiac angiogenesis. Hence, QYDP could be a promising alternative for the treatment of Doxo-induced cardiotoxicity.

MeSH Keywords: **Angiogenesis Inducing Agents • Apoptosis • Doxorubicin • Fibrosis**

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Background

Qishen Yiqi Dropping Pills (QYDP) is a Chinese traditional medicine and it has been applied to treat coronary heart disease and ischemic heart failure in China [1,2]. However, few studies have explored whether QYDP exerted an effect on doxorubicin (Doxo)-induced cardiotoxicity. Some results showed that QYDP could effectively improve Doxo-induced cardiac dysfunction [3,4]. However, its mechanism remains inconclusive.

The anthracycline antibiotic Doxo is widely used to treat a variety of cancers, but its cumulative cardiotoxicity seriously limits its clinical use [5]. Doxo-induced cardiotoxicity often leads to irreversible cardiomyopathy and heart failure [5]. Many studies have been carried out to explore the potential mechanisms of its cardiac toxicity and some hypotheses have been proposed to explain its cardiotoxicity, such as oxidative stress [6] and topoisomerase 2-beta [7]. However, the pertinent interventions failed to prevent cardiac toxicity caused by Doxo [8].

Emerging evidence suggests that the disturbance of myocardial angiogenesis may be closely associated with the development of Doxo-induced cardiotoxicity [9–11]. Myocardial angiogenesis plays a pivotal role in maintain cardiac function [12]. Cardiac angiogenesis is mainly regulated by vascular endothelial growth factors (VEGFs). Overexpression of VEGF enhances cardiac function by increasing myocardial microvessel density while inhibiting VEGF signaling leads to impaired capillary density and results in cardiac dysfunction [12,13]. Interestingly, some studies reported that QYDP could promote cardiac angiogenesis through increasing VEGF expression level in heart [14,15].

Hence, we hypothesized that QYDP could ameliorate Doxo-induced cardiotoxicity through enhancing cardiac angiogenesis. The purpose of the present study was to explore the mechanisms of QYDP in the treatment of Doxo-induced cardiotoxicity in mice, which could provide a promising way for the treatment of Doxo-induced cardiotoxicity.

Material and Methods

Animals

Mice with C57BL/6 background (male, 20–25 g, 8–10 weeks old) were housed in the Laboratory Animal Center of Wuhan University of Science and Technology (WUST, Wuhan, China). The mice were raised for 1 week before the beginning of the experiment. Then, they were randomly assigned to 4 groups ($n \geq 6$ mice per group): Control group, QYDP group, Doxo group, and QYDP+Doxo group. The mice in Doxo group and QYDP+Doxo group were intraperitoneal injected with Doxo at a dose of 5 mg/kg weekly for 4 week as described previously [16],

and the Control group were subjected to saline. QYDP was obtained from Tasly Pharmaceutical Company Limited (Tianjin, China). It was dissolved in normal saline solution and intragastrically administered (35 mg per kg of body weight daily) in the QYDP and QYDP+Doxo groups. All experimental protocols were approved by the Animal Research Ethics Committee of WUST. All animals were sacrificed under anesthetization.

Echocardiography

After Doxo injections for 4 weeks, echocardiographic analysis was conducted, as described previously [17]. Briefly, the mice were tied under anesthetization, and their chest hairs were removed. Then, echocardiography was carried out using a VisualSonics Vevo1100 ultrasound machine (VisualSonics Inc., Toronto, Canada). And left ventricular (LV) internal diameter in diastole (LVlDd), LV internal diameter in systole (LVlDs), ejection fraction (EF), and fractional shortening (FS) were recorded.

Immunohistochemical staining

To assess cardiac angiogenesis, anti-CD31 antibody (ABclonal Biotech, 1: 100 dilution) staining by immunohistochemistry was used to display myocardial microvessel density, and the myocardial microvessel density was analysis using Image-Pro Plus 6. To determine cardiac apoptosis, TUNEL (terminal deoxynucleotidyl transferase dUTP nick end labeling) staining was carried out according to TACS TdT In Situ Apoptosis Detection Kit – DAB (R&D Systems, Minneapolis, MN, USA). For Masson staining, Masson staining kit (Jiancheng Bioengineering Institute, Nanjing, China) was applied to evaluate cardiac fibrosis following the manufacturer's guidance. Images were acquired under optical microscope (Nikon, Tokyo, Japan). In order to display the structure of the cardiac muscle fibers, anti-actinin-2 antibody (GeneTex Inc., 1: 100 dilution) staining by immunofluorescence were used. Briefly, primary antibody incubations were performed in heart sections overnight at 4°C. Subsequently, after 90-minute incubation with a secondary antibody, the nuclei were stained with DAPI solution for 5 minutes. Then, images were acquired under fluorescence microscope (Nikon, Tokyo, Japan).

For quantitative assessment of cardiac fibrosis, 5 randomly selected fields were collected under the microscope at 100× magnification. Then, LV fibrosis was evaluated in Image-Pro Plus 6 and the average ratio of stained fibrotic area to total ventricular area was calculated. The myocardial microvessel density were evaluated in 5 randomly selected microscopic fields under the microscope at 400× magnification. Then, the average ratio of CD31 positive cell to all area was calculated with Image-Pro Plus 6. Quantitative assessment of cardiac apoptosis was similar to CD31 staining, the average ratio of apoptotic cell stained with TUNEL to total ventricular area was calculated.

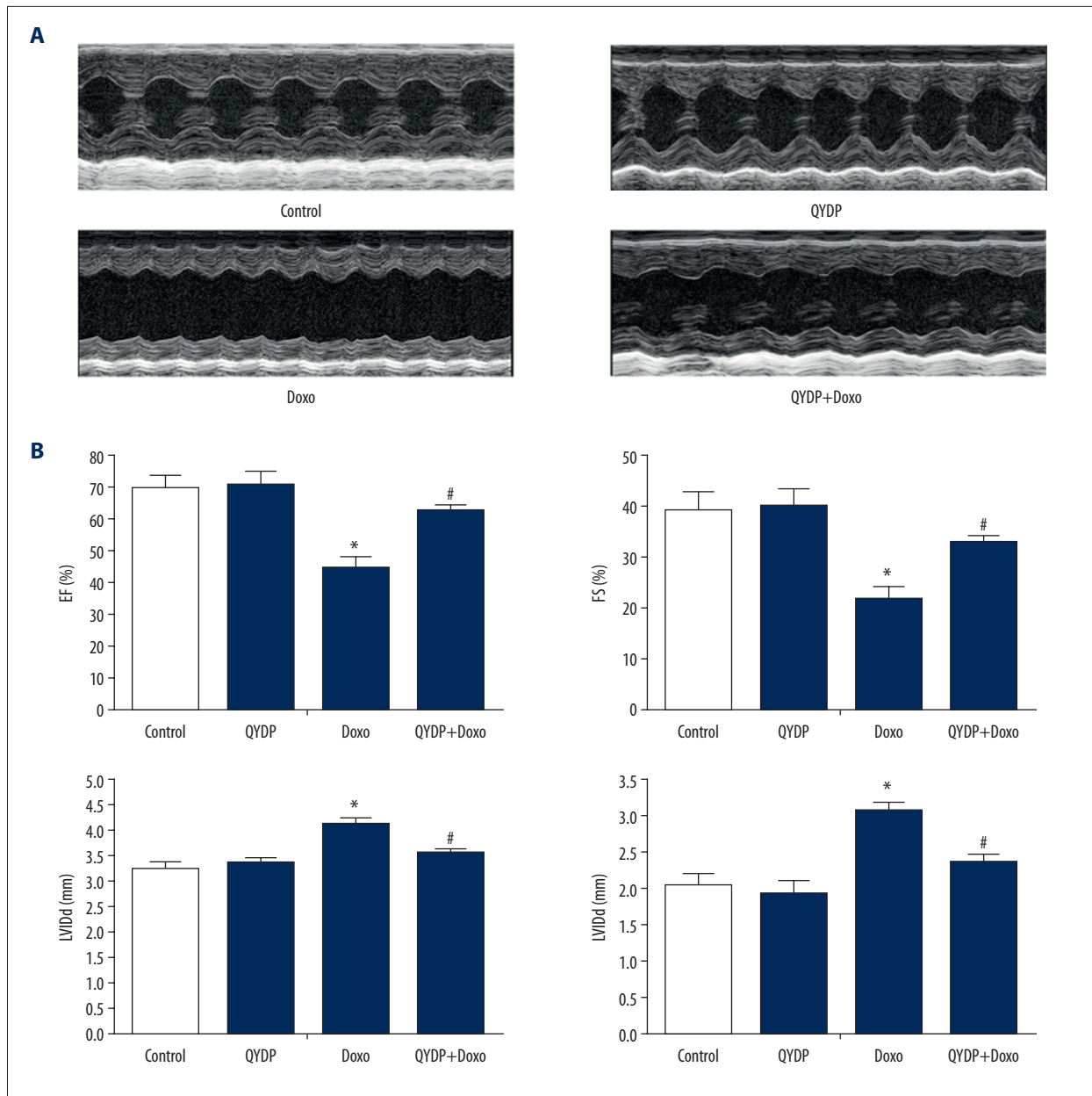


Figure 1. QYDP protected against cardiac dysfunction in Doxo treated mice. **(A)** Representative short-axis images from M-mode echocardiography in the Control group, QYDP group, Doxo group, and QYDP+Doxo group, which indicated that LV dilatation existed in Doxo group and that LV dilatation in Doxo group was improved after QYDP treatment. **(B)** Quantitative analysis of mice after Doxo injection for 4 weeks. All data are presented by means \pm SEM (n=7). * $P<0.05$ versus Control group; # $P<0.05$ versus Doxo group. QYDP – Qishen Yiqi Dropping Pills; Dox, – doxorubicin; LV – left ventricular; SEM – standard error of the mean.

Western blot analysis

Proteins were extracted from cardiac lysates as described previously [18]. Protein samples were subjected to SDS/PAGE, transferred onto a polyvinylidene fluoride membrane. The membranes were incubated in a 5% bovine serum albumin blocking buffer for 1 hour at room temperature and followed

by incubation with primary antibodies overnight. The antibodies were used as follows: rabbit anti-total-Akt (Cell signaling; 1: 1000 dilution); rabbit anti-total-eNOS (Affinity; 1: 1500 dilution); rabbit anti-phospho-Akt (Cell signaling; 1: 2000 dilution); rabbit anti-phospho-eNOS (Affinity; 1: 1000 dilution), rabbit anti-VEGF (Abcam 1: 1000; dilution), rabbit anti-cleaved caspase-3 (Cell signaling; 1: 1000 dilution), rabbit anti- α -SMA (Cell

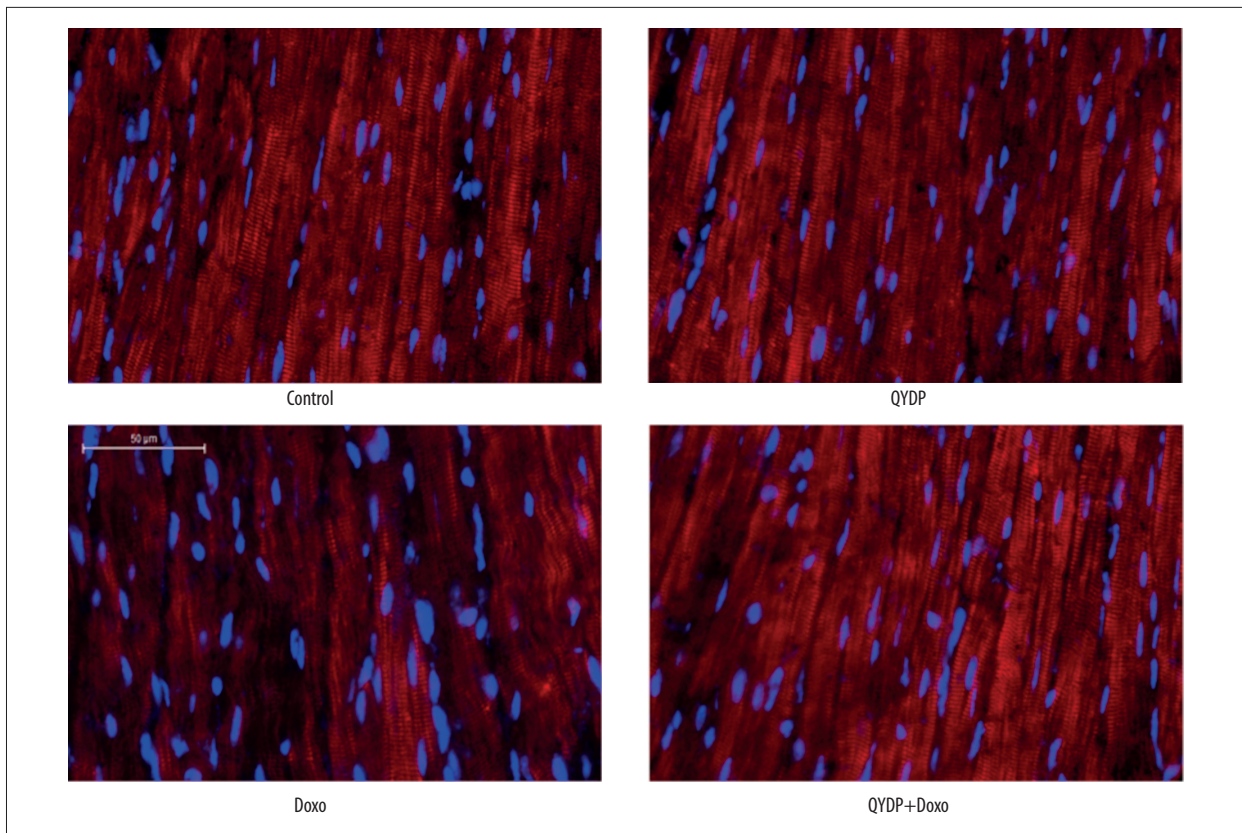


Figure 2. QYDP improved disrupted cardiac muscle fibers in Doxo treated mice. Representative images of anti-actinin-2 antibody staining in conjunction with Cy3-conjugated secondary antibody in heart sections. The Control group showed ordered structure of the cardiac muscle fibers while the Doxo group showed loss organization of cardiac muscle fibers. After QYDP treatment, cardiac muscle fibers disruption significantly improved in the Doxo group. QYDP – Qishen Yiqi Dropping Pills; Dox – doxorubicin.

signaling; 1: 1000 dilution). Then, membranes were incubated with secondary antibodies conjugated with the horseradish peroxidase for 1 hour at room temperature. Subsequently, the protein signal detection was carried out using chemiluminescence reagent and band intensity were analyzed by Image-Pro Plus 6.

Quantitative real-time polymerase chain reaction (qRT-PCR)

Total RNAs from the frozen hearts were extracted using the TRIzol reagent (Invitrogen) and cDNA was synthesized using the RT-PCR kit (Invitrogen). Then, the VEGF mRNA expression level was determined by SYBR Green (Applied Biosystems) qRT-PCR with a 7900HT RT-PCR System (Applied Biosystems). GAPDH was used as the endogenous control. The sequences of primers were as follows: 5'-TCGTCCAACCTCTGGGCTCTT-3' (VEGF forward) and 5'-CCCCTCTCTCTCTCTCTCT-3' (VEGF reverse); 5'-ATGGGTGTGAACCACGAGA-3' (GAPDH forward) and 5'-CAGGGATGATGTTCTGGGCA-3' (GAPDH reverse).

Statistical analysis

Data are expressed the mean \pm standard error of the mean (SEM). Data were analyzed with SPSS statistical software (version 19.0, SPSS Inc., Chicago, USA). The statistical analysis was performed using 2-way analysis of variance. The level of significance was considered at $P < 0.05$.

Results

QYDP attenuates cardiac dysfunction induced by Doxo

M-mode echocardiography was applied to evaluate the LV function and LV size in mice 4 weeks after Doxo injection. Figure 1A presents the representative short-axis images from M-mode echocardiography in the Control group, QYDP group, Doxo group, and QYDP+Doxo group, which indicated that LV dilatation existed in the Doxo group and that LV dilatation in the Doxo group was improved after QYDP treatment. Specifically, Figure 1B shows that EF and FS was significantly decreased

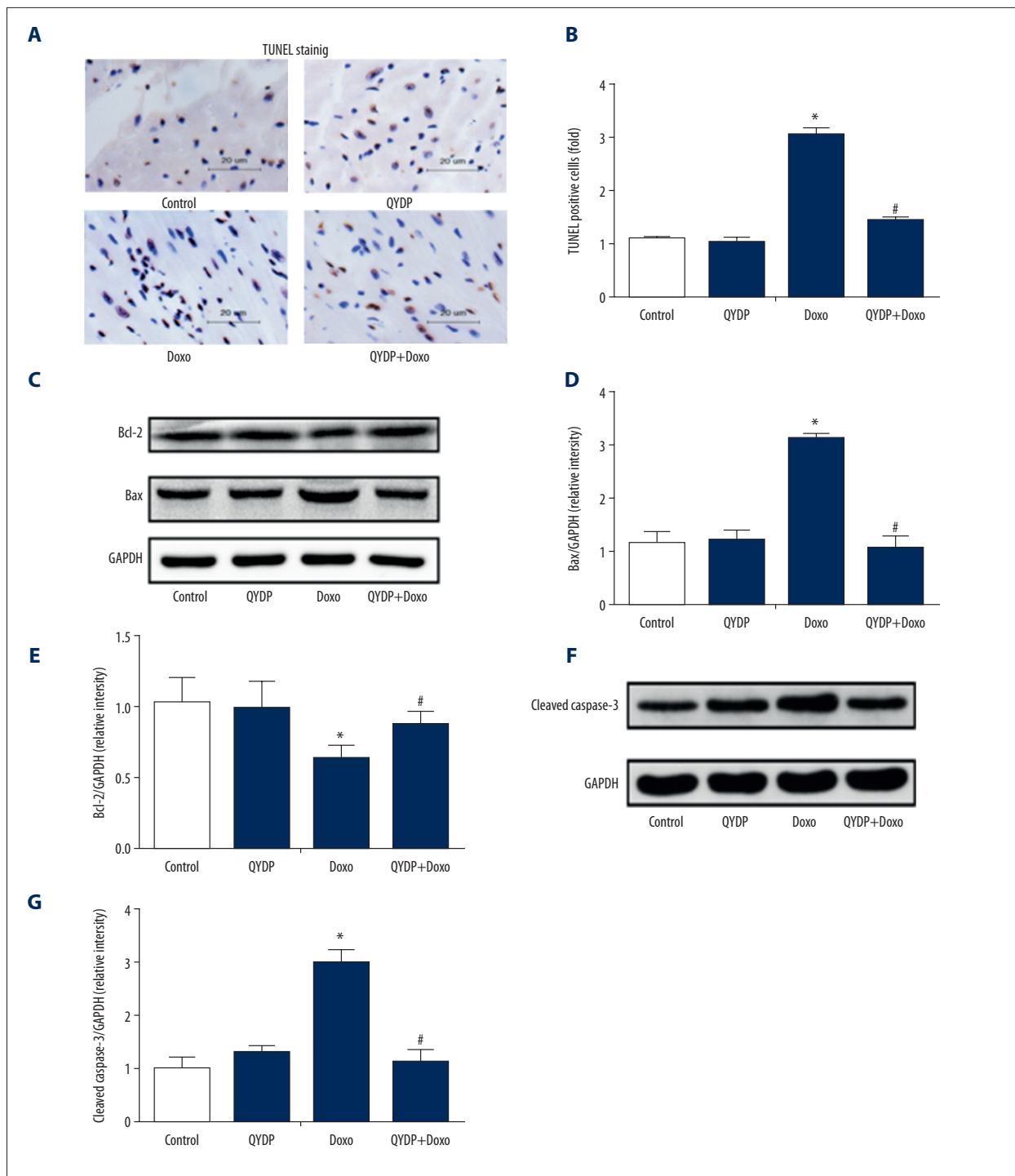


Figure 3. QYDP attenuated myocardial apoptosis in Doxo treated mice. **(A)** Representative images of TUNEL staining in myocardial sections. **(B)** Quantitation of TUNEL staining in myocardial sections (* $P < 0.05$ versus Control group; # $P < 0.05$ versus Doxo group); All data are presented by means \pm SEM (n=6). **(C)** Representative western blot image of Bax and Bcl-2 in myocardial tissue. **(D, E)** Quantitative analysis of Bax and Bcl-2 protein levels in myocardial tissue (* $P < 0.05$ versus Control group; # $P < 0.05$ versus Doxo group); **(F)** Representative western blot image of cleaved caspase-3 in myocardial tissue. **(G)** Quantitation of cleaved caspase-3 protein levels in myocardial tissue. (* $P < 0.05$ versus Control group; # $P < 0.05$ versus Doxo group); All data are presented by means \pm SEM (n=3). QYDP – Qishen Yiqi Dropping Pills; Dox – doxorubicin; TUNEL – terminal deoxynucleotidyl transferase dUTP nick end labeling; SEM – standard error of the mean.

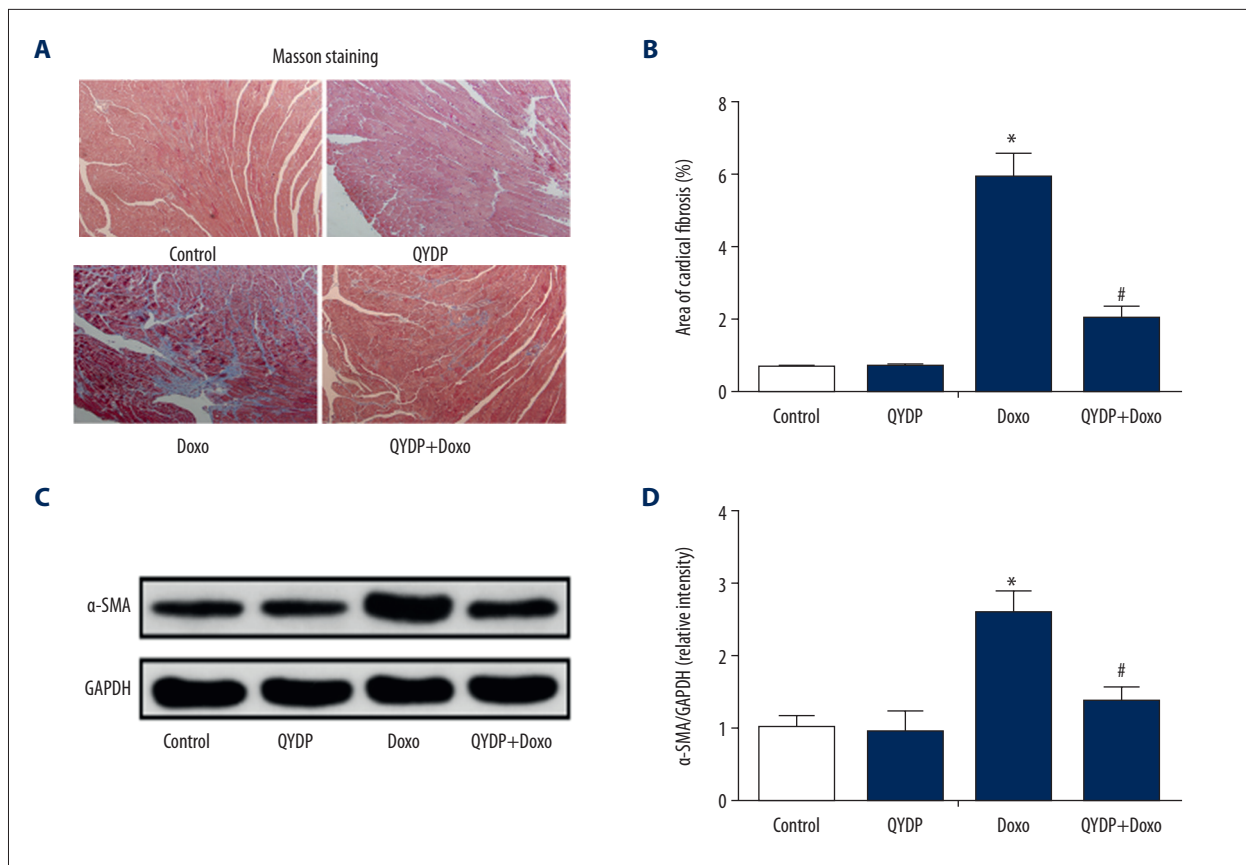


Figure 4. QYDP reduced myocardial fibrosis in Doxo treated mice. **(A)** Representative images of Masson trichrome staining in myocardial sections. Blue fibers indicate cardiac fibrosis. **(B)** Quantitation of cardiac fibrosis in the Control group, QYDP group, Doxo group, and QYDP+Doxo group. * $P < 0.05$ versus Control group; # $P < 0.05$ versus Doxo group; **(C)** Representative western blot image of α -SMA in myocardial tissue. **(D)** Quantitation of α -SMA protein levels in myocardial tissue. (* $P < 0.05$ versus Control group; # $P < 0.05$ versus Doxo group); All data are presented by means \pm SEM (n=6). QYDP – Qishen Yiqi Dropping Pills; Dox – doxorubicin; SEM – standard error of the mean.

in the Doxo group, and LVIDd and LVIDs was also significantly increased in the Doxo group, which suggested that the mice in the Doxo group developed heart failure after Doxo injection. After QYDP treatment, EF and FS, as well as LVIDd and LVIDs, significantly improved in the Doxo group. Heart rate in the 4 groups was not significantly different. Echocardiographic analysis suggested that QYDP protected against cardiac dysfunction induced by Doxo.

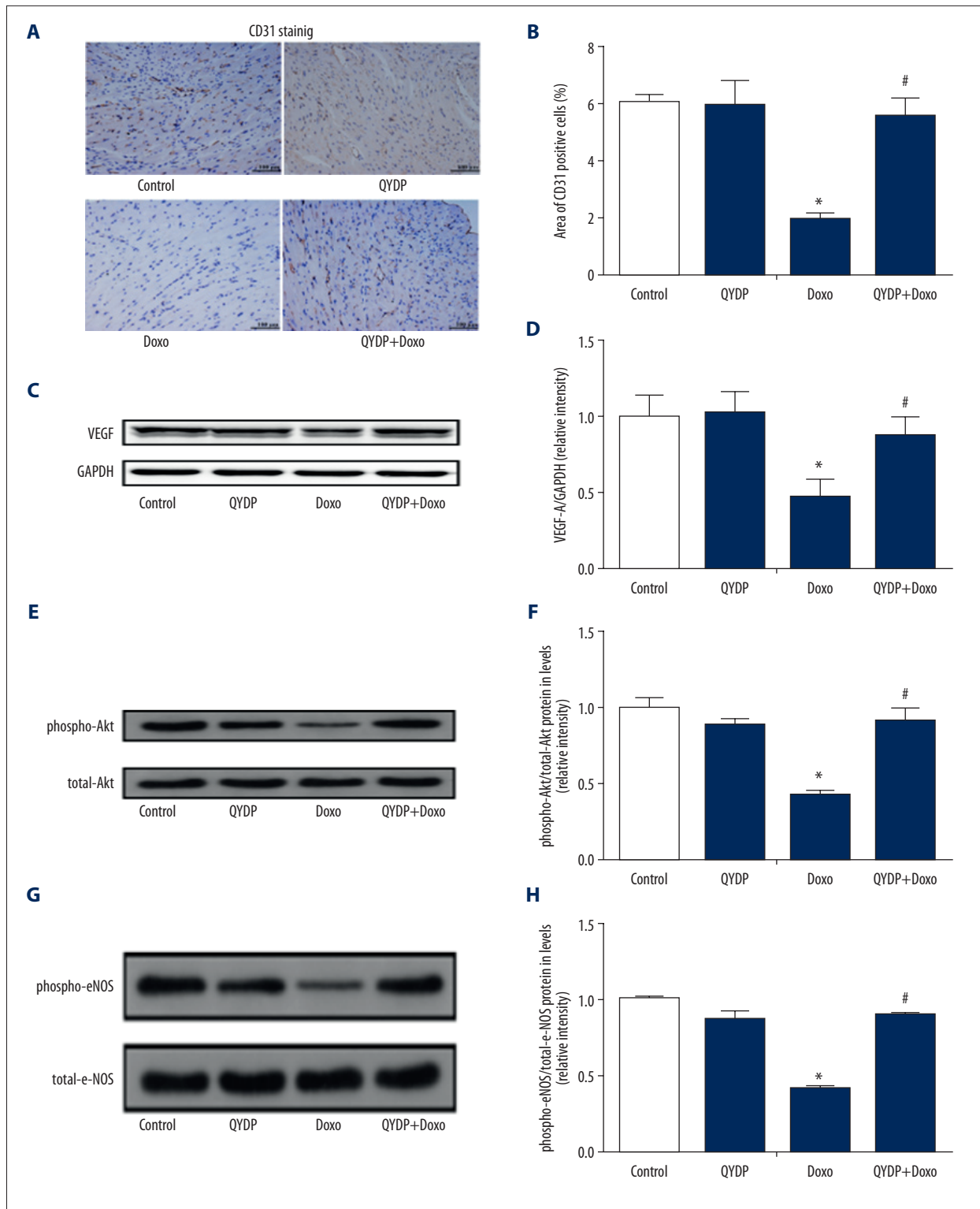
QYDP improves disrupted cardiac muscle fibers in Doxo treated mice

In order to further explore the effect of QYDP on the organization of cardiac muscle fibers in Doxo treated mice, anti-actinin-2 antibody staining by immunofluorescence was carried out to display the structure of cardiac muscle fibers. In Figure 2, the Control group showed ordered structure of cardiac muscle fibers while the Dox group showed loss organization of cardiac

muscle fibers. After QYDP treatment, cardiac muscle fibers disruption significantly improved in the Doxo group.

QYDP attenuates cardiac apoptosis in Doxo treated mice

To assess whether QYDP exerts effect on myocardial apoptosis, myocardial tissue was stained with TUNEL. The control group existed extremely few apoptotic cell stained with TUNEL. In contrast, apoptotic cell stained with TUNEL in the Doxo group was significantly increased, which suggested obvious cardiac apoptosis. However, treatment with QYDP significantly decreased the apoptotic cell stained with TUNEL in the Doxo group (Figure 3A, 3B). Additionally, western blotting was applied to detect the Bax protein, Bcl-2 protein, and cleaved caspase-3 protein expression levels. Figure 3C–3G shows that, compared with the Control group, the Bax and cleaved caspase-3 expression levels in Doxo group was higher and the Bcl-2 expression levels was lower, thereby suggesting increased cardiac apoptosis. However, QYDP treatment significantly decreased Bax



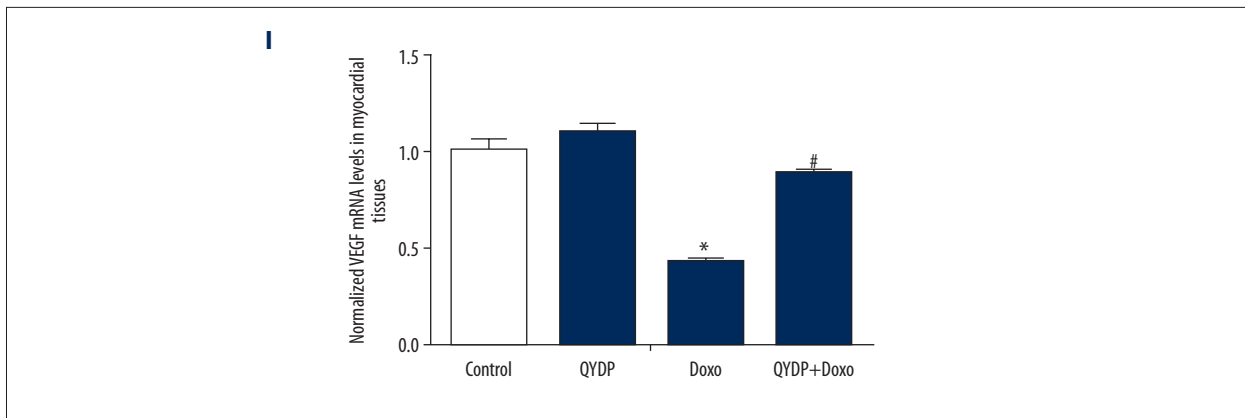


Figure 5. QYDP promote cardiac angiogenesis in Doxo treated mice. (A) Representative images of anti-CD31 antibody staining by immunohistochemistry in myocardial sections (scale bar, 100 μ m). (B) Quantitative analysis of CD31 expression level in myocardial sections (* $P < 0.05$ versus Control group; # $P < 0.05$ versus Doxo group); All data are presented by means \pm SEM ($n \geq 4$). (C) Representative western blot image of VEGF in myocardial tissue. (D) Quantitation of VEGF protein levels in myocardial tissue. (* $P < 0.05$ versus Control group; # $P < 0.05$ versus Doxo group); All data are presented by means \pm SEM ($n = 3$). (E) Representative western blot image of total-Akt and phospho-Akt in myocardial tissue. (F) Quantitation of total-Akt and phospho-Akt protein levels in myocardial tissue. (G) Representative western blot image of total-eNOS and phospho-eNOS in myocardial tissue. (H) Quantitation of total-eNOS and phospho-eNOS protein levels in myocardial tissue. * $P < 0.05$ versus Control group; # $P < 0.05$ versus Doxo group; (I) Quantitation real-time PCR for VEGF mRNA expression level in the heart; (* $P < 0.05$ versus Control group; # $P < 0.05$ versus Doxo group); All data are presented by means \pm SEM ($n = 3$). QYDP – Qishen Yiqi Dropping Pills; Dox – doxorubicin; SEM – standard error of the mean; VEGF – vascular endothelial growth factor; PCR – polymerase chain reaction.

protein and cleaved caspase-3 protein expression levels and increased Bcl-2 protein expression levels, thereby suggesting decreased myocardial apoptosis.

QYDP reduces cardiac fibrosis in Doxo treated mice

Masson staining in heart sections was carried out to whether QYDP exerts effect on myocardial fibrosis. As shown in Figure 4A and 4B, the Control group existed extremely few blue fibers, which suggested insignificant cardiac fibrosis. Conversely, a statistically significant increase of blue fiber was observed in the Doxo group, thereby suggesting apparent cardiac fibrosis. However, after QYDP treatment, the blue fiber in the Doxo group remarkably decreased. Besides, Figure 4C and 4D shows that the protein expression levels of α -SMA in the Control group, a biomarker for myocardial fibrosis, was increased in Doxo group, which suggested increased cardiac fibrosis. However, QYDP treatment significantly decreased α -SMA protein expression levels, thereby suggesting decreased myocardial fibrosis.

QYDP promote cardiac angiogenesis in Doxo treated mice

To investigate the effect of QYDP on myocardial microvessel, anti-CD31 antibody staining by immunohistochemistry was carried out. Figure 5A and 5B shows that the CD31 expression level in myocardial tissue, a critical indicator for evaluating myocardial microvessel density, was remarkably decreased in the Doxo group. After treatment with QYDP, the expression

of CD31 was significantly increased, which suggested that QYDP attenuated the reduction in myocardial capillary density induced by Doxo.

In additionally, compared with the Control group, a significant decrease of VEGF protein expression levels was found in the Doxo group; whereas QYDP treatment remarkably elevated protein levels of VEGF in Doxo treated mice (Figure 5C, 5D). Similar results were determined by qRT-PCR (Figure 5I). Interestingly, Figure 5E–5H show that compared with the Control group, the ratio of phospho-Akt/total-Akt and phospho-eNOS/total-eNOS in the Doxo group significantly decreased, thereby suggesting reduced activation of Akt and eNOS. Surprisingly, QYDP treatment significantly increased the Akt and eNOS activation.

These results suggested that compared with the Control group, the VEGF-induced activation of Akt/eNOS signaling pathway was significantly suppressed in the Doxo group, and QYDP treatment promote VEGF-induced activation of Akt/eNOS signaling pathway.

Discussion

The present study aimed to investigate the role of QYDP on cardiotoxicity in Doxo treated mice and its potential mechanism. Our study found that QYDP treatment improved cardiac dysfunction and cardiac muscle fibers disruption induced by Doxo.

Furthermore, QYDP effectively alleviated myocardial apoptosis and myocardial fibrosis in Doxo treated mice. Additionally, we also observed that QYDP treatment increase VEGF protein levels and myocardial microvessels density in Doxo treated mice.

In our study, M-mode echocardiography was carried out to assess LV function and anti-actinin-2 antibody staining by immunofluorescence was applied to show the structure of cardiac muscle fibers. Previous studies reported that QYDP attenuate cardiac dysfunction in Doxo-induced cardiomyopathy, which could be attributed to reduction of cardiac apoptosis [3]. Besides, QYDP was also reported to significantly improve Doxo-induced cardiac muscle fibers disruption and its effect might be attributed to the elevation in myocardial ATP content [4]. Consistent with these studies, our results indicated that QYDP could protected against cardiac dysfunction and cardiac muscle fibers disruption. However, its probable mechanism may be related to enhancement of myocardial microvessel density, which few studies have reported.

Moreover, myocardial apoptosis and myocardial fibrosis were detected to assess the cardiotoxicity induced by Doxo in mice. Previous studies reported that QYDP attenuate cardiac dysfunction in Doxo-induced cardiomyopathy, and the potential mechanism could be reduction of cardiac apoptosis [3]. Consistent with these studies, we also observed that QYDP could effectively attenuate Doxo-induced cardiac apoptosis. However, we also found that QYDP could alleviate Doxo-induced cardiac fibrosis, which few studies have reported.

Considering that cardiac angiogenesis plays a critical role in the development of heart failure [19,20], we also investigate whether QYDP exerts effect on QYDP on myocardial angiogenesis in heart failure induced by Doxo. Few studies reported whether QYDP exerts effect on myocardial angiogenesis in heart failure induced by Doxo. Our study found that QYDP treatment significantly ameliorate myocardial apoptosis in

Doxo treated mice, accompanied with increased VEGF protein levels and myocardial microvessel density. These results suggested that the probable mechanism of QYDP in protecting against the Doxo-induced cardiotoxicity might be closely associated with enhanced myocardial angiogenesis. Furthermore, we also observed that the activation of Akt/eNOS in the Doxo group was significantly suppressed compared with the Control group. Interestingly, the activation of Akt/eNOS was remarkably elevated in Doxo group after QYDP treatment. These results suggested that the probable mechanism of QYDP in enhancing myocardial angiogenesis might be closely associated with the VEGF-induced activation of Akt/eNOS signaling pathway in the heart.

Our research comprehensively explored the cardioprotective effects of QYDP on the cardiotoxicity induced by Doxo. Furthermore, our study simultaneously explored the probable mechanism. However, the association between enhanced cardiac angiogenesis and the cardioprotective role of QYDP on cardiotoxicity induced by Doxo is extremely weak, and the association between VEGF-induced activation of Akt/eNOS and proangiogenic effects of QYDP on Doxo-induced heart failure is also extremely weak. Therefore, more rigorous studies should be conducted before convincing conclusions can be drawn.

Conclusions

In general, our results indicated that QYDP could alleviate cardiotoxicity in Doxo treated mice, and the probable mechanism may be closely associated with enhanced myocardial angiogenesis by QYDP. Hence, treatment with QYDP could be a promising treatment for Doxo-induced cardiotoxicity.

Conflicts of interest

None.

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