Hydrogen sulfide ameliorates rat myocardial fibrosis induced by thyroxine through PI3K/AKT signaling pathway

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Abstract. This study aims to investigate the role and regulatory mechanism of the Hydrogen sulfide (H2S) in amelioration of rat myocardial fibrosis induced by thyroxine through interfering the autophagy via regulating the activity of PI3K/AKT1 signaling pathway and the expression of relative miRNA. 40 adult male SD rats were randomly divided into 4 groups (n = 10): the control group, the thyroxine model group (TH group), the model group with H2S intervention (TH + H2S group) and the normal group with H2S intervention (H2S group). Pathological changes were observed via H&E staining and Masson staining, Expressions of MMPs/TIMPs, PI3K/AKT, autophagy-related proteins in myocardial tissues were detected via Western blotting, and the expressions of miR-21, miR-34a, miR-214 and miR-221 were detected via RT-qPCR. Compared with the control group, in the TH group, myocardial fibrosis was more significant, the expressions of proteins in PI3K/AKT and autophagy-related proteins were significantly decreased, as well as the expression of miR-221; while the expressions of miR-21, miR-34a and miR-214 were significantly elevated. By contrast, all above-mentioned changes were obviously reversed with H2S treatment, which demonstrated the positive function of H2S in amelioration of rat myocardial fibrosis induced by thyroxine. The mechanism of such amelioration may be correlated with autophagy activated by the upregulation of expression of PI3K/AKT signaling pathway and downregulation of expressions of miR-21, miR-34a and miR-214.

Key words: Hydrogen sulfide, Myocardial fibrosis, Autophagy, Thyroxine, PI3K/AKT

Hypothyroidism refers to the thyrotoxicosis caused by excessive generation of thyroxine from thyroid. It always brings damages to organs, tissues and cells at varying degrees. As a key target organ sensitive to thyroxine, heart under direct or indirect action of excessive thyroxine in the circulation suffers hyperthyroid heart disease with apparent clinical symptoms and pathological changes, such as myocardial hypertrophy, myocardial fibrosis, arrhythmia or cardiac insufficiency [1, 2]. Among them, myocardial fibrosis is a key marker indicating myocardial remodeling in hyperthyroid heart disease, and also is the major cause leading to insufficiency of left heart [3, 4]. Although the pathogenesis of myocardial fibrosis has not been fully understood due to its complexity, it has been suggested by existing evidences that such pathogenesis is associated with many factors, including the metabolic disorder in myocardial cells, activation of Renin-angiotensin-aldosterone System (RAAS), imbalance between the sympathetic tone and vagal tone, and excessive inflammatory responses and stress responses in myocardium [5-7].

As a specific metabolic process, autophagy in eukary-
ote can degrade the damaged, degenerated and aged proteins, and organelles in cells with the involvement of lysosome. Therefore, it is critically significant to maintain the regular intracellular homeostasis and self-renewal in cells [8]. Moderate autophagy is a kind of self-protective mechanism surviving cells in adverse environment [9, 10]. Current studies have shown that cell autophagy involves in the pathogenesis of myocardial fibrosis and myocardial remodeling. However, few studies focused on the relation between autophagy and the occurrence and progression of myocardial fibrosis caused by hyperthyroid heart disease.

PI3K/AKT signaling pathway, one of the signaling pathways widely existing in animals, not only acts as classic signaling pathway that regulates the cell autophagy, but also involves the regulation of a series of pathophysiological processes, such as cell proliferation, apoptosis and stress response [11].

In addition, the PI3K/AKT signaling pathway is also important for insulin signaling and glucose metabolism, lymphocyte migration, proliferation and differentiation, and TGF-β signaling regulation. Thus it is an crucial therapeutic target for the treatment of various disease, including cancer, diabetes and others [12]. Actually, many studies indicated the presence of activation of PI3K/AKT signaling pathway in many cancers. For instances, Gamal Badr et al. reported that apoptosis of human breast carcinoma cells are induced by samsum ant venom through PI3K/AKT signaling pathway [13]. Pei Li et al. suggested that resveratrol apoptosis and senescence of attenuates high glucose-induced nucleus pulposus cell through activating the ROS-mediated PI3K/Akt pathway [14].

It has been confirmed that H₂S, a novel gas signal module, has a wide variety of biological effects, including protecting the myocardium in various cardiovascular diseases, such as hypertension and myocardial ischemia-reperfusion injuries through expanding the vessels, and regulating the mechanisms of autophagy, anti-oxidation, anti-inflammation and anti-apoptosis [15]. However, when it comes to the question of whether H₂S can exert the protective effect on hyperthyroid heart disease and myocardial fibrosis induced by high-concentration thyroxine, and its signaling regulation mechanism, more efforts are required. Therefore, a rat myocardial fibrosis model induced by thyroxine at a high concentration was established in this study to investigate the effect of H₂S on rat myocardial fibrosis induced by high-concentration thyroxine, and to determine the role of H₂S on the regulation of cell autophagy and expressions of myocardial fibrosis-related miRNAs (miR-21, miR-34a, miR-214 and miR-221). In conclusion, the research aimed to provide experimental evidence for further understanding of pathogenesis of hyperthyroid heart disease and discovery of new therapeutic targets.

Materials and Methods

Animals and reagents

The experimental protocol, which had been approved by the Animal Ethics Committee of University of South China (Hengyang, China), complied with the provisions on experimental animal management in the People’s Republic of China. 40 adult male Sprague Dawley (SD) rats, weighing 140–160 g, were purchased from the SJA Experimental Animal Center of Changsha (Changsha, Chinese), and then kept in separate cages under controlled temperature and allowed to access to food and water freely. Artificial lighting was alternated every 12 hours.

Sodium hydrogen sulfide (NaHS) was supplied by Sigma-Aldrich (St. Louis, MO, USA). L-Thyroxine was from Merck Drugs & Biotechnology (Darmstadt, Germany). Cell lysis buffer was bought for western blotting, and phenylmethylsulfonyl fluoride (PMSF), bicinchoninic acid (BCA) protein assay kit, tris buffered saline, SDS-PAGE gel preparation kit and chloral hydrate were purchased from Beyotime Biotechnology Co., Ltd. (Shanghai, Chinese). Polyvinylidene fluoride (PVDF) membrane and pre-stained protein molecular marker were supplied from Merck Millipore (Billerica, MA, USA); Rabbit polyclonal anti matrix metalloproteinase 11 (MMP11), rabbit polyclonal anti-MMP12, rabbit polyclonal anti-MMP14, rabbit polyclonal anti-TIMP-1, TIMP-4 and rabbit, rabbit polyclonal anti-MMP17 and polyclonal anti-glyceraldehyde 3-phosphate dehydrogenase (GAPDH) were procured from Wuhan Boster Biological Technology, Ltd. (Wuhan, China). The dilution rate of above these antibodies was 1:400. Rabbit anti-autophagy related gene 5 (ATG5), rabbit monoclonal anti-ATG7, rabbit monoclonal anti-A16L1, rabbit monoclonal anti-Beclin1, rabbit monoclonal anti-LC3A, rabbit polyclonal anti-P13K, rabbit polyclonal anti-AKT1 were provided by Cell Signaling Technology, Inc. (Danvers, MA, USA), and the dilution rate was 1:1,000. Furthermore, anti-rabbit secondary antibody and anti-rat secondary antibody were also purchased from Proteintech Group, and the dilution rate for which was 1:2,000.
Establishment of model

These experimental animals were randomly divided into four groups (n = 10 for each group): normal group (control group), L-thyroxine (TH)-treated group (TH group), and hyperthyroidism treated with H$_2$S group (TH + H$_2$S group), normal rats treated with H$_2$S group (H$_2$S group). TH group and TH + H$_2$S group were injected intraperitoneally with L-thyroxine (2.5 mg/kg/day). Rats in the control group and H$_2$S group were injected intraperitoneally with normal saline. While TH + H$_2$S group and H$_2$S group was treated with intraperitoneally injected sodium hydrogen sulfide (100 μmol/kg). The rats in the control group and TH group were daily treated with PBS. After 4 weeks, all rats were weighed, then they were anesthetized with chloral hydrate (350 mg/kg) to lavage their hearts with ice-cold normal saline; afterward the hearts were removed and weighed, and then reserved at −80°C for experiment.

Pathological analysis of myocardial fibers

Myocardial tissue was firstly rinsed with sterile saline, and then was fixed to 4% paraformaldehyde (Beyotime Institute of Biotechnology, Shanghai, China), alcohol dehydration, Paraffin embedded. After that, they were made into 4 μm thick slices and stained with Hematoxylin and Eosin staining kits and Masson kits. Finally, these slices were observed under optical microscope.

Transmission electron microscope (TEM) observation

The myocardial tissue was firstly fixed with 2.5% glutaraldehyde (Sinopharm Chemical Reagent Co., Ltd.). Then it was sequentially fixed with 1% osmium tetroxide (Beyotime Institute of Biotechnology, Shanghai, China), rinsed with Phosphoric acid rinse solution (Beyotime Institute of Biotechnology, Shanghai, China), dehydrated at different concentrations of acetone (Beyotime Institute of Biotechnology, Shanghai, China), and solidified into 50–100 nm thick slices. After being stained with 3% uranyl acetate (Johnson Biotechnology Co., Ltd., Shanghai, China) and lead nitrate (Tianyuan Industrial Fine Chemical Co., Ltd., Yingkou, China), the ultrathin slices were observed under TEM.

Western blot analysis

Proteins were extracted by cell lysis buffer containing protease inhibitors, and then quantified by BCA protein test kit. Denatured proteins were separated by SDS-PAGE electrophoresis, and then transferred to the PVDF membrane by wet transfer method. The Tris membrane was blocked in Tris buffered saline containing Twain 20 and 5% skim milk at 37°C, and then it was incubated with blocking solution containing primary antibody for 1 hour, and stayed overnight at 4°C. After washing with TBST for three times, the membrane was sequentially incubated with horseradish peroxidase-conjugated secondary antibody for 1 hour. After that, the membrane was again rinsed for three times with TBST buffer. Finally, the membranes was subjected to chemiluminescence detection assay. The bands were analyzed with a Molecular Imager VersaDoc MP 5000 system (Bio-Rad Laboratories, Inc., Hercules, CA, USA) and Alpha Imager (San Leandro, USA) analysis strip, with GAPDH as a reference.

RT-qPCR analysis

Total RNA was extracted from myocardial tissue of mice from each group using Trizol reagent (Invitrogen, California, USA). The concentration of the extracted RNA was measured by ultraviolet Spectrophotometer (Agilent Technologies, CA, USA). and the integrity of RNA was analyzed by gel imaging system (Bio-Rad Laboratories, Inc., Hercules, CA, USA). Reverse transcription reaction was manipulated with mi-RNA specific RT primer (GenScript USA Inc., Nanjing, China) using reverse transcription polymerase chain reaction kit (MBI Fermentas, the Republic of Lithuania). The expression levels of miR-21-TAGCTTATCAGACTGATGTTGA, miR-34a-TGGCAGTGTCTTAGCTGGTTGT, miR-214-AGAGTTGTCATGTGTCT and miR-221-ACCTGGCATACAATGTAGATTTC were detected by Real-time PCR using Taqman mi-RNA assay probe (Applied Biosystems, Shanghai, China), and normalized by endogenous U6 small RNA. Reverse transcription was manipulated under following circumstances: 37°C for 15 minutes, 42°C for 50 minutes and 85°C for 5 minutes. The cDNA obtained was subjected to real-time PCR under the following circumstances: 50°C for 2 minutes, 95°C for 10 minutes, 95°C for 5 seconds, and 60°C for 30 seconds.

Statistical analysis

Data were expressed as mean ± standard deviation (SD). Group differences were assessed by one-way analysis of variance using SPSS software 18.0 (Chicago, USA). Differences between two groups were analyzed using the Student-Newman-Keuls test. p < 0.05 was considered as statistically significant.
Results

Effect of hydrogen sulfide on body weight, heart weight and heart weight/body weight ratio

Eight weeks later, 10, 9, 8, 10 mice were survived in control group, TH group, TH + H\textsubscript{2}S group, H\textsubscript{2}S group respectively. As shown in Table 1, the differences in body weight, heart weight and heart weight/body weight ratio between groups were not significant (Table 1).

Effect of hydrogen sulfide on myocardial fibrosis induced by thyroxine

To observe the morphological changes in myocardium of heart tissues, HE-staining (Fig. 1a), Masson-staining (Fig. 1b) and collagen Volume Fraction (CVF) of the Masson staining (Fig. 1c) were respectively conducted to identify the pathological changes in myocardial tissues, and the morphology of myocardial fiber and collagen deposition in myocardial tissues. As shown in the Fig. 1a, purplish blue area stands for the composition of cell nucleus, while red is for the compositions in cytoplasm and extracellular matrix. In the control group, the morphology of myocardium with a clear structure was normal; in TH group, the diameter of myocardial cells were increased, and the myocardial tissues were irregularly arranged; compared with TH group, the diameter of myocardial cells was shortened and the structure of myocardial tissues was improved in TH + H\textsubscript{2}S group. However, changes in myocardial tissue structure and diameter of myocardial cells in H\textsubscript{2}S group were similar to those in the control group. The figure shows that the regions in blue between myocardial cells are collagen fibers. It can be seen from the Fig. 1b that in comparison to the control group, myocardial fibers of rats in TH group were arranged irregularly, and the quantity of blue-stained fibers was increased with obvious myocardial fibrosis. In comparison with TH group, disordered arrangement of myocardial cells was significantly improved, the quantity of blue-stained collagen fibers was decreased, and the myocardial fibrosis was improved in TH + H\textsubscript{2}S group, while there was no significant difference in changes of myocardial tissues between the control group and H\textsubscript{2}S group was observed.

Effects of H\textsubscript{2}S on collagen III expressions of thyroxine rats

The expression of collagen III in myocardium tissues, which can be used to reflect fibrosis in some degree, was measured by immunohistochemically analysis. As a result, it is found that when compared with the control group, the positive expression of collagen III was significantly decreased in TH group, and the myocardial expression of collagen III was remarkably reduced in TH + H\textsubscript{2}S group. While as for TH group and TH + H\textsubscript{2}S group, there is no significant difference was observed. (Fig. 2).

H\textsubscript{2}S content in each group

To discover the correlation of the myocardial fibrosis induced by high-concentration thyroxine with the changes in endogenous H\textsubscript{2}S, the level of endogenous H\textsubscript{2}S in myocardial tissues of all groups by ELISA was analyzed in the very study. Results indicate that in comparison to the control group, the expression level of H\textsubscript{2}S in myocardial tissues of rats in TH group was dramatically down-regulated; however, compared with TH group, H\textsubscript{2}S expression in myocardial tissues of rats in TH + H\textsubscript{2}S group was significantly upregulated. Overall, the expression level of H\textsubscript{2}S in myocardial tissues is the highest in TH group, and there is no statistical difference between TH group and TH + H\textsubscript{2}S group (Fig. 3).

Effects of H\textsubscript{2}S on myocardial ultrastructural in thyroxine rats

To observe the changes in myocardial ultrastructural, and effect of H\textsubscript{2}S on autophagy of myocardial cells and

<table>
<thead>
<tr>
<th>Group</th>
<th>Number</th>
<th>BW (g)</th>
<th>HW (mg)</th>
<th>HW/BW (mg/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>10</td>
<td>191.60 + 6.17</td>
<td>766.20 + 47.00</td>
<td>4.00 + 0.32</td>
</tr>
<tr>
<td>TH</td>
<td>9</td>
<td>187.33 + 8.56</td>
<td>765.78 + 68.77</td>
<td>4.10 + 0.48</td>
</tr>
<tr>
<td>TH + H\textsubscript{2}S</td>
<td>8</td>
<td>196.50 + 10.20</td>
<td>766.62 + 59.12</td>
<td>3.90 + 0.21</td>
</tr>
<tr>
<td>H\textsubscript{2}S</td>
<td>10</td>
<td>188.00 + 11.55</td>
<td>756.10 + 38.15</td>
<td>4.04 + 0.35</td>
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Values are expressed as means ± SD.
myocardial fibers in rats with hyperthyroidism induced by H$_2$S, autophagosomes and myocardial fibers in all groups were examined under TEM. As a result, autophagosomes were found in control group, TH + H$_2$S group and H$_2$S group, while absent in TH group. Myocardial fibers were clearly observed in normally arranged without swelling in the control group. In the TH group, the myocardial fibers from some myocardial tissues were arranged disorderly and dropsically; and swelling of the myocardial fibers were observed as well. However, all these changes observed in TH group were obviously augmented in TH + H$_2$S group. Similarly, there was no significant changes were found between the control group and H$_2$S group (Fig. 4).

**Fig. 1** H$_2$S improves myocardial fibrosis in thyroxine rats. Morphological changes in myocardium assessed by eosin (HE) staining (a), Masson staining (b) and Collagen Volume Fraction (CVF) of the Masson staining (c). Images were acquired at ×400 magnification.

**Fig. 2** Effects of H$_2$S on collagen III expressions of thyroxine rats measured using immunohistochemical analysis.

**Effects of H$_2$S on MMP11, MMP12, MMP14, MMP17, TIMP1 and TIMP4 expressions of thyroxine rats**

Since dynamic balance between MMPs and TIMPs is closely correlated with the formation and degradation of collagen in myocardial tissues, Western blotting assay was performed to detect the changes in expressions of MMPs/TIMPs-related proteins in myocardial tissues. This then helps reveal the correlation between the colla-
Gen deposition in myocardial tissues and interstitial fibrosis. Western blotting assay results indicated protein expressions of MMP11, MMP12, MMP17, TIMP1 and TIMP4 were significantly upregulated, and however the protein expression of MMP14 was significantly decreased in TH group when compared with the control group. Compared with TH group, all the protein expressions of MMP11, MMP12, MMP17, TIMP1 and TIMP4 were significantly decreased, except the MMP14, which was upregulated in TH + H$_2$S group; However, in comparison between H$_2$S group and control group, there were no statistically significant differences were observed (Fig. 5).

**Effect of H$_2$S on autophagy protein markers expression in thyroxine rats**

Similarly, Western blotting assay covering detection of the protein expressions of ATG5, ATG7, ATG16L1, Beclin1 and LC3A in all groups, was also conducted to observe the level of autophagy in myocardial tissues and effect of H$_2$S on expression level of autophagy. The results showed that compared with the control group, protein expressions of ATG5, ATG7, ATG16L1, Beclin1 and LC3A in myocardial tissues of rat in TH group were significantly decreased; and on the contrary expressions of all these proteins in myocardial tissues of rat were significantly increased in TH + H$_2$S group when compared to TH group. While the comparison between H$_2$S group and the control group suggested that these two groups possess statistically comparable protein expressions of ATG5, ATG7, ATG16L1, Beclin1 and LC3A (Fig. 6).

**Effects of H$_2$S on the PI3K/AKT1 signalling pathway in thyroxine rats**

To determine the potential role of PI3K/AKT1 signaling pathway in the regulatory mechanism of myocardial fibrosis caused by high-concentration thyroxine, and autophagy, Western blotting assay was performed to measure the expressions of PI3K and AKT1 proteins, and the key proteins in PI3K/AKT1 signaling pathway. The results indicated that the protein expressions of PI3K and AKT1 in the myocardial tissues of rat in control group, TH group and TH + H$_2$S group were firstly significantly increased and then significantly decreased. However, the expression between H$_2$S group and the control group were almost same without statistical difference (Fig. 7).

**Effects of H$_2$S on miRNA21, miRNA34a, miRNA214, miRNA221 expression in thyroxine rats**

To figure out the potential role of miRNA21, miRNA34a, miRNA214 and miRNA221 in the occurrence and progression of hyperthyroid heart disease, and the effect of H$_2$S on expressions, RT-qPCR was
employed to detect the expressions of these miRNAs in myocardial tissues of rats in all groups. The original data of miRNA was provided in the supplementary file. The results revealed that when compared with the control group, the expressions of miRNA21, miRNA34a and miRNA214 in the myocardial tissues of rat in TH group

Fig. 5  Effects of H$_2$S on MMP11, MMP12, MMP14, MMP17, TIMP1 and TIMP4 expressions of thyroxine rats. Data are expressed as mean ± SD (n = 3). *p < 0.05 vs. Control group; †p < 0.05 vs. TH group.
were significantly increased, while the expression of miRNA221 was downregulated. Similar tendency for these all miRNAs was observed from TH group to TH + H\textsubscript{2}S group. Specifically, miRNA21, miRNA34a and miRNA214 were statically significantly increased and the expression of miR-221 was insignificantly increased. Similarly, the changes in expressions of all miRNAs in H\textsubscript{2}S group and the control group were statistically equivalent (Fig. 8).

**Discussion**

Hyperthyroidism may augment metabolism, and affect almost all of the physiological systems, especially the cardiovascular system, resulting in symptoms of hypertension, arrhythmia and cardiac hypertrophy. In some severe conditions, patients even suffer from hyperthyroid heart disease [16-18]. As for hyperthyroid heart disease, major pathological changes are associated with the direct toxicity and indirect effect of thyroxine on myocardium, including cardiac hypertrophy, myocardial fibrosis and dysfunction of left heart. Hyperthyroidism may activate the sympathetic nervous-catecholamine system and the RAAS system to induce the proliferation of cardiac fibroblast and mass deposition of collagen, thereby leading to the interstitial fibrosis in myocardium. Myocardial fibrosis, the major pathological change in myocardial remodeling, is closely associated with the microvascular injuries and damages of the myocardial structures, and also plays a critical role in the occurrence and progres-

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*Fig. 6 Effect of H\textsubscript{2}S on autophagy protein markers expression in thyroxine rats. Data are expressed as mean ± SD (n = 3). *p < 0.05 vs. Control group; #p < 0.05 vs. TH group.*
sion of hyperthyroid heart disease [19, 20]. In this study, a rat model of myocardial fibrosis was prepared under the induction of mass thyroxine through intraperitoneal injection of L-Thy. Based on this, it was then found that the collagen deposition was significantly increased in the myocardial tissues of rat with the induction of thyroxine through Masson-staining; meanwhile, imbalance in MMPs/TIMPs was also identified in the myocardial tissues of rat. Furthermore, immunohistochemistry result indicated that the expression of collagen III in the myocardial tissues was significantly increased after induction of thyroxine. As a whole, significant interstitial fibrosis occurred in the myocardial tissues after induction of thyroxine, which resulted in remodeling in myocardial

Fig. 7  Effects of H₂S on the PI3K/AKT1 signalling pathway in thyroxine rats. Data are expressed as mean ± SD (n = 3). *p < 0.05 vs. Control group; †p < 0.05 vs. TH group.

Fig. 8  Effects of H₂S on miRNA21, miRNA34a, miRNA214, miRNA221 expression in thyroxine rats. Data are expressed as mean ± SD (n = 3). *p < 0.05 vs. Control group; †p < 0.05 vs. TH group.
tissues and collagen deposition. Myocardial fibrosis has been considered as the key link in identifying the occurrence and progression of hyperthyroid heart disease, but the pathogenesis of myocardial fibrosis was induced by high concentration of thyroxine remains unclear.

Autophagy is a process of self-elimination of damaged proteins and organelles in cells, through which the content in cytoplasm could be degraded and reused by eukaryotes. Recent evidences show that in basal conditions, autophagy serves as a key homeostasis mechanism to maintain normal cardiovascular functions and morphology, as well as a major way of cell protection [21-23]. Dysfunction in autophagy is correlated with the occurrence of some myocardium-related diseases. The decreased autophagy usually results in the aggregation of abnormal proteins, thereby inducing dysfunction of cytoskeleton proteins and decrease in cellular functions, which finally contributes to cell death [24]. Xiao Y et al. reported that cucurbitacin-b can ameliorate the hyperthyroid and myocardial fibrosis in pressure-overload mouse cells [25]. Liu S et al. also suggested that activated autophagy can improve the myocardial fibrosis induced by angiotensin II [26]. Therefore, it is believed that autophagy is involved in cellular homeostasis and maintenance of cellular function, and plays a critical role in mechanisms of occurrence and progression of myocardial remodeling. ATG5 and ATG7 are the necessary genes that participate in autophagy in mammals [27]. In fact, due to insufficient energy [28], ATG5 and ATG7 gene-knockout mice are very likely to die. Although some mice survived, they may still suffered severe dilated cardiomyopathy. As key components in autophagy, LC3 in microtubule-associated protein, which makes up the isolating membrane of autophagosome, and Beclin1 exert critical roles in regulation of cell autophagy and serve as the markers of cell autophagy [29-31]. In this experiment, the rat models of myocardial fibrosis were prepared through the induction of thyroxine at high concentration, and upon which, a series of assays were performed to detect the levels of autophagy-related proteins of ATG5, ATG7, ATG16L1, Beclin1 and LC3A. The results showed that compared with the control group, the expressions of all these proteins were significantly decreased in myocardial tissues of rats, suggesting that the decrease of autophagy may trigger the occurrence and progression of myocardial fibrosis in rat with hyperthyroidism heart disease.

PI3K/AKT signaling pathway is a kind of classic pathways regulating cell autophagy, and is the key signaling transduction pathway in cells with extremely significant biological functions in cell apoptosis and autophagy [32, 33]. It can directly regulate the level of downstream mTOR to inhibit autophagy, thus participating in the regulation of cell proliferation, migration and differentiation [34, 35]. Existing studies showed that up-regulated expression of PI3K/AKT signaling pathway can suppress autophagy [36]. In the very study, obvious myocardial fibrosis and suppression in autophagy were found in myocardium of Hyperthyroidism rats, and the protein expressions of PI3K and AKT in PI3K/AKT signaling pathway were obviously upregulated. Therefore, it could be concluded that the mechanism of high-concentration thyroxine leading to myocardial fibrosis may be associated with the decreased autophagy caused by upregulated expression of PI3K/AKT signaling pathway.

Current studies suggest that miRNA involves in the development of heart and the differentiation of stem cells through translational repression [37]. While studies also indicated that miR-34a and miR-21 participate in the regulatory mechanism of cell autophagy. Some studies revealed that miR-21 can ameliorate hypoxia/reoxygenation-induced myocardial injuries by inhibiting cell autophagy via AKT/mTOR signaling pathway [38]. Similarly, there are studies confirming that the mechanism of miR-21 inhibiting autophagy is correlated with the downregulation of PI3K-AKT-mTOR signaling pathway [39]. Moreover, miR-34a inhibits cell autophagy by regulating the activity of TNF-α [40]. In addition, as demonstrated by a large number of studies, all miRNAs of miR-21, miR-34a and miR-214 are all involved in the regulatory mechanism of myocardial fibrosis [41-43]. The results of this study showed that under the stimulation of high-concentration thyroxine significant fibrosis was found in myocardium of rat, significant upregulations of the expression levels of miR-21, miR-34a and miR-214 in myocardial tissues were observed, as well as evident decrease of autophagy in myocardial tissues. Consequently, it is evident that myocardial fibrosis in rat induced by high-concentration thyroxine may be correlated with the inhibition of cell autophagy and myocardial fibrosis through the up-regulation of miR-21 and miR-34a expressions.

H₂S, a colorless gas with the characteristic odor of rotten eggs, is the third endogenous gas signal molecule with extensive physiological functions secondary to NO and CO. Existing studies have revealed that endogenous H₂S is involved in regulation of a series of physiological and pathological processes, and has extensive effects in
cardiovascular system to protect the heart, such as the anti-inflammation, anti-fibrosis and pro-angiogenesis [44]. Meanwhile, our previous studies also demonstrated that H$_2$S can improve the myocardial fibrosis in rat with diabetes mellitus, which is associated with the activation of cell autophagy [45]. However, there is no study focused on the role and relevant regulatory mechanism of myocardial fibrosis induced by high-concentration thyroxine. The results of this study showed that after H$_2$S intervention, collagen deposition in myocardial interstitium was significantly decreased under the stimulation of thyroxine at high concentration, and at the same time, the imbalance of MMPs/TIMPs was obviously ameliorated. Thus, it can be concluded that H$_2$S can improve the myocardial fibrosis of rat under the induction of high-concentration thyroxine. Additionally, researches also showed that under the induction of high-concentration thyroxine, the autophagy level in the myocardial tissues of rat was decreased, but after the intervention of H$_2$S, the expression of autophagy-related proteins in myocardial tissues of rat were significantly elevated. Moreover, some studies reported that in H$_2$S group, the protein expressions of PI3K and AKT, the key proteins in PI3K/AKT signaling pathway, were significantly decreased in rats compared with the model group. In other words, the mechanism of H$_2$S upregulating the autophagy is possibly associated with the downregulation of PI3K/AKT signaling pathway, and H$_2$S may activate cell autophagy by suppressing the PI3K/AKT signaling pathway. Furthermore, the study also found that the intervention of H$_2$S can significantly downregulated the expression levels of miR-21, miR-34a and miR-1 in myocardial tissues. Namely, H$_2$S may inhibit cell autophagy and myocardial fibrosis by downregulating the expressions of miR-21, miR-34a and miR-1.

In this study, it is found that by up-regulating the autophagy level, H$_2$S can ameliorate the myocardial fibrosis induced by thyroxine at high concentration. The mechanism of this may be associated with the downregulation of PI3K/AKT signaling pathway and expressions of miR-21, miR-34a and miR-214. All these results provide new evidence for better understanding of the pathogenesis of hyperthyroid heart disease, and suggest that H$_2$S, an endogenous gas signal, may be a new intervention target for prophylaxis and treatment of hyperthyroid heart disease and myocardial fibrosis. On the one hand, with these new knowledge a new drug that can release the endogenous gas signal of H$_2$S in the myocardial tissue may be developed as a novel approach to improve the heart function and to alleviate the myocardial fibrosis of hyperthyroid heart disease in some degree. On the other hand, PI3K/AKT and relevant miRNAs may be a potential a novel target for the treatment of hyperthyroid heart disease. For example, an inhibitor or activator of the PI3K/AKT signaling pathway which can regulate PI3K/AKT and relevant miRNAs may be developed, along with the gene knockout technology, to jointly intervene the signaling pathway related to our finding. However, more studies are further required to discover the specific molecular mechanism by which H$_2$S regulates the activity of PI3K/AKT signaling pathway and the expressions of relevant miRNAs and interferes with cell autophagy.

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**Competing Financial Interests Statement**

The authors declare no conflict of competing financial interest.

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