Technology and Health Care 29 (2021) S195–S209
DOI 10.3233/THC-218020
IOS Press

H_2S improves doxorubicin-induced myocardial fibrosis by inhibiting oxidative stress and apoptosis via Keap1-Nrf2

Yaling Li[a,1], Thakur Prakash Chandra[a,1], Xiong Song[a,1], Liangui Nie[a], Maojun Liu[a], Jiali Yi[a], Xia Zheng[a], Chun Chu[b,*] and Jun Yang[a,*]
[a]*Department of Cardiology, The First Affiliated Hospital of University of South China, Hengyang, Hunan 421001, China*
[b]*Department of Pharmacy, The Second Affiliated Hospital of University of South China, Hengyang, Hunan 421001, China*

Abstract.
OBJECTIVE: We waimed to investigate whether H_2S can relieve the myocardial fibrosis caused by doxorubicin through Keap1-Nrf2.
METHODS: Sprague-Dawley (SD) rats were randomly divided into four groups: normal control group (Control); DOX model group (DOX); H_2S intervention model group (DOX+H_2S); H_2S control group (H_2S). DOX and DOX+H_2S group were injected with doxorubicin (3.0 mg/kg/time) intraperitoneally. Both of the Control group and H_2S groups were given normal saline in equal volume, 2 weeks later, DOX+H_2S and H_2S group were controlled with NaHS (56 μmol/kg/d) through the abdominal cavity, while the Control and DOX group were injected with normal saline of the same dosage intraperitoneally.
RESULTS: Myocardial injury and myocardial cell apoptosis were significantly increased, the H_2S content in myocardial tissue was remarkably down-regulated, the expression levels of MDA, Keap1, caspase-3, caspase-9, TNF-α, IL1β, MMPs and TIMP-1 in rat myocardial tissue was significantly up-regulated ($P < 0.05$), and the expression levels of GSH, NQO1, Bcl-2 were down-regulated compared with those of control group. The above results can be reversed by the DOX+H_2S group. There is no statistically significant difference between the Control group and the H_2S control group.
CONCLUSIONS: These results suggest that H_2S can improve DOX-induced myocardial fibrosis in rats, and the keap1/Nrf2 signaling pathway, oxidative stress, inflammation, and apoptosis may be involved in the mechanism.

Keywords: Hydrogen sulfide, doxorubicin, myocardial fibrosis, keap1/Nrf2, apoptosis

1. Introduction

Doxorubicin is the first-choice anti-tumor chemotherapy drug for various malignant tumors (e.g., acute leukemia, lymphoma, sarcoma, and breast cancer) as effectively improving patients' survival rate. Yet doxorubicin may also lead to cardiomyopathy and congestive heart failure (CHF) in a dose-dependent manner. As statistics suggest, nearly 10% of patients would develop cardiomyopathy after being treated

[1]Yaling Li, Thakur Prakash Chandra and Xiong Song contributed equally to this work.
*Corresponding authors: Chun Chu, Department of Pharmacy, The Second Affiliated Hospital of University of South China, Hengyang, Hunan 421001, China. E-mail: yjchuchun@163.com. Jun Yang, Department of Cardiology, The First Affiliated Hospital of University of South China, Hengyang, Hunan 421001, China. E-mail: yangjunincn@163.com.

0928-7329 © 2021 – The authors. Published by IOS Press. This is an Open Access article distributed under the terms of the Creative Commons Attribution-NonCommercial License (CC BY-NC 4.0).

with doxorubicin. The use of doxorubicin will lead to obvious cardiotoxicity, to a certain degree, as well as limiting its clinical use and curative benefits. Furthermore, the clinical manifestations of doxorubicin-induced cardiomyopathy in the animal model are very similar to those of dilated cardiomyopathy (DCM) in human beings as well. Thus, it has become the most commonly used and most classic animal model in DCM-related studies [1,2]. As a type of heterogeneous cardiomyopathy characterized by systolic dysfunction in the left ventricle or both ventricles, DCM is considered the leading cause of sudden cardiac death and cardiac failure [3,4]. The pathogeneses of DCM and doxorubicin-induced cardiomyopathy still need further studies. Yet it has been found that myocardial fibrosis and the resulting myocardial remodeling are the key links in the occurrence and development of cardiomyopathy and cardiac failure. Accordingly, it is of great scientific significance and necessary to discuss the internal mechanism of myocardial fibrosis induced by doxorubicin.

The clinical pathological change of doxorubicin-induced myocardial fibrosis is very similar to DCM. It has now been found that oxidative stress and cell apoptosis are the major mechanisms that induce myocardial injury and myocardial remodeling. Oxidative stress means that excessive generation of reactive oxygen species (ROS) causes damage to tissue cells [5]. ROS may trigger an inflammatory response and lead to cell apoptosis. Also, it may lead to lipid peroxidation, thereby up-regulating malondialdehyde (MDA) expression. The current study has suggested that oxidative stress, inflammatory response, and cell apoptosis are closely correlated with the mechanism of doxorubicin-induced myocardial fibrosis and DCM [6–8].

A Keap1-Nrf2 signaling pathway is a signaling pathway that has been found resistant to oxidative damage in and outside the organism recently. It is critical for anti-oxidative stress, anti-apoptosis, anti-inflammatory response, anti-tumor, and anti-atherosclerosis [9–11]. Hydrogen sulfide (H_2S) is a newly-found thirdly endogenous gaseous signal molecule after NO and CO. As an endogenous antioxidant, H_2S is capable of exerting numerous biological effects such as anti-oxidative stress, anti-apoptosis, anti-inflammation and anti-endoplasmic reticulum stress [12]. The role of gaseous signal molecule H_2S in doxorubicin-induced myocardial fibrosis in rats and its internal mechanism has been rarely studied. Some studies have revealed that H_2S can relieve doxorubicin-induced myocardial damage, thereby improving the cardiac function of doxorubicin-induced myocardial fibrosis in rats [13,14]. Thus, we assume that H_2S may improve doxorubicin-induced myocardial fibrosis in rats, and the relevant mechanism may be correlated with the inhibition of oxidative stress, inflammatory response, and apoptosis by regulating the Keap1-Nrf2 signaling pathway.

This study aimed to preliminarily discuss the potential mechanism of doxorubicin-induced myocardial damage and myocardial fibrosis and to investigate the effect of H_2S in doxorubicin-induced myocardial fibrosis in rats by establishing a model of rats with doxorubicin-induced myocardial fibrosis. The present study is expected to provide experimental evidence for the in-depth understanding of doxorubicin-induced cardiotoxicity and the pathogenesis of DCM and a new target for preventing and treating doxorubicin-induced myocardial cardiomyopathy and DCM.

2. Experiment material

2.1. Experiments with animals

Forty male adult SD rats, weighing 200 ± 20 g, purchased from Changsha Lake Animal Experimental Center, kept in a constant environment at a suitable temperature (22 ± 2°C), 12 h day and night, allowed to eat freely, with drinking water adapted for 1 wk.

2.2. Main experimental reagents

Doxorubicin hydrochloride was purchased from Meilun Biotech Co., Ltd., China; TGF-β (BA0290), CSE (A04496), MMP8 (BM4427), MMP16 (A05065), TIMP1 (A00561), Collagen III (A00788), and primary antibody, anti-rabbit secondary antibody were purchased from Boster Company, China; TNF-α (17590-1-AP), Keap1 (10503-2-AP), caspase-3 (19677-1-AP), Caspase-9 (10380-1-AP), Bax (50599-2-Ig), IL1β (16806-1-AP), IL6 (21865-1-AP), COX2 (66351-1-Ig), Nrf2 (16396-1-AP), NQO1 (11451-1-AP), HO-1 (10701-1-AP), GCLC (12601-1-AP) and Bcl-2 (12789-1-AP) were purchased from Proteintech; These antibodies are diluted 1:1000. BCA protein quantification kit and cell lysate were purchased from Biyuntian Company, China; sodium hydrosulfide (the H2S donor) was purchased from Sigma, USA; the H2S test kit was purchased from Mlbio; the Masson staining kit was purchased from Maixin Company, China; the MDA (ab118970), GSH (ab138881) and SOD (ab65354) test kits were purchased from Abcam, China.

3. Experiment methods

3.1. Establishing the animal models and grouping

After 1 week of adaptive feeding at a constant temperature, 40 rats were randomly stratified into Control group, DOX group, DOX+H_2S group and H_2S group, with 10 rats in each group. Rats in the DOX group and DOX+H_2S group was injected doxorubicin (3 mg/kg/time, diluted with physiological saline to 0.5 mg/ml, freshly prepared before use) [15,16] intraperitoneally, 3 times a week for 2 weeks. The DOX+H_2S and the H_2S group were injected with NaHS solution (56 μmol/kg/d) intraperitoneally. The Control and the DOX group were injected with the same dose of normal saline intraperitoneally.

3.2. Echocardiographic detection

After 8 weeks, all rats were examined using the ultrasound instrument probe to measure the left ventricular long-axis view of the sternum at the level of the long-axis papillary muscles. We analyzed and measured the parameters of three consecutive cardiac cycles. The average value was calculated and recorded: left ventricular end-diastolic Internal diameter (LVEDD), left ventricular end-systolic diameter (LVESD), left ventricular ejection fraction (LVEF), left ventricular short-axis shortening rate (LVFS).

3.3. Calculating cardiac body mass index

All rats were weighed before sacrifice. All animals were anesthetized with chloral hydrate (350 mg/kg). The heart was washed out with saline and then washed with filter paper. Next, the heart weight was weighed. The heart weight index (heart weight/body weight, HW/BW, mg/g) was calculated.

3.4. Masson staining

We observed the deposition of collagen in the myocardial tissue of each group. Samples of heart tissue fixed with formaldehyde were collected, then impregnated with 70% and 90% alcohol until the water in the tissue was removed, then transparent with xylene for about 1 hour, and then paraffin was added around the myocardial tissue until the tissue was completely immersed, cooled and embedded for

0.5 hour. Then the wax block was sliced under a slicer, the thickness of the slice was about 4 μm, then dewaxed to hydration, and stained with hematoxylin dye for about 5–10 minutes. Rinse thoroughly with double-distilled water, rinse off the excess dye solution that has not been combined with the slice, then treat it with 1% hydrochloric acid-alcohol for about 15 seconds, then rinse in double distilled water for 25 minutes. Dye the Masson Ponceau Red acid magenta solution for 5–10 minutes, then rinse a little in double distilled water, and then differentiate with 1% phosphomolybdic acid aqueous solution. The fiber staining can be observed under the microscope and can be used directly without cleaning. After dyeing with aniline blue solution for 5 minutes, the stained sections were soaked in 1% glacial acetic acid for about 1 minute, then dehydrated with 95% alcohol and anhydrous ethanol, and finally stained for collagen fibers in the dyed area under the light microscope.

3.5. Transmission electron microscopy observation of myocardial fibers and ultrastructure

2.5% glutaraldehyde fixed heart tissue was fixed, acetone was dehydrated, pure acetone + embedding solution were soaked, embedded and solidified with ultra-thin slicer sliced 50–100 nm, 3% uranyl acetate and lead nitrate double were stained, observation and filming were conducted under Hitachi H7700 transmission electron microscope.

3.6. Detecting the target protein expressions via Western blotting

The left ventricle myocardium was taken from each group, and the protein was extracted and quantified under BCA colorimetry. The protein sample was boiled at 99°C for 10 min. The 10% SDS-PAGE separation gel was placed in electrophoresis buffer in line with the proportion of the instructions. The target band was transfected; TBST was blocked; primary antibodies of Keap1, caspase-3, caspase-9, Bax, TNF-α, IL1β, IL6, COX2, MMP-8, MMP-16, TIMP-1, TGF-β, Nrf2, NQO1, HO-1, GCLC, Bcl-2 and GAPDH (1:1000) were added on the membrane for incubation at 37°C followed by incubation at 4°C overnight; after being washed with TBST, HRP-labeled anti-rabbit secondary antibody (dilution 1:2000) After incubation at 37°C; membrane washed with TBST; ECL development; exposure, scanning; optical density analysis of target protein bands using Image J software. Taking the primary antibody as an internal reference, the gray ratio of the target band to the internal reference suggested the protein expression level, respectively.

3.7. Detection of H_2S, GSH and MDA contents and SOD activity in myocardial tissue

All procedures for H_2S, GSH, and MDA content and SOD activity detection were performed in strict accordance with the kit instructions. In this experiment, the activity of SOD in myocardial tissue was determined using the xanthine oxidase method. The content of H_2S in myocardial tissue was detected under ELISA. The content of GSH in myocardial tissue was determined using the micro-enzyme method. The content of MDA in myocardial tissue was determined using thiobarbital method. Finally, the measured absorbance of each tube was measured with a luminometer, and the contents of H_2S, GSH, and MDA and the activity of SOD in myocardial tissue were calculated.

3.8. Statistical analysis the experimental data were statistically analyzed with SPSS18.0 software

The observed index values were expressed as mean ± standard deviation (mean ± SD). Student-t was used to analyze comparisons between groups and one-way analysis of variance (ANOVA) was used to analyze comparisons among multiple groups. $P < 0.05$ indicates statistical significance.

Table 1
Body weight, heart weight and ratio of heart weight and body weight in each group (mean ± SD)

Groups	Number	BW (g)	HW (mg)	HW/BW (mg/g)
Control	10	354.17 ± 21.77	1050.00 ± 99.60	2.96 ± 0.16
DOX	6	265.00 ± 8.94*	1041.67 ± 87.04	3.93 ± 0.24*
DOX+H_2S	7	310.00 ± 14.72#	1002.71 ± 80.23	3.23 ± 0.19#
H_2S	10	353.33 ± 18.07	1028.17 ± 60.01	2.91 ± 0.17

Note: $^*P < 0.05$ *vs* Control group; $^\#P < 0.05$ *vs* DOX group.

Table 2
Comparison of the echocardiographic parameters in each group (mean ± SD)

Group	LVEDD (mm)	LVESD (mm)	EF (%)	FS (%)
Control	5.18 ± 0.40	2.65 ± 0.42	85.18 ± 4.04	49.03 ± 5.25
DOX	6.83 ± 0.37*	4.80 ± 0.24*	63.57 ± 5.01*	30.03 ± 3.60*
DOX+H_2S	5.84 ± 0.26#	3.73 ± 0.19#	73.07 ± 4.11#	35.93 ± 3.45#
H_2S	5.30 ± 0.29	2.90 ± 0.36	83.08 ± 4.66	46.03 ± 4.41

Note: $^*P < 0.05$ *vs* Control group; $^\#P < 0.05$ *vs* DOX group. Values are expressed as mean ± SD. Left ventricular end-diastolic dimension (LVEDD); left ventricular end-systolic diameter (LVESD); ejection fraction (EF); fractional shortening (FS).

4. Experimental results

4.1. Rat body weight (BW), heart weight (HW), and heart weight to body weight ratio (HW/BW)

The BW, HW and HW/BW of each group 8 weeks later are shown below. BW of DOX group was declined, and HW/BW was increased, showing a statistically significant difference ($P < 0.05$), compared with those of the Control group. BWs of DOX+H_2S group and H_2S were remarkably increased, and HW/BWs were significantly decreased, showing statistically significant differences ($P < 0.05$), compared with those of the DOX group. There were no statistical significances in HW change between different groups ($P > 0.05$), as shown in Table 1.

4.2. Comparisons of ECG results between different groups

As the results suggested, LVEDD and LVSED of DOX group were obviously increased, and EF and FS were substantially decreased compared with those of Control group; LVEDD and LVSED of DOX+H_2S group were reduced, showing statistically significant differences ($P < 0.05$) compared with those of DOX group. The Control group and H_2S group had no obvious differences in LVEDD, LVSED, EF, and FS ($P > 0.05$), as shown in Table 2.

4.3. Masson staining results of rat myocardial tissue of different groups

In Masson staining, collagen fiber and muscle fiber were blue and red, respectively. The myocardial cells of the DOX group were misarranged, blue collagen fiber in myocardial interstitium was remarkably increased, and there was obvious myocardial fibrosis compared with those of the Control group. The myocardial cells of DOX+H_2S were relatively better arranged, and collagen fiber in myocardial interstitium was lower significantly compared with those of the DOX group. The myocardial cells of

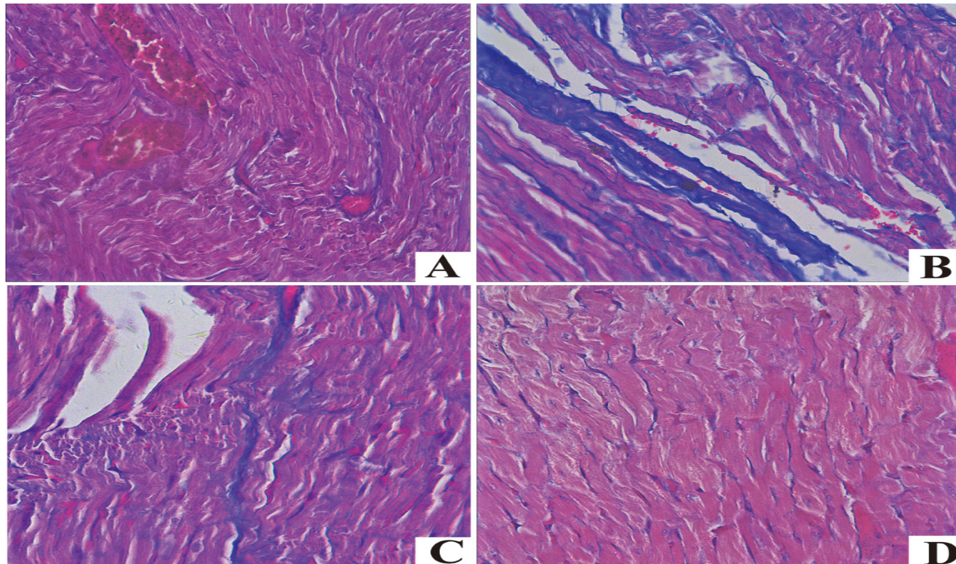

Fig. 1. Masson staining of myocardial tissues in each group. A. Control; B. DOX; C. DOX+H_2S; D. H_2S (100 μm).

the DOX+H_2S group were orderly arranged, and the area of blue collagen fiber was declined compared with those of the DOX group, suggesting that myocardial fibrosis of the DOX+H_2S group was improved. The Control group and H_2S group showed no statistically significant difference in the deposition of myocardial collagen fiber, as shown in Fig. 1.

4.4. Observation results of rat myocardial tissue of different groups under TEM

TEM results are shown in the Figure. Myocardial fibers of the Control group and H_2S group were arranged orderly, without edema or lytic necrosis; there was no swelling or vacuolation in mitochondria. The myocardial fiber of the DOX group was misarranged and swollen, and there is some lytic necrosis occurred in some sites, mitochondria were abundant, edema and vacuolation occurred in some mitochondria, and apoptosis was observed in some cells compared with those of Control group. The disarrangement and swelling of myocardial fiber were improved in the DOX+H_2S group, and vacuolation and edema in mitochondria were also improved compared with those in the DOX group. The above results indicated that H_2S can alleviate DOX-induced myocardial injury and mitochondrial pathology. See Fig. 2 for the H_2S contents in rat myocardial tissue of different groups.

The H_2S content in the myocardial tissue of different groups were detected using the ELISA method. H_2S expression in the DOX group was down-regulated compared with that in the Control group, suggesting that the H_2S content of the DOX group was reduced ($P < 0.05$). H_2S expression in rat myocardial tissue was significantly up-regulated in the DOX+H_2S group compared with that in the DOX group, suggesting that the H_2S content of the DOX+H_2S group was increased, showing a statistically significant difference ($P < 0.05$), as shown in Fig. 3.

4.5. Expression of MDA, SOD, and GSH in rat myocardial tissue of different groups

MDA expression in myocardial tissue was up-regulated in the DOX group, and SOD and GSH expression was significantly down-regulated compared with those of the Control group, showing statistically

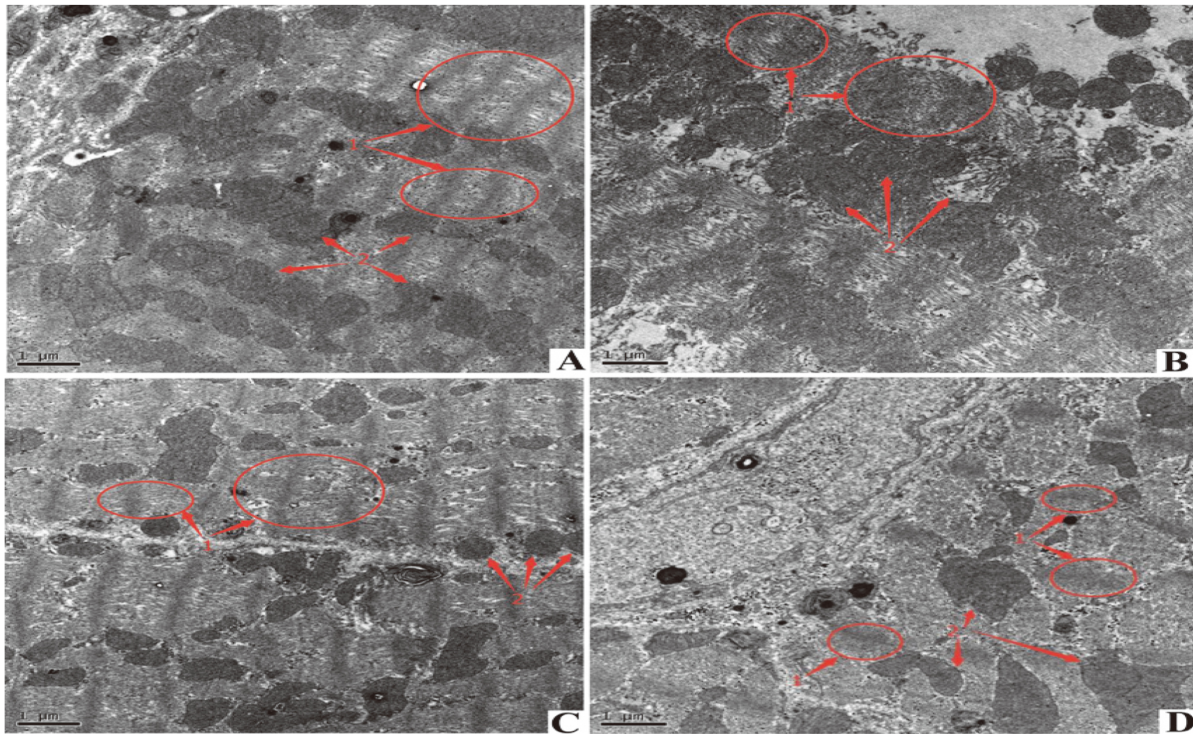

Fig. 2. Electron microscopic observation of mitochondrial swelling and vacuole formation in the selected area of the DOX group. H_2S pretreatment could reverse the DOX-induced mitochondrial ultrastructural changes in SD rats, while the mitochondria in the cells of the control and H_2S treatment groups in the selected area were normal. A. Control; B. DOX; C. DOX+H_2S; D. H_2S (1 μm).

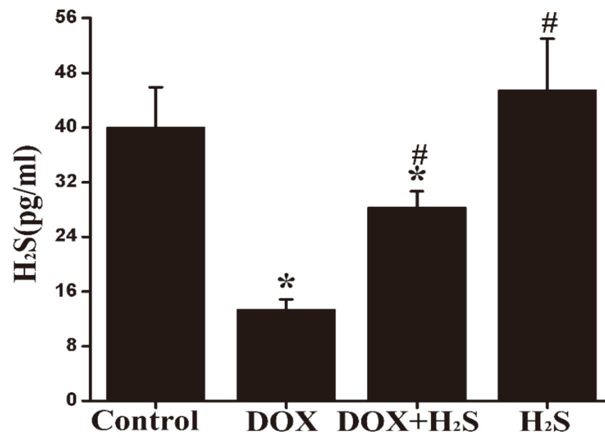

Fig. 3. Quantification of myocardial H_2S content in rats from each group. $^*P < 0.05$ *vs* Control group; $^\# P < 0.05$ *vs* DOX group.

significant differences ($P < 0.05$). MDA expression in myocardial tissue DOX+H_2S group was substantially down-regulated, while SOD and GSH expression in myocardial tissue was up-regulated compared with those of the DOX group, showing statistically significant differences ($P < 0.05$), as shown in Fig. 4.

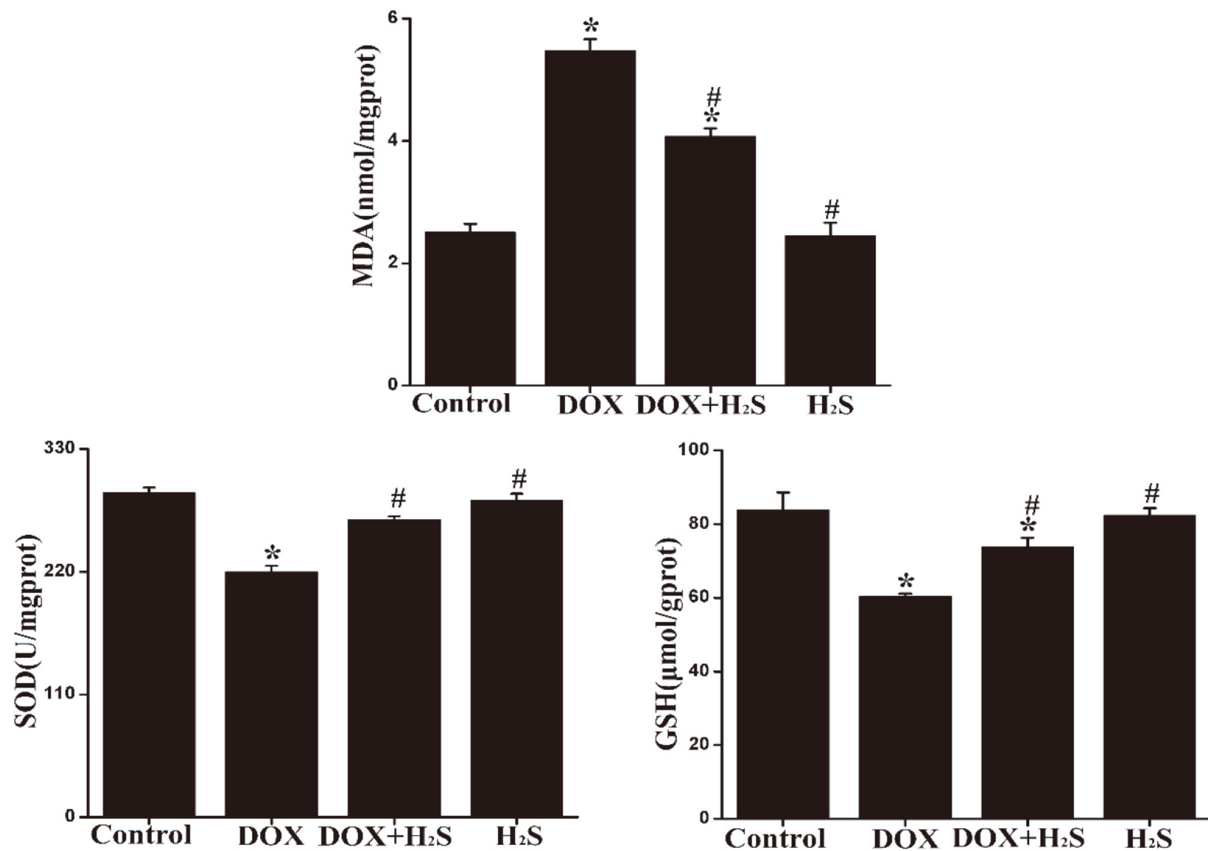

Fig. 4. The expression levels of MDA, SOD and GSH in myocardial tissues from each group. $^{*}P < 0.05$ *vs* Control group; $^{\#}P < 0.05$ *vs* DOX group.

4.6. Expression levels of Collagen III, MMP-8, MMP-16, and TIMP-1 in rat myocardial tissue of different groups

The expression levels of Collagen III, MMP-8, MMP-16 and TIMP-1 in rat myocardial tissue of DOX group were significantly down-regulated ($P < 0.05$) compared with those of Control group; the expression levels of Collagen III, MMP-8, MMP-16 and TIMP-1 in rat myocardial tissue of DOX+H_2S group were down-regulated ($P < 0.05$) compared with those of DOX group. See Fig. 5.

4.7. Expression levels of TNF-α, TGF-β, IL1β, IL6, Bcl-2, caspase-3, caspase-9, Bax, and COX2 in rat myocardial tissue of different groups

The expression levels of caspase-3, caspase-9, Bax, TNF-α, IL1β, IL6, TGF-β and COX2 in rat myocardial tissue of DOX group were up-regulated ($P < 0.05$), and the expression level of Bcl-2 was remarkably down-regulated ($P < 0.05$) compared with those of Control group. The expression levels of caspase-3, caspase-9, Bax, TNF-α, IL1β, IL6, TGF-β, and COX2 in rat myocardial tissue of DOX+H_2S group were decreased ($P < 0.05$), and the expression level of Bcl-2 was significantly increased ($P < 0.05$) compared with those of DOX group (Fig. 6).

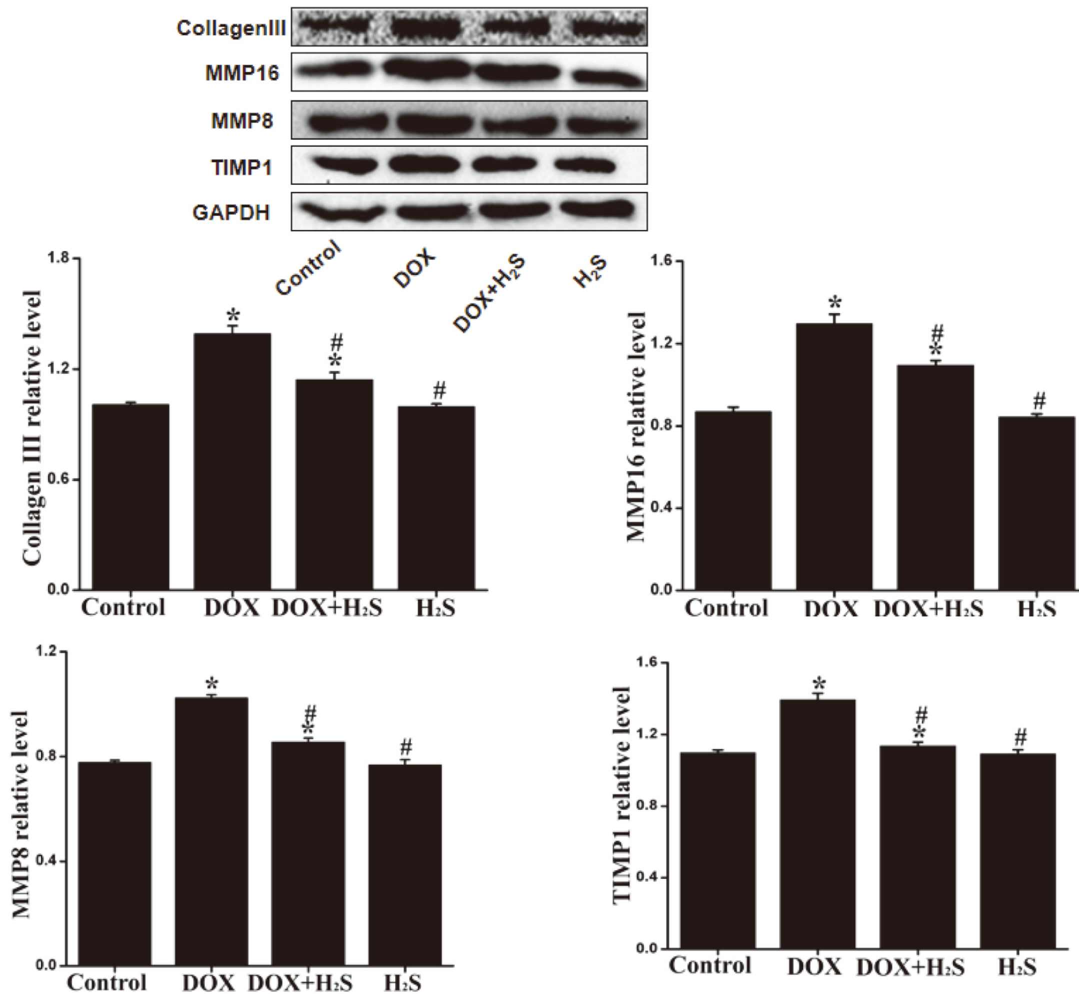

Fig. 5. The expression levels of Collagen III, MMP-8, MMP-16 and TIMP-1 in rat myocardial tissue of different groups. $^*P <$ 0.05 *vs* Control group; $^\# P < 0.05$ *vs* DOX group.

4.8. Expression levels of Keap1, Nrf2, SOD, NQO1, HO-1, and GCLC in rat myocardial tissue of different groups

The expression levels of Keap1 in rat myocardial tissue of DOX group were up-regulated ($P < 0.05$), and those of Nrf2, SOD, NQO1, HO-1, and GCLC were significantly decreased ($P < 0.05$) compared with the expression levels of Control group. the expression level above the detection indicator of the DOX+H_2S group was reversed compared with the DOX group (Fig. 7).

5. Discussion

Myocardial fibrosis is a vital pathological change in the occurrence and development of DCM and doxorubicin-induced cardiomyopathy. It is a key link in myocardial remodeling following myocardial

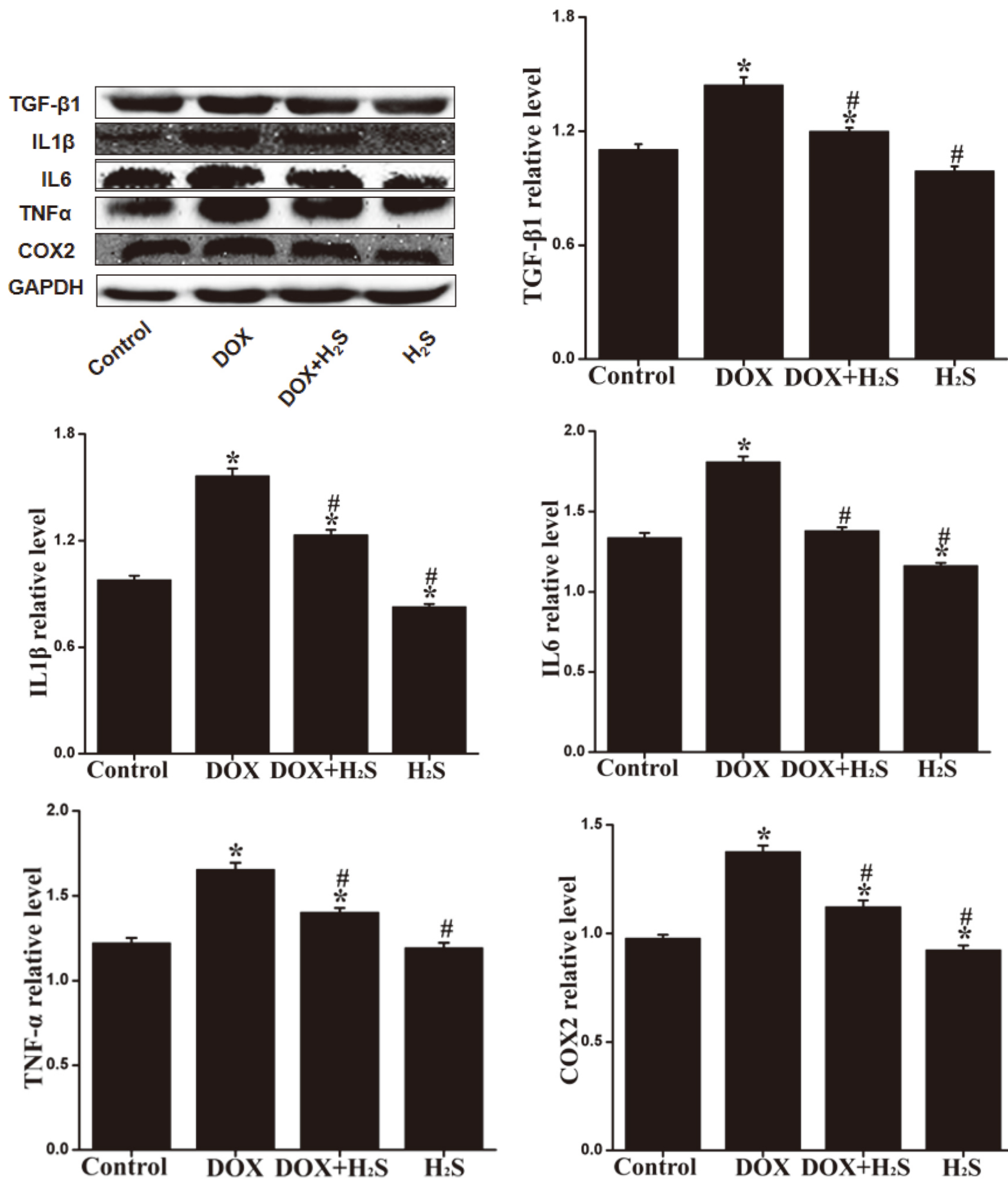

Fig. 6. Expression of TNF-α, TGF-β1, IL1β, IL6, Bcl-2, caspase-3, caspase-9, Bax protein in myocardial tissue of each group. $^{*}P < 0.05$ *vs* Control group; $^{\#}P < 0.05$ *vs* DOX group.

injury as well. Myocardial fibrosis is the result of the imbalance between the synthesis and degradation of extracellular matrix (ECM), resulting in the extensive deposition of ECM in the myocardial interstitium. Matrix metalloproteinases (MMPs) are the major enzymes degrading extracellular matrix components, while tissue inhibitor of metalloproteinases (TIMPs) serves as the endogenous specific inhibitor of MMPs. The interaction between MMPs and TIMPs is critical for maintaining the dynamic equilibrium

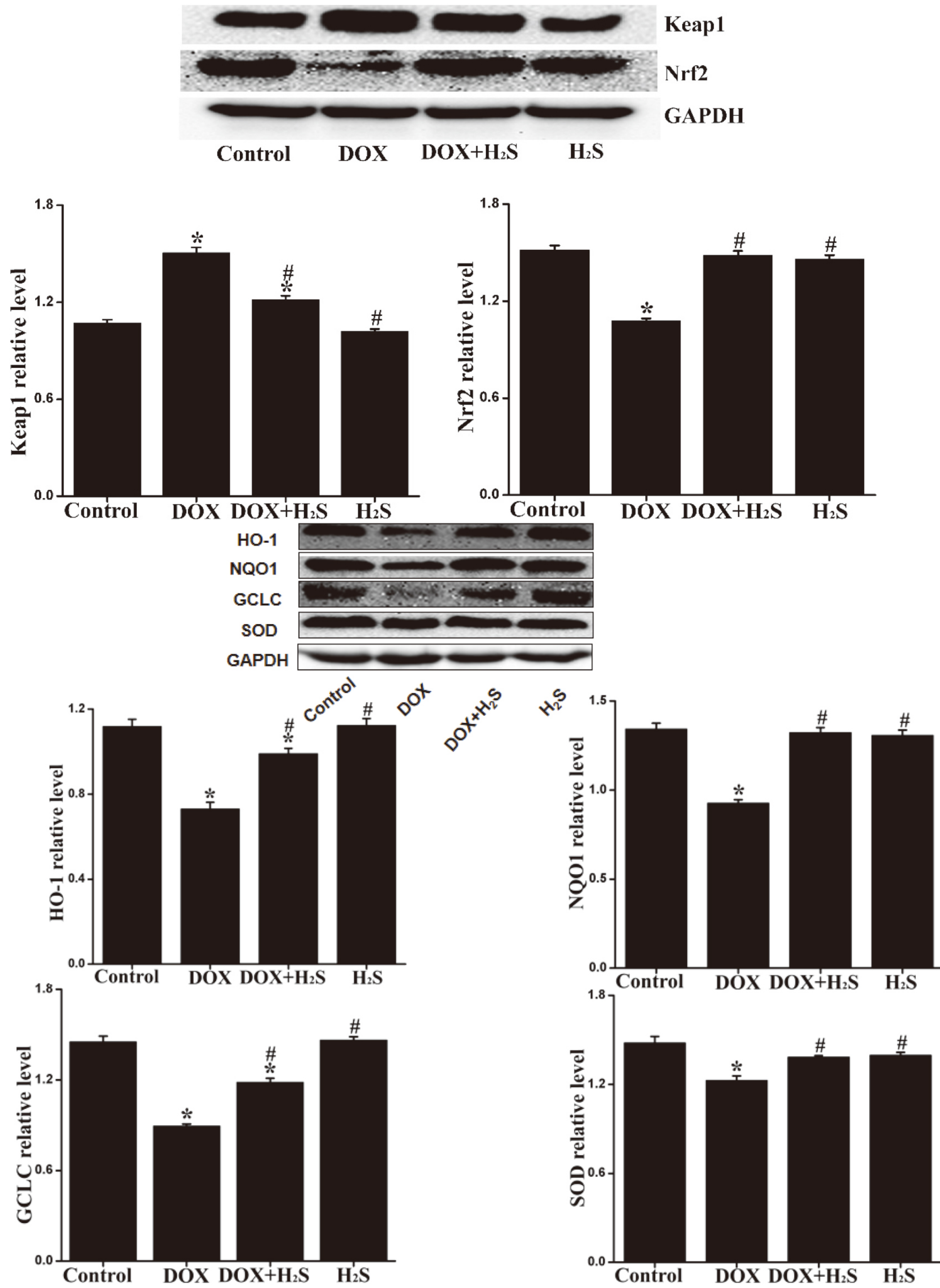

Fig. 7. Expression of Keap1, Nrf2, SOD, NQO1, HO-1, and GCLC proteins in myocardial tissue of each group. $^*P < 0.05$ *vs* Control group; $^\# P < 0.05$ *vs* DOX group.

of ECM. Thus, the formation of myocardial fibrosis is closely associated with MMPs/TIMPs interaction [17,18]. Also, the inflammatory reaction is involved in the occurrence mechanism of myocardial fibrosis, and in particular, TGF-β (Transforming growth factor-β) can activate cardiac fibroblasts, promote the differentiation of cardiac fibroblasts towards myofibroblasts, and result in a considerable amount of ECM deposition and myocardial fibrosis formation [19]. An inflammatory response is another important mechanism for the occurrence of myocardial fibrosis, which has been broadly recognized at home and abroad. The level of inflammatory factors can even, to some extent, help to predict the occurrence of cardiovascular events [20]. TNF-α, IL1β, IL6, and COX-2 are critical inflammatory factors in inflammatory responses in the body, and these factors play extremely important roles in the formation of myocardial remodeling [21,22]. The present study found that doxorubicin-induced obvious myocardial fibrosis in the DOX group, MMPs/TIMPs were significantly down-regulated, and such inflammatory factors as TGF-β, TNF-α and IL6 were also remarkably up-regulated, suggesting that inflammatory response and MMPs/TIMPs dysregulation are involved in the mechanism of doxorubicin-induced myocardial fibrosis.

Oxidative stress is a significant mechanism in the occurrence and development of cardiomyopathy causing myocardial damage. Oxidative stress usually suggested a serious imbalance between the generation of ROS and the elimination of ROS in the antioxidant defense system in the body, causing substantial accumulation of ROS that may lead to lipid peroxidation and up-regulate the expression levels of malondialdehyde (MDA), 4-hydroxynonenal (4-HNE) and other RNS metabolites. As a result, the heart is very sensitive to oxidative damage [23,24]. Some antioxidant enzymes (e.g., superoxide dismutase (SOD) and glutathione (GSH) in the body) can reduce or eliminate ROS, being critical for preventing oxidative stress damage. Doxorubicin is a clinically commonly-used anthraquinone broad-spectrum anti-tumor drug. A large number of literature have suggested that doxorubicin can significantly up-regulate the level of oxidative stress in myocardial tissue and lead to myocardial apoptosis [25]. In this study, we found that the DOX group induced by doxorubicin had obvious myocardial fibrosis, the level of oxidative stress was up-regulated, the expression levels of MDA and 4-HNE were up-regulated, and those of SOD, NQO1, HO-1, GCLC, and other antioxidants were significantly down-regulated. Existing studies have verified that ROS can also lead to apoptosis as a major factor in inducing oxidative stress in the body. Apoptosis has a significant impact on the occurrence and development of myocardial fibrosis. The Caspases cascade pathways in myocardial tissue will be activated separately with the action of various pro-apoptotic factors, thereby inducing apoptosis. Bax has been found as a common pro-apoptotic protein, while Bcl-2 has been proven to block caspase activation and inhibit apoptosis. In this study, it is being suggested that the level of apoptosis in myocardial tissue induced by doxorubicin was up-regulated significantly with the level of oxidative stress, Caspases and Bax were up-regulated, and Bcl-2 was down-regulated, suggesting that apoptosis is also involved in the mechanism of doxorubicin-induced myocardial fibrosis.

Keap1/Nrf2 signaling pathway is an important antioxidant stress pathway in the body. Numerous studies have suggested that the Keap1/Nrf2 signaling pathway is critical for anti-oxidative stress, anti-inflammation, anti-apoptosis, anti-cell proliferation, and protection of myocardial cells in the body [26]. In the Keap1/Nrf2 signaling pathway, Keap1 primarily modulates the transcriptional activity of Nrf2 and negatively regulates the function of Nrf2 [27]. When the body is damaged by oxidative stress, inflammatory reaction, etc., the activation of the Keap1/Nrf2 signaling pathway in the body may start transcript downstream phase II detoxifying enzymes, antioxidant enzymes, etc., including catalase (CAT), superoxide (SOD), glutathione S-transferase (GST), oxidoreductase (NQO1) and heme oxygenase (HO-1) [28], to alleviate cell damage caused by active oxygen and electrophilic substances, keep the cells in a stable state, and be critical for anti-oxidative stress, anti-apoptosis, and anti-inflammatory. A study made by Siyin et al. revealed that Nrf2 deficiency aggravates doxorubicin-induced cardiotoxicity and

cardiac dysfunction [29]. In this study, we found that doxorubicin-induced the remarkable up-regulation of Keap1 and inhibition of Nrf2 expression, as well as the down-regulation of the downstream phase II detoxifying enzymes and antioxidant enzymes of Keap1/Nrf2 signaling pathway, e.g., NQO1, HO-1, and GCLC. This suggested that the Keap1/Nrf2 signaling pathway may be involved in the mechanism of doxorubicin-induced myocardial fibrosis, oxidative stress, and apoptosis.

As a colorless, smelly rotten-egg gas, H_2S may also be produced in the body in the same way as NO, CO, etc. are produced, and it has important biological effects in the cardiovascular system. H_2S has been found to have such biological activities as anti-oxidation, anti-apoptosis, anti-inflammatory response, angiogenesis promotion, and vasodilatation. Also, this gas is critical for myocardial protection in cardiovascular diseases [30]. The production of most of endogenous H_2S in mammalian tissues is catalyzed by cystathionine-gamma-lyase (CSE), 3-mercaptopyruvate sulfurtransferase (3-MST) and cystathionine-β-synthase, cystathionine-β-synthase (CBS), among which CSE is the dominant H_2S-producing enzyme in the cardiovascular system. Some studies have found that H_2S can reduce oxidative stress by reducing the production of ROS, thereby relieving isoproterenol-induced myocardial damage [29]. One of our previous studies also found that H_2S also improved diabetic myocardial fibrosis, which is associated with the inhibition of oxidative stress [31]. In an experiment *in vitro*, H_2S was also found to improve the cardiotoxicity of doxorubicin, and H_2S sustained release agent Diallyl trisulfide inhibited doxorubicin-induced myocardial apoptosis [13,14]. The results of this experiment revealed that after the intervention with H_2S donor NaHS, the cardiac function of the rats in the DOX+H_2S group was improved, EF and FS were increased, LVEDD and LVESD were significantly decreased, and HW/BW was significantly decreased, compared with DOX group. The results of immunohistochemistry showed that muscle cells in the DOX+H_2S group were arranged neatly, myocardial collagen fibers were significantly reduced, and collagen III expressions were substantially decreased in the myocardium. The expression levels of MDA, TNF-α, IL1β1, IL6, Cox-2, caspase-2, caspase-3, caspase-9, Bax, and Keap1 were down-regulated significantly, and those of SOD, GSH, Bcl-2, Nrf2, HO-1, and NQO1 were up-regulated obviously. These results suggested that H_2S can ultimately alleviate the myocardial fibrosis induced by doxorubicin in rats by reducing inflammation and inhibiting apoptosis and excessive oxidative stress, and H_2S may improve the internal mechanism of doxorubicin-induced myocardial fibrosis in rats, which is likely to be associated with its regulation of Keap1/Nrf2 signaling pathway.

In conclusion, the findings of the present study suggest that H_2S may inhibit oxidative stress, reduce the inflammatory response, and improve apoptosis by regulating the Keap1/Nrf2 signaling pathway to inhibit doxorubicin-induced myocardial fibrosis in rats. The endogenous gas signaling molecule H_2S may also protect the myocardial remodeling and development of cardiomyopathy. This study may lay a basis for an in-depth understanding of the pathogeneses of doxorubicin-induced cardiomyopathy and DCM and a new target for the prevention and treatment of cardiomyopathy.

Acknowledgments

This study was supported by grants from the National Natural Science Foundation of China (Grant No. 81202830) and the Hunan Provincial Innovation Foundation For Postgraduates (Grant No. CX20200973).

Availability of data and materials

All data generated or analyzed during the study period is included in this article.

Conflict of interest

None to report.

References

[1] Nithipongvanitch R, Ittarat W, Cole MP, et al. Mitochondrial and nuclear p53 localization in cardiomyocytes: redox modulation by doxorubicin (Adriamycin). Antioxid Redox Signal. 2007; 9: 1001-1008.
[2] Gu R, Bai J, Ling L, et al. Increased expression of integrin-linked kinase improves cardiac function and decreases mortality in dilated cardiomyopathy model of rats. PLoS ONE. 2012; 7: e31279.
[3] Luk A, Ahn E, Soor GS, Butany J. Dilated cardiomyopathy: a review. J Clin Pathol. 2009; 62: 219-225.
[4] Lakdawala NK, Winterfield JR, Funke BH. Dilated cardiomyopathy. Circulation Arrhythmia & Electrophysiology. 2013; 6: 228-37.
[5] Wang J, Xue Z, Lin J, et al. Proline improves cardiac remodeling following myocardial infarction and attenuates cardiomyocyte apoptosis via redox regulation. Biochem Pharmacol. 2020; 178: 114065.
[6] Zare MFR, Rakhshan K, Aboutaleb N, et al. Apigenin attenuates doxorubicin induced cardiotoxicity via reducing oxidative stress and apoptosis in male rats. Life Sci. 2019; 232: 116623.
[7] Garg S, Narula J, Chandrashekhar Y. Apoptosis and heart failure: clinical relevance and therapeutic target. J Mol Cell Cardiol. 2005; 38: 73-79.
[8] Fang L, Ellims AH, Beale AL, et al. Systemic inflammation is associated with myocardial fibrosis, diastolic dysfunction, and cardiac hypertrophy in patients with hypertrophic cardiomyopathy. Am J Transl Res. 2017; 9: 5063-5073.
[9] Choi WS, Jeong JW, Kim SO, et al. Anti-inflammatory potential of peat moss extracts in lipopolysaccharide-stimulated RAW 264.7 macrophages. Int J Mol Med. 2014; 34: 1101-1109.
[10] Guo S, Cheng X, Lim JH, et al. Control of antioxidative response by the tumor suppressor protein PML through regulating Nrf2 activity. Mol Biol Cell. 2014; 25: 2485-2498.
[11] Sandra V, Anne S, Michael J. Parnhamet al: Nrf2, the master regulator of anti-oxidative responses. Int. J. Mol. Sci. 2017; 18: 2772.
[12] Calvert JW, Coetzee WA, Lefer DJ. Novel insights into hydrogen sulfide-mediated cytoprotection. Antioxidants & Redox Signaling. 2010; 12: 1203-1217.
[13] Liu MH, Lin XL, Yuan C, et al. Hydrogen sulfide attenuates doxorubicin-induced cardiotoxicity by inhibiting the expression of peroxiredoxin III in H9c2 cells. Mol Med Rep. 2016; 13: 367-372.
[14] Wen SY, Tsai CY, Pai PY, et al. Diallyl trisulfide suppressesdoxorubicin-induced cardiomyocyte apoptosis by inhibiting MAPK/NF-κB signaling through attenuation of ROS generation. Environ Toxicol. 2018; 33: 93-103.
[15] Renu K, Abilash VG, Tirupathi PP, et al. Molecular mechanism of doxorubicin-induced cardiomyopathy-AnUpdate. European Journal of Pharmacology. 2017; 818(2018): 241-253.
[16] Xia Y, Chen Z, Chen A, et al. LCZ696 improves cardiac function via alleviating Drp1-mediated mitochondrial dysfunction in mice with doxorubicin-induced dilated cardiomyopathy. Journal of Molecular & Cellular Cardiology. 2017; 108: 138-148.
[17] Li Y, Li L, Zeng O, Liu JM, Yang J. H_2S improves renal fibrosis in STZ-induced diabetic rats by ameliorating TGF-β1 expression. Renal Failure. 2017; 39: 265-272.
[18] Ma J, Ma S-Y, Ding C-H. Curcumin reduces cardiac fibrosis by inhibiting myofibroblast differentiation and decreasing transforming growth factor β1 and matrix metalloproteinase 9/tissue inhibitor of metalloproteinase 1. Chinese Journal of Integrative Medicine. 2017; 23: 362-369.
[19] Seeland U, Haeuseler C, Hinrichs R, et al. Myocardial fibrosis in transforming growth factor-beta(1) (TGF-beta(1)) transgenic mice is associated with inhibition of interstitial collagenase. Eur J Clin Invest. 2002; 32: 295-303.
[20] Ayinapudi K, Samson R, Le Jemtel TH, et al. Weight reduction for obesity-induced heart failure with preserved ejection fraction. Curr Hypertens Rep. 2020; 22(8): 47.
[21] Fang L, Ellims AH, Beale AL, et al. Systemic inflammation is associated with myocardial fibrosis, diastolic dysfunction, and cardiac hypertrophy in patients with hypertrophic cardiomyopathy. Am J Transl Res. 2017; 9: 5063-5073.
[22] Westermann D, Van Linthout S, Dhayat S, et al. Tumor necrosis factor-alpha antagonism protects from myocardial inflammation and fibrosis in experimental diabetic cardiomyopathy. Basic Res Cardiol. 2007; 102: 500-507.
[23] Deepa M. Gopal, flora sam: new and emerging biomarkers in left ventricular systolic dysfunction – insight into dilated cardiomyopathy. J Cardiovasc Transl Res. 2003; 6: 516-527.
[24] Herbet M, Korga A, Gawrońska-Grzywacz M, et al. Chronic variable stress is responsible for lipid and DNA oxidative disorders and activation of oxidative stress response genes in the brain of rats. Oxidative Medicine & Cellular Longevity. 2017; 2017: 7313090.

[25] Octavia Y, Tocchetti CG, Gabrielson KL, et al. Doxorubicin-induced cardiomyopathy: from molecular mechanisms to therapeutic strategies. J Mol Cell Cardiol. 2012; 52: 1213-1225.
[26] Syed Minhaj Uddin Ahmed, Lin Luo, Akhileshwar Namani: Nrf2 signaling pathway: Pivotal roles in inflammation. Biochimica et Biophysica Acta. 2016; 1863: 585-597.
[27] McMahon M, Itoh K, Yamamoto M, et al. Keap1-dependent proteasomal degradation of transcription factor Nrf2 contributes to the negative regulation of antioxidant response element-driven gene expression. The Journal of Biological Chemistry. 2003; 278: 1592-21600.
[28] Xiao L, Liang S, Ge L, et al. 4, 5-di-O-caffeoylquinic acid methyl ester isolated from Lonicera japonica Thunb. targets the Keap1/Nrf2 pathway to attenuate H2O2-induced liver oxidative damage in HepG2 cells. Phytomedicine. 2020; 70: 153219.
[29] Li S, Wang W, Niu T. Nrf2 deficiency exaggerates doxorubicin-induced cardiotoxicity and cardiac dysfunction. Oxid Med Cell Longev. 2014; 2014: 748524.
[30] Pan L-L, Qin M, Liu X-H, et al. The role of hydrogen sulfide on cardiovascular homeostasis: an overview with update on immunomodulation. Front Pharmacol. 2017; 8: 686.
[31] Liu M, Li Y, Liang B, et al. Hydrogen sulfide attenuates myocardial fibrosis in diabetic rats through the JAK/STAT signaling pathway. Int J Mol Med. 2018; 41: 1867-1876.