**Original Article**

**H₂S improves myocardial fibrosis caused by hyperthyroidism by downregulating stat pathways and regulating mir-21 and mir-29a expression**

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Received September 16, 2018; Accepted February 8, 2019; Epub May 15, 2019; Published May 30, 2019

**Abstract:** Objective: The aim of this study was to explore the effects of H₂S on myocardial fibrosis induced by hyperthyroxine and expression of STAT signal pathways, miR-21, and miR-29a in the myocardium. Methods: A total of 40 rats were selected and randomly stratified into 4 groups, including the control group (Control), HCM model group (Thy), H₂S-intervention model group (Thy+H₂S), and H₂S-intervention normal group (H₂S). Hyperthyroidism rat models were prepared by intraperitoneal injections of L-thyroxine. Rats in the control group received intraperitoneal injections of normal saline. NaHS solution was added to rats in the Thy+H₂S group and H₂S group. LVs, LVd, LVPWs, and EF values were measured by echocardiography, Pathological changes in myocardial cells and myocardial interstitium were observed by H&E staining. Collagen deposition in the myocardium was also observed by Masson’s staining. Protein expression of STAT1, STAT3, AKT1, TGF-β1, NFKB p65, PI3K, TIMP1, TIMP4, MMP12, MMP13, MMP16, and MMP24 was detected by Western blotting and expression of miR-21 and miR-29a in myocardial tissues was measured by real-time fluorescence quantitative PCR (RT-qPCR). Results: Compared with the control group, rats in the Thy group showed significant decreases in weight and rapid acceleration in heart rates (P<0.05). Findings of pathological examinations revealed that myocardial fibers were in disordered arrangement. Collagen deposition in the myocardium was significantly increased and values of LVs and LVd were significantly increased (P<0.05). Moreover, protein expression of STAT1, STAT3, AKT1, TGF-β1, NFKB p65, PI3K, TIMP1, TIMP4, MMP12, MMP13, and MMP24 in myocardial tissues was significantly upregulated (P<0.05). Expression of MMP12 and MMP16 was significantly downregulated (P<0.05). Comparisons between the Thy group and Thy+H₂S group suggested that the weight of rats in Thy+H₂S was significantly increased, accompanied by a reduction in heart rates (P<0.05). Also, the pathological examination showed that some improvement was identified in myocardial fibers in disordered arrangement and collagen deposition in the myocardium was significantly reduced. Moreover, values of LVs and LVd were significantly decreased (P<0.05), protein expression of STAT1, STAT3, AKT1, TGF-β1, PI3K, TIMP1, TIMP4, MMP13, and MMP24 was obviously downregulated (P<0.05), and expression of MMP12 and MMP16 was significantly upregulated (P<0.05). Conclusion: H₂S can ameliorate myocardial fibrosis induced by hyperthyroxine. Relevant mechanisms might be correlated with regulation of STAT3 signal pathways, downregulation of expression of miR-21, and upregulation of expression of miR-29a.

**Keywords:** H₂S, hyperthyroid cardiomyopathy, STAT, miR-21, miR-29a

**Introduction**

Hyperthyroid cardiomyopathy (HCM), a type of endocrinological cardiomyopathy, is characterized by a series of cardiovascular systems and vital signs caused by direct or indirect toxicity on the heart generated by excessive thyroid hormones. It can lead to variations, such as hypertension and arrhythmia or even structural reconstructions, like a decrease in systolic functions of heart and myocardial fibrosis [1-3]. However, signal transducers and activators of transcription (STAT) are involved in cardiomyocyte hypertrophy, regulation of myocardial energy metabolism, balance in extracellular matrix, and regulatory mechanisms of myocardial reconstruction [4]. One study showed that, compared with rats in the control group, expression
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of p-STAT in rats of the diabetic cardiomyopathy group was significantly upregulated, with obvious fibrosis in the myocardial interstitium [5]. Some studies have shown that STAT signaling pathway can promote the activation of PI3K/AKT signaling pathways [6] and inflammatory factors were found overexpressed. It was found that micro-RNAs, a type of short non-coding endogenous RNAs, are involved in the regulation of myocardial fibrosis [7]. A study found that miR-29 might be involved in the expression of collagen-I via Smad3-dependent TGF-β1 pathways [8]. Hydrogen sulfide (H\textsubscript{2}S) is a type of newly found endogenous gaseous signaling molecule. Its multiple biological effects include resistance to oxidative stress, fibrosis, and myocardial remodeling. Whether H\textsubscript{2}S can exert its protective effects in hyperthyroid cardiomyopathy or function in regulation via STAT pathways or miRNA remains unclear. Thus, the hyperthyroid cardiomyopathy rat models were built in this study to investigate the effects of H\textsubscript{2}S on myocardial fibrosis induced by hyperthyroidism and expression of STAT, miRNA-21, and miR-29a.

**Experimental material**

**Experimental animals**

A total of 40 adult male SD rats, with an average weight of (180±40) g, were purchased from Laboratory Animal Center of University of South China. Rats were fed in separate cages in a clean laboratory at (23±1)°C, during which the day and night were set to 12 hours, respectively. All rats could freely access water and food.

**Major experimental reagents**

NaHS, L-thyroxine, EDTA, Tris, and DEPC were purchased from Sigma, USA; BCA protein quantitation kit was purchased from Solarbio Co., Ltd., China; RIPA was provided by Beyotime Biotech Co., Ltd., China; Horseradish peroxidase-labelled goat anti-mouse IgG was purchased from Proteinintech Group, USA; Primary antibodies of GAPDH, T1MP1, T1MP4, MMP12, MMP13, MMP16, and MMP24 were provided by Boster Biological Technology Co., Ltd., China; STAT1, STAT3, AKT, NFKB p65, PI3K, and TGF-β1 were purchased from Cell Signaling Co., Ltd., USA; Reverse transcription kit of miRNA and reagent for extracting the free RNA were purchased from Cwbiotech, China; Taq enzyme, DL2000 DNA Marker and dNTP were purchased from Genestar, China; Design and synthesis of primers were performed by Sangon Biotech, Shanghai, China.

**Experimental methods**

**Establishing the animal models and grouping**

After being fed for 1 week, SD rats were randomly stratified into 4 groups, the control group (Control), HCM model group (Thy), H\textsubscript{2}S-intervention model group (Thy+H\textsubscript{2}S), and H\textsubscript{2}S-intervention normal group (H\textsubscript{2}S). Hyperthyroidism rat models were prepared by intraperitoneal injections of L-thyroxine (100 µg/kg/day). Rats in the control group underwent intraperitoneal injections of normal saline. NaHS solution (100 umol/kg) was given to rats for 4 weeks in the Thy+H\textsubscript{2}S group and H\textsubscript{2}S group, then left ventricular posterior wall thickness (LVPWs), left ventricular end diastolic diameter (LVd), left ventricular end systolic diameter (LVs), and ejection fraction (EF) were measured by echocardiography. After the continuous measurement of three cardiac cycle parameters, the mean value was calculated.

**HE staining detection**

Myocardial tissues in left ventricle were collected for rats in each group. They were then fixed in 4% paraformaldehyde solution, followed by rinsing with running water, dehydration, clearing with xylene, and embedding with paraffin. Subsequently, sections were then regularly dewaxed and hydrated for staining with hematoxylin. After free hematoxylin was removed by rinsing, 1% hydrochloric-alcohol solution was added onto the section for differentiation. Next, the sections were washed by warm water until turning blue. Moreover, 1% eosin was applied for counterstaining. Sections were then observed under the light microscope.

**Detecting collagen deposition via Masson’s staining**

Myocardial tissues in the left ventricle were collected for rats in each group. They were then fixed in 4% paraformaldehyde solution, followed by rinsing with running water, regular dehydra-
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Detection of target protein expression via Western blotting

From the -80°C refrigerator, rat hearts in each group were taken out to extract proteins. After quantification using the BCA method, protein samples were boiled for 5 minutes at 100°C. Next, 10% SDS-PAGE separation gel was prepared strictly following the proportions in instructions and then placed into the buffer for electrophoresis. Targeted stripes were transferred onto a membrane, which was then blocked with TBST. Next, primary antibodies of STAT1, STAT3, AKT, TGF-β1, NFKB p65, PI3K, TIMP1, TIMP4, MMP12, MMP13, MMP16, and MMP24 and GAPDH (1:1000) were added onto the membrane for incubation at 37°C. This was followed by incubation at 4°C overnight. Membranes were then washed with TBST and incubated with secondary antibody (1:2000) at 37°C. Subsequently, membranes were washed with TBST and treated by ECL for color development, followed by exposure and scanning. In this section, the primary antibody of GAPDH was selected as internal reference.

Detecting expression of miR-21 and miR-29a via RT-QPCR

Tissues preserved in TRIzol were ground and cracked. Subsequently, tissues were transferred into the chloroform for vibration, followed by standing and centrifugation. This study prepared the reaction solutions in accordance with the requirements of the kit and according to the instructions on reverse transcription. Moreover, cRNA was synthesized with the total RNA as the template. Also, negative control was set. Reverse transcription was performed as follows: 37°C for 15 minutes and 85°C for 5 seconds; cDNA reaction solution for synthesis was directly employed for fluorescence quantitative detection, in which the sequences of primers were as follows. Quantitative PCR reaction procedures were set as follows: 95°C for 10 minutes for 1 cycle; 95°C for 10 seconds, 59°C for 50 seconds, for a total of 40 cycles. Temperature was collected in real-time with the melting curve: 60°C-95°C. Results were studied.

Statistical analysis

SPSS 18.0 software was employed for statistical analysis. Data of observation indexes are denoted as mean ± SEM. LSD-t test was performed for intergroup comparisons and one-way ANOVA was conducted for analysis of randomized grouping design. P<0.05 suggests that differences are statistically significant.

Results

General conditions of rats in each group

Four weeks later, compared with the control group, rats in Thy group showed significant decreases in weight and rapid increases in heart rates (P<0.05). Compared with the Thy group, the weights and heart rates of rats in the Thy+H₂S group were respectively increased and slowed down (P<0.05). By comparing the control group and H₂S group, differences in weights and heart rates of rats showed no statistical significance (P>0.05) (Table 1). These results indicate that H₂S could increase BM and decrease HR caused by hyperthyroidism.

Detection results of echocardiography

LVs and LVD rose significantly in the Thy group and EF decreased significantly (P<0.05), com-

Table 1. Body weight and heart rate in each group (mean ± SD)

<table>
<thead>
<tr>
<th>Groups</th>
<th>Number</th>
<th>BW (g)</th>
<th>HR (/min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>10</td>
<td>191.60±6.17</td>
<td>343.10±34.58</td>
</tr>
<tr>
<td>Thy</td>
<td>9</td>
<td>173.33±7.76</td>
<td>542.56±21.16</td>
</tr>
<tr>
<td>Thy+H₂S</td>
<td>8</td>
<td>196.50±10.20*</td>
<td>445.25±22.82*</td>
</tr>
<tr>
<td>H₂S</td>
<td>10</td>
<td>188.00±11.55</td>
<td>343.00±29.95</td>
</tr>
</tbody>
</table>

Mean ± SD: *P<0.05 vs. the Control group; #P<0.05 vs. the Thy group.
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Table 2. Comparison of echocardiographic parameters in each group (mean ± SD)

<table>
<thead>
<tr>
<th>Groups</th>
<th>Number</th>
<th>LVd (mm)</th>
<th>LVs (mm)</th>
<th>LVPW (mm)</th>
<th>EF (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>10</td>
<td>4.33±0.09</td>
<td>2.67±0.12</td>
<td>0.90±0.05</td>
<td>69.90±6.14</td>
</tr>
<tr>
<td>Thy</td>
<td>9</td>
<td>5.63±0.03</td>
<td>4.10±0.12</td>
<td>1.00±0.08</td>
<td>65.44±5.55</td>
</tr>
<tr>
<td>Thy+H₂S</td>
<td>8</td>
<td>4.93±0.09</td>
<td>3.50±0.06</td>
<td>1.03±0.03</td>
<td>67.88±6.45</td>
</tr>
<tr>
<td>H₂S</td>
<td>10</td>
<td>4.30±0.11</td>
<td>2.40±0.07</td>
<td>0.87±0.33</td>
<td>68.80±5.75</td>
</tr>
</tbody>
</table>

Mean ± SD; *P<0.05 vs. the Control group; #P<0.05 vs. the Thy group.

Figure 1. Analysis of HE staining results for myocardial tissues; The result of HE staining in rats from each group (×400).

Analysis of H&E staining results for myocardial tissues

According to H&E staining results, in the control group, myocardium was in regular morphology and the structure was visible. In the Thy group, the diameter of myocardial cell increased and myocardial cells were disordered. Disordered arrangement in myocardial cells in the Thy+H₂S group was somehow ameliorated, compared with that in the Thy group (Figure 1). Results showed that the improvement of heart fibrosis caused by hyperthyroidism after H₂S intervention was significantly improved.

Effects of H₂S on protein expression of STAT3, TGF-β1, TIMP1, MMP12, and MMP16 in myocardium in rats

Western blotting detection revealed that, compared with the control group, significant upregulation was seen in the protein expression of STAT3, TGF-β1, and TIMP1 in myocardium in rats in the Thy group (P<0.05). Protein expression of MMP12 and MMP16 was significantly downregulated (P<0.05). Compared with the Thy group, significant downregulation was seen in the protein expression of STAT3, TGF-β1, and TIMP1 in myocardium in rats in the Thy group (P<0.05). Protein expression of MMP12 and MMP16 was significantly up-regulated (P<0.05) (Figure 3).

Detecting expression of miR-21 and miR-29a in myocardium of rats in all groups

According to RT-qPCR results, miR-21 expression in myocardium of rat in the Thy group was
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Significantly higher than that in the control group (P<0.05) and expression of miR-29a was significantly down-regulated (P<0.05). However, when compared with the Thy group, expression of miR-21 in myocardium of rats in the Thy+H₂S group showed a significant decrease (P<0.05) and expression of miR-29a was up-regulated (P<0.05). Comparing the H₂S group and the control group, there existed no statistically significant differences in expression of miR-21 and miR-29a (P>0.05) (Figure 4). Results indicate that H₂S could down-regulate miR-21 and up-regulate miR-29a to improve myocardial fibrosis.

Discussion

In general, pathological changes of hyperthyroid cardiomyopathy include cardiac hypertrophy, myocardial fibrosis, and left ventricular dysfunction, which are mainly associated with the toxicity of excessive thyroid hormones on myocardium. During the disease course of hyperthyroidism, renin-angiotensin-aldosterone system is activated to induce the proliferation of cardiac fibroblasts and synthesis and deposition of collagen, thus resulting in fibrosis in myocardial interstitium [9]. Matrix metalloproteinases are a Zn²⁺-dependent endopeptidase family and TIMPs are specific inhibitors of MMPs. Disproportionate MMPs/TIMPs will cause disorders in extracellular matrix, further leading to myocardial fibrosis. Existing studies have confirmed that disproportionate MMPs/TIMPs will induce myocardial fibrosis [10]. TGF-β1, a member of transforming growth factor family, can affect the synthesis and metabolism of extracellular matrix and bring about the excessive aggregation of extracellular matrix and potent pro-fibrosis effects. Occurrence of myocardial fibrosis is also associated with disproportionate MMPs/TIMPs and regulation of TGF-β1 signal pathways. Thus, TGF-β1 can induce myocardial fibrosis through facilitating the deposition of extracellular matrix. PI3K/AKT (phosphodylinsitol-3-kinase-series/thru kinase) signaling pathways play an important role in the prevention of myocardial ischemia-reperfusion injury and inhibition of apoptosis [11]. At the same time, it was found that STAT signaling pathways can promote the activation of PI3K/AKT signaling pathways. NFKB p65 (nuclear transcription factor-kb p65) is a group of nucleoprotein factors that regulate expression of a wide range of genes. NFKB p65 is vital for the regulation of gene transcription related to inflammatory response, cell proliferation, differentiation and apoptosis, immune response, and tumor formation [12]. In this study, significant upregulation was seen in the protein expression of STAT1, STAT3, TGF-β1, PI3K, AKT, TIMP1, TIMP4, MMP13, and MMP24 in myocardium in rats in the Thy group. Protein expression of MMP12 and MMP16 was significantly down-regulated, suggesting that myocardial fibrosis in rats with hyperthyroid cardiomyopathy might be correlated with the upregulation of TGF-β1 and disproportionate MMPs/TIMPs.

Moreover, miRNAs are a group of short, non-coding, and endogenous RNAs, in which miR-21, miR-26, miR-29, miR-30, and miR-133a are correlated with fibrosis [13]. Several studies revealed that miR-21 can promote myocardial fibrosis, yet significant improvement can be seen in myocardial fibrosis after the addition of antagonist of miR-21 [14]. The positive effects of miR-21 on myocardial fibrosis might be correlated with its involvement in extracellular matrix metabolism through adjusting the
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MMPs/TIMPs proportion [15]. In addition, a study reported the down-regulation of expression of miR-29 in the marginal zone of acute myocardial infarction, which can also induce the deposition of extracellular matrix and myocardial fibrosis [16]. In a study by Yuqing Huang et al., they further verified that, in patients with atrial fibrillation and CHF, expression of miR-29b is down-regulated, while expression of miR-29 in patients with myocardial remodeling is also down-regulated [17]. STAT, as a type of transcriptional regulatory factor, is closely correlated with cellular hypertrophy, cell regeneration, extracellular matrix metabolism, and regu-

Figure 3. Effects of H₂S on protein expression of STAT3, TGF-β1, TIMP1, MMP12, and MMP16 in myocardium in rats; Expression of MMP12, MMP16, TIMP1, TGF-β1, and STAT3 protein in the four groups (*P<0.05 vs. the Control group; #P<0.05 vs. the Thy group).
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A. Amplification curve

B. Amplification curve

C. Amplification curve

Logarithm RFU
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Hydrogen sulfide improves myocardial fibrosis induced by hyperthyroidism

Figure 4. Detecting expression of miR-21 and miR-29a in myocardium of rat in all groups; Expression of miR-21 and miR-29a in myocardial tissues from each group; Amplification curve of U6 (B) Melting curve of U6 (C) Amplification curve of miR-21 (D) Melting curve of miR-21 (E) Amplification curve of miR-29a (F) Melting curve of miR-29a (G) Comparison of expression of miR-21 and miR-29a. Means ± SD, *P<0.05 vs. 1, #P<0.05 vs. 2.
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lation in myocardial energy metabolism [18]. STAT can deliver the extracellular signals to cell nucleus, thereby affecting the transcription of targeted genes and regulating cell proliferation, differentiation, apoptosis and immunoregulation. Thus, it is one of the key downstream signal pathways of cytokines. Existing studies have found that STAT3 is involved in the regulation of miRNA expression, including miR-21, and participates in the fibrosis mechanism through affecting the activity of miR-21 to regulate MMPs/TIMPs levels [19, 20]. Results here revealed that, besides the significant myocardial fibrosis and disproportionate MMPs/TIMPs, rats in the Thy group also exhibited obvious upregulation in expression of miR-21 in myocardium and the downregulation in miR-29a. Yet, there was no significant increase in protein expression of STAT3, suggesting that STAT signal pathways and STAT-mediated changes in expression of miR-21 and miR-29a are probably involved in the pathogenesis of myocardial fibrosis in HCM rats.

H₂S is a type of newly found endogenous gaseous signaling molecule and its multiple biological effects include resistance to oxidative stress, fibrosis, and myocardial remodeling. Some studies have shown that, in myocardial ischemia reperfusion injury, H₂S can dramatically suppress the activity of STAT, thereby alleviating myocardial injuries [21]. By regulating the expression of miR-21, myocardial injuries caused by ischemia or inflammatory responses can now be ameliorated [22]. In the present study, it was found that, in comparison with the Thy group, in the Thy+H₂S group, diameter of myocardial cells of rats was reduced, disorder arrangement and deposition of myocardial fibers were improved, protein expression of STAT1, STAT3, TGF-β1, PI3K, AKT, TIMP1, TIMP4, MMP13, and MMP24 and expression of miR-21 was significantly decreased, and expression of miR-29a and protein expression of MMP12 and MMP16 was obviously up-regulated. Results suggest that H₂S can ameliorate myocardial fibrosis and disproportionate MMPs/TIMPs induced by hyperthyroidism, which might be correlated with downregulation of miR-21 and upregulation of miR-29a via regulating STAT signal pathways. Current results provide new evidence for investigating the pathogenesis of hyperthyroid cardiomyopathy and new intervention targets. However, further studies are needed to clarify its intrinsic regulation mechanisms.

Acknowledgements

This study was supported by grants from the National Natural Science Foundation of China, Hunan Provincial Graduate Research and Innovation Project, and the Science Foundation Project for Postgraduate student of the University of South China (Grant No. 81202830, Grant No. 81270181, Grant No. CK2018B635, and Grant No. 2018KY2063).

Disclosure of conflict of interest

None.

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References

[7] Younce CW, Wang K, Kolattukudy PE. Hyperglycaemia-induced cardiomyocyte death is medi-
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