Hydrogen Sulfide Regulates Cardiac Function and Structure in Adriamycin-Induced Cardiomyopathy

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Background: The present study was designed to investigate if hydrogen sulfide (H$_2$S), a novel gasotransmitter, might have a regulatory effect on cardiac function and structure, as well as oxidative stress, in adriamycin (ADR)-induced cardiomyopathy.

Methods and Results: Hemodynamic measurements, histopathological examination and stereological ultrastructural analysis of mitochondria in ADR-treated rats showed characteristics of cardiomyopathy with remarkable greater size and smaller number of cardiomyocytic mitochondria and a significantly low H$_2$S content in plasma and myocardium, but increased levels of thiobarbituric acid reactive substance (TBARs) and decreased superoxide dismutase (SOD) and glutathione peroxidase (GSH-Px) activities in plasma and myocardium compared with controls (P<0.01). However, administration of the H$_2$S donor, NaHS, markedly improved cardiac function, as demonstrated by elevated left ventricular developed pressure (+/–LVPd/dtmax; P<0.01) with ameliorated morphological alterations in the myocardium. Myocardial TBARs content decreased, whereas the activities of SOD and GSH-Px increased (P<0.01 and P<0.05, respectively).

Conclusions: Downregulation of endogenously-generated H$_2$S is probably involved in the pathogenesis of ADR-induced cardiomyopathy, whereby H$_2$S reduces lipid peroxidation, increases antioxidation, and inhibits oxidative stress injury. (Circ J 2009; 73: 741–749)

Key Words: Adriamycin; Dilated cardiomyopathy; Hydrogen sulfide; Oxidative stress

Adriamycin (ADR; doxorubicin) is an antitumor, broad-spectrum drug that has the potential for severe toxic side-effects on the heart, which may lead to dilated cardiomyopathy and congestive heart failure (CHF). One of the most important hypotheses offered to explain ADR-induced cardiomyopathy (ADR-CM) is that free reactive oxygen species (ROS) mediate cardiomyocyte damage and increased oxidative stress. ADR contains a quinone functional group that can undergo redox cycling between quinone and semiquinone. The semiquinone form interacts with molecular oxygen, which then initiates a chain reaction leading to the generation of free radical species, followed by cardiomyocyte injury and cardiomyopathy.

Hydrogen sulfide (H$_2$S) has been considered a toxic gas for some time, but is now being considered as a novel gasotransmitter, exerting various effects in the cardiovascular system. H$_2$S is produced endogenously from cysteine by pyridoxal-5'-phosphate-dependent enzymes, including cystathionine $\beta$-synthase (CBS) and/or cystathionine $\gamma$-lyase (CSE). Recent studies have shown that H$_2$S has important physiologic functions and a deficient CSE/H$_2$S pathway is involved in the development of some cardiovascular diseases. Whether the CSE/H$_2$S pathway is involved in the pathogenesis of cardiomyopathy is unclear.

The present study was undertaken to determine the effects of the CSE/H$_2$S pathway on cardiac function, the micro- and ultrastructure of myocardial tissue, generation of thiobarbituric acid reactive substance (TBARs, the marker of lipid peroxidation) and activities of the antioxidant enzymes, superoxide dismutase (SOD) and glutathione peroxidase (GSH-Px), in plasma and myocardial tissues of rats with ADR-CM. We thus aimed to investigate the regulatory role of the novel gasotransmitter, H$_2$S, in the pathogenesis of ADR-CM and the possible mechanisms involved.

**Materials**

Male Wistar rats (150–200g) were provided by the Animal Department of the Health Science Center of Peking University. All animal experiments were undertaken with the approval of the Animal Care Committee of Peking University First Hospital. ADR was produced by Wanle Pharmaceutical (Batch No. SFDA approval number: H44024359; Shenzhen, China). The H$_2$S donor, NaHS, was obtained from Sigma (St Louis, MO, USA). A stock solution of NaHS (100 mmol/L) was freshly prepared by dissolving NaHS immediately before use. Detection kits for TBARs content, and SOD and GSH-Px activities were purchased from Jiancheng BioEngineering (Nanjing, China). Other chemicals and reagents were of analytical grade.

**Methods**

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Animal Model of ADR-CM

Weight-matched adult male Wistar rats (n=54) were randomly divided into 5 groups based on treatment for 10 weeks as follows: (1) ADR group (n=12; 2.5 mg/kg ADR injected intraperitoneally (IP) at 09:00 h once a week); (2, 3) combined ADR and NaHS groups (n=12 each; 2.5 mg/kg ADR IP at 09:00 h once a week and NaHS (2.8 μmol·kg⁻¹·day⁻¹ as small dose; 14 μmol·kg⁻¹·day⁻¹ as large dose) once a day) immediately after the ADR was injected; (4) Control group (n=9; IP injections of an equivalent volume of physiological saline 1.25 ml/kg once a week); (5) NaHS-alone group (n=9; IP injections of 14 μmol/kg NaHS solution once a day).

After treatment, myocardial structure and cardiac function were determined, then the animals were killed and tissue samples were extracted and stored at −70°C.

Hemodynamic and Echocardiographic Measurements

After treatment, all animals were anesthetized with 2.5% pentobarbital sodium (40 mg/kg, IP), then fixed in the supine position. Catheterization was on 1 side into the left ventricle (LV) from the carotid artery, and the other side was connected to a polygraph system (PowerLab4S; AD Instruments Pty Ltd, Bella Vista, NSW, Australia) by a pressure transducer. The LV systolic pressure (LVSP), LV diastolic pressure (LVEDP), LV end-diastolic pressure (LVEDP), intraventricular pressure difference (ΔLVPP=LVSP−LVEDP), and LV peak rate of contraction and relaxation developed pressure (+/−LV dp/dtmax) were measured.

Preparation of Myocardial Samples for Optical and Electron Microscopy

The rats were weighed before death, and the hearts were dissected and weighed immediately after death. The free wall of the right ventricle (RV) was separated, detached from the septum and resected following the plane of curvature of the interventricular septum (IVS). The LV, LV + RV and LV + IVS were weighed separately with an electronic balance, and then the LV weight to body weight (LV/BW) ratio could be calculated as the LV mass index, while the heart weight to BW (HW/BW) ratio was taken as the heart mass index.

Samples of apical ventricular muscle were fixed and dehydrated with 10% formalin, imbedded in paraffin, sectioned in 5-μm increments, stained with hematoxylin–eosin, and observed using optical microscopy. Fresh myocardial tissues were cut into pieces 1 mm³, fixed with 3% glutaraldehyde, and flushed with phosphate-buffered saline, then fixed with 1% perosomic acid and dehydrated with acetone.

Ultrathin sections from each rat were selected at random for further stereological analysis in the transmission electron microscopy. Ten microscopic fields were randomly chosen per section and photographed. Estimation of the number mitochondrial per unit area (N) was performed by the point counting method as described previously.

Based on this method, the average diameter (D), volume density (VV), surface density (SV), numerical density (NV) and specific surface (Rsv, surface-to-volume ratio) of mitochondria were further calculated following B100 double-square lattice system with the number of test points Pt=400²

All the stereological evaluations were conducted with assistance from the Motic Digital Medical Image Analysis System (Motic Med 6.0; Motic Image Technology Co, Ltd, Beijing, China).

Measurement of H2S Concentration in Plasma

A modified sulfide electrode (PXS-270 ionometer, Pag/S1 Modified Sulfide Electrode; Shanghai Precision & Scientific Instrument Co, Ltd, Shanghai, China) was used for measurement of H2S. For the preparation of antioxidant and standard H2S solution, samples of plasma were obtained from fasting rats and combined with antioxidant solution of equal volume. The total volume encompassed the electrode (usually >0.8 ml). The electrode was activated in deionized water for at least 2 h. Next, the modified sulfide electrode and reference electrode were dipped into the sample solution. The electrode was flushed with deionized water after sample determination, and the activated state was maintained by dipping the electrode in the deionized water. The standard curve was determined with the standard S²⁻ solution before each determination, and the H2S concentration was calculated according to the standard curve.

Measurement of H2S Content in Myocardial Tissue

In this procedure, 50 mg of rat LV tissue was freshly stripped, and the fatty tissues on the outer membrane were removed. Saline was added to prepare a 10% homogenate (w/v). Next, 500 μl of homogenate was added to the reaction bottle (10 ml conical flask with a medium pore at the bottom, diameter 1 cm, height 0.8 cm), then 0.5 ml of 1 mol/L HCL was added to release H2S from the tissues. NaOH (0.5 ml of 1 mol/L) was added to the medium pore to absorb H2S, the flask was sealed quickly and then incubated in a 37°C shaking bath for 4 h. The medium pore solution was taken to determine the S²⁻ solution of the standard curve. The protein concentration was determined according to Coomassie blue staining in the tissue homogenate, and the H2S content in the tissue was expressed in nanomoles per milligram of protein.

Determination of lipid Peroxides and Antioxidant Enzyme Activity

The extent of lipid peroxidation (LPO) was determined by analyzing the levels of TBARs in the myocardial homogenate and plasma following the thiobarbituric acid reaction (TBA) assay. A commercially available kit for this assay was used. SOD and GSH-Px activities in the myocardial homogenate and plasma were determined according to the xanthine oxidase method and 5,5'-dithiobis-2-nitrobenzoic acid (DTNB) method provided by standard assay kits, as described previously.

Statistical Analysis

The results are expressed as the means±standard deviation. For comparison among groups, ANOVA with a Bonferroni correction was used with SPSS, version 11.0 (SPSS; Chicago, IL, USA). A value of P<0.05 was considered significant.

Results

Effect on BW and HW, and Their Ratio

After completion of the protocol, ADR-treated rats showed a conspicuous loss in BW, HW, LVW, LVW+IVS weight (IVSW) and LVW+RVW (39%, 32%, 30%, 25% and 26%...
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reductions from control rats, all, P<0.01, respectively). Co-treatment with NaHS (2.8 or 14.0 μmol · kg⁻¹ · day⁻¹, respectively, for 10 weeks) did not statistically increase any of these parameters mentioned (comparable to ADR group, P>0.05). The NaHS alone group had similar parameters to those of the control rats (P>0.05, respectively). Treatment with ADR resulted in remarkable elevation in both the HW/BW ratio and LVW/BW ratio of the ADR group (14% and 17% increase from control rats, both P<0.05, respectively). In the combined ADR+NaHS groups, the ratios of HW/BW and LVW/BW were approximately comparable to the ADR group values (P>0.05). NaHS treatment alone did not alter either HW/BW or LVW/BW in comparison with control rats (P>0.05) (Table 1).

H2S Content of Plasma and Myocardium

Compared with the control group, ADR group rats had a noteworthy reduction in the H2S content of both plasma and myocardium (32.32±2.57 vs 49.15±3.42 μmol/L in plasma, decreased by 34%, P<0.01) and (10.96±3.96 vs 20.22±3.03 μmol/mg protein in myocardium, 46% decline, P<0.01), respectively. Administration of NaHS (2.8 or 14.0 μmol · kg⁻¹ · day⁻¹, for 10 weeks) for ADR-treated rats elevated H2S levels by 46% in plasma (47.14±6.43 vs 32.32±2.57 μmol/L for

Table 1.  Comparison of HW Among the 5 Groups of Rats (±SD)

| Group | n  | Weight (g) | HW (mg) | LVW (mg) | LVW+IVSW (mg) | LVW+RVW (mg) | HW/BW (1×10⁻³) | LVW/BW (1×10⁻³) |
|-------|----|------------|---------|----------|---------------|--------------|----------------|----------------|----------------|
| Control | 9  | 458±36    | 1,056±119 | 821±70 | 0.79±0.08 | 0.98±0.05 | 2.192±0.094 | 1.73±0.124 |
| ADR    | 8  | 281±24    | 715±87    | 578±82 | 0.59±0.08 | 0.73±0.09 | 2.494±0.333 | 2.018±0.285 |
| Combined ADR+small-dose NaHS | 12 | 313±38    | 825±190 | 613±92 | 0.63±0.10 | 0.83±0.21 | 2.649±0.593 | 1.96±0.19 |
| Combined ADR+large-dose NaHS | 12 | 276±31   | 697±70.6 | 550±105 | 0.57±0.10 | 0.72±0.76 | 2.502±0.145 | 1.98±0.244 |
| NaHS   | 9  | 453±24    | 991±44.5 | 784±74 | 0.83±0.07 | 1.06±0.13 | 2.315±0.253 | 1.79±0.154 |
| F      |   | 62.89     | 17.75    | 16.871 | 15.221     | 15.521     | 1.924     | 2.120     |
| P value |     | <0.01     | <0.01    | <0.01   | <0.01      | <0.01      | <0.01     | <0.01     |

αP<0.01, βP<0.05 vs control group; ααP<0.01, αβP<0.05 vs combined ADR and small-dose NaHS group; αααP<0.01, ααβP<0.05 vs NaHS group.

HW, heart weight; LVW, left ventricular weight; IVSW, interventricular septum weight; RVW, right ventricular weight; HW/BW, HW to body weight ratio; LVW/BW, LVW to body weight ratio; ADR, adriamycin.
The myocardial H$_2$S contents in the combined ADR + small-dose and ADR + large-dose NaHS groups were significantly increased by 72% and 81%, respectively, compared with those of the ADR group (18.80 ± 2.1 vs 10.96 ± 3.96 and 19.81 ± 3.59 vs 10.96 ± 3.96 μmol/mg protein, P<0.01). NaHS treatment alone had no apparent effect on either plasma or myocardial H$_2$S content compared with the control group (P>0.05) (Figure 1).

As compared with the control group, cardiac functions were significantly inhibited in the ADR group. LVSP, ΔLVP, +LVdp/dtmax and –LVdp/dtmax of the ADR-treated rats were distinctly decreased by 30%, 44%, 56% and 59%, respectively, relative to control rats (all, P<0.01). Notable elevations of LVDP and LVEDP occurred in the rats receiving ADR treatment (with 162% and 67% increase from control values, both, P<0.01, respectively). Administration of NaHS (2.8 and 14.0 μmol · kg$^{-1}$ · day$^{-1}$, respectively, for 10 weeks) markedly prevented ADR-induced cardiac dysfunction, as demonstrated by the significant elevation of +LVdp/dtmax and –LVdp/dtmax in the combined ADR + small-dose (70% and 61% increase, P<0.01 and P<0.05, respectively) and +large-dose NaHS groups (67% and 62% increase, P<0.01 and P<0.05, respectively) compared with the ADR group. There was no significant difference in cardiac function between the NaHS alone group and control group or between the ADR + small-dose and ADR + large-dose NaHS groups (Table 2).

**Effect of NaHS Treatment on ADR-Induced Depression of Cardiac Function**

As compared with the control group, cardiac functions were significantly inhibited in the ADR group. LVSP, ΔLVP, +LVdp/dtmax and –LVdp/dtmax of the ADR-treated rats were distinctly decreased by 30%, 44%, 56% and 59%, respectively, relative to control rats (all, P<0.01). Notable elevations of LVDP and LVEDP occurred in the rats receiving ADR treatment (with 162% and 67% increase from control values, both, P<0.01, respectively). Administration of NaHS (2.8 and 14.0 μmol · kg$^{-1}$ · day$^{-1}$, respectively, for 10 weeks) markedly prevented ADR-induced cardiac dysfunction, as demonstrated by the significant elevation of +LVdp/dtmax and –LVdp/dtmax in the combined ADR + small-dose (70% and 61% increase, P<0.01 and P<0.05, respectively) and +large-dose NaHS groups (67% and 62% increase, P<0.01 and P<0.05, respectively) compared with the ADR group. There was no significant difference in cardiac function between the NaHS alone group and control group or between the ADR + small-dose and ADR + large-dose NaHS groups (Table 2).

**Table 2. Comparison of Cardiac Function Parameters Among the 5 Groups of Rats (±SD)**

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>LVSP (mmHg)</th>
<th>LVP (mmHg)</th>
<th>ΔLVP (mmHg)</th>
<th>LVEDP (mmHg)</th>
<th>+dp/dtmax (mmHg/S)</th>
<th>–dp/dtmax (mmHg/S)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>9</td>
<td>121.8±10.6</td>
<td>11.9±8.5</td>
<td>129.9±14.3</td>
<td>12.4±9.9</td>
<td>5,881.2±1,093.9</td>
<td>5,322.8±1,026.4</td>
</tr>
<tr>
<td>ADR</td>
<td>8</td>
<td>86.2±23.7a</td>
<td>31.2±11.4a</td>
<td>72.9±30.6a</td>
<td>20.4±14.4a</td>
<td>2,567.0±1,007.2a</td>
<td>2,157.1±1,036.5a</td>
</tr>
<tr>
<td>Combined ADR + small-dose NaHS</td>
<td>12</td>
<td>102.1±20.0a</td>
<td>27.3±14.1a</td>
<td>79.0±42.7a</td>
<td>12.3±8.4</td>
<td>4,370.9±1,137.7a</td>
<td>3,469.7±685.9a</td>
</tr>
<tr>
<td>Combined ADR + large-dose NaHS</td>
<td>12</td>
<td>103.3±13.7</td>
<td>33.6±13.7a</td>
<td>94.8±16.9</td>
<td>12.9±6.5</td>
<td>4,275.9±1,201.1a</td>
<td>3,494.3±1,075.0a</td>
</tr>
<tr>
<td>NaHS</td>
<td>9</td>
<td>136.2±15.3a</td>
<td>7.9±11.1</td>
<td>131.8±51.5</td>
<td>8.3±3.4</td>
<td>7,333.3±1,165.5a</td>
<td>5,724.1±1,083.4</td>
</tr>
<tr>
<td>F</td>
<td>6.21</td>
<td>8.934</td>
<td>3.874</td>
<td>2.721</td>
<td>5.8</td>
<td>7,333.3±1,165.5a</td>
<td>5,724.1±1,083.4</td>
</tr>
</tbody>
</table>

$^{a}P<0.01, ^{b}P<0.05$ vs control group; $^{a}P<0.01, ^{b}P<0.05$ vs ADR group; $^{a}P<0.01, ^{b}P<0.05$ vs NaHS group.

LVSP, left ventricular systolic pressure; LVP, left ventricular diastolic pressure; ΔLVP, intraventricular pressure difference; LVEDP, left ventricular end-diastolic pressure; +dp/dtmax, left ventricular peak rate of contraction; –dp/dtmax, left ventricular peak rate of relaxation. Other abbreviation see in Table 1.

**Figure 2.** Histopathological changes in the rat hearts. (A) Control group: myocardial architecture is intact with cardiomocytes lined up in order (HE, ×200). (B) Adriamycin (ADR) group: myofibrils are widely disintegrated, accompanied by multifocal degeneration and myocytolysis with diffused lymphocytes and monocytes infiltration (×200). (C) Combined ADR + small-dose NaHS group: most myofibrils have a clear structure, but there is sporadic focal necrosis with mild cellular edema (×200). (D) Combined ADR + large-dose NaHS group: myocardial morphology closely resembles that of the small-dose NaHS group (×200); (E) NaHS alone treatment shows no abnormalities in the myocardium (×200).
Effect of NaHS Treatment on ADR-Induced Myocardial Damage

Under optical microscopic observation, focal and diffuse areas of myocardial degeneration characterized by multiple vacuolation of cardiomyocytes, disorganization of myofibrils, disappearance of the myocardial pattern, and homogenization of the sarcoplasm were observed in the ADR group; the interstitium was edematous with extensive lymphocyte and monocyte infiltration. In the combined ADR + NaHS treatment rats, the cardiac structure disturbances were markedly improved as compared with the ADR group; the myocardial structure was clear and only sporadic focal necrosis with mild interstitial edema could be observed. No clear difference in the staining results was found between both groups of ADR + NaHS treatment. In the control group, the cardiomyocytes were regularly arranged with a clear structure of the myocardium, and no inflammatory cells were seen. Administration of NaHS (14 μmol·kg⁻¹·day⁻¹) alone did not cause any histological changes in rat myocardium compared with controls (Figure 2).

Under electron microscopic observation, degenerative changes with prominent cytoplasmic vacuolation and extensive interstitial edema were found predominantly in the myocardium of ADR-treated rats; myofibrils lost their parallel orientation and individual cardiomyocytes were hypertrophic with an irregular cell nucleus; chondrosomes were swollen and deformed with fractured or dissolved cristae; lamellar bodies and dense granules were observed in consumedly swollen chondrosomes; the sarcoplasmic reticulum and transverse tubular system were distinctly dilated and many packed cytolyosomes, lamellar bodies, a few lipid droplets and heaped vacuoles were seen. Combined

Figure 3. Ultrastructure of the rat cardiomyocytes. Control group (A×20,000 and B×40,000): normal histological structure of the myocardium. Adriamycin (ADR) group: (C×20,000): prominent myofilament disarray, in addition to marked cytoplasmic vacuolization accompanied by pronounced distension of the sarcoplasmic reticulum and focal lysis of cristae in the mitochondria; (D×40,000): at a higher magnification, hypertrophied cardiomyocytes with extensive interstitial edema, significant mitochondrial swelling, and lamellar bodies are seen. Combined ADR + small-dose NaHS group (E×20,000 and F×40,000): myofibrils show regular array of myofilaments, but the sarcoplasmic reticulum is slightly flattened with slightly swollen mitochondria and focal dissolution of cristae. Combined ADR + large-dose NaHS group (G×20,000 and H×40,000): mitochondria and myofibrils are better maintained but the sarcoplasmic reticulum is dilated. NaHS group (I×20,000 and J×40,000): ultrastructural morphology does not differ from that of the control group.
Table 3. Effects of NaHS on Parameters of Myocardial Mitochondria Among the 5 Groups of Rats (±SD)

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>N</th>
<th>(D) (μm)</th>
<th>(V_v) (μm^3)</th>
<th>(S_v) (μm^2)</th>
<th>(N_v) (μm^3)</th>
<th>(R_s) (μm^2)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>9</td>
<td>21.3±1.35</td>
<td>0.96±0.08</td>
<td>0.32±0.02</td>
<td>2.15±0.09</td>
<td>0.47±0.03</td>
<td>6.82±0.16</td>
</tr>
<tr>
<td>ADR</td>
<td>8</td>
<td>15.5±2.72</td>
<td>1.29±0.11</td>
<td>0.40±0.03</td>
<td>1.31±0.07</td>
<td>0.33±0.02</td>
<td>4.55±0.46</td>
</tr>
<tr>
<td>Combined ADR+small-dose NaHS</td>
<td>12</td>
<td>19.3±3.56</td>
<td>1.03±0.05</td>
<td>0.32±0.01</td>
<td>2.07±0.09</td>
<td>0.43±0.03</td>
<td>6.36±0.36</td>
</tr>
<tr>
<td>Combined ADR+large-dose NaHS</td>
<td>12</td>
<td>20.5±3.93</td>
<td>0.89±0.09</td>
<td>0.30±0.04</td>
<td>2.13±0.04</td>
<td>0.46±0.09</td>
<td>7.05±0.33</td>
</tr>
<tr>
<td>NaHS</td>
<td>9</td>
<td>21.8±1.87</td>
<td>0.97±0.08</td>
<td>0.36±0.01</td>
<td>2.22±0.04</td>
<td>0.48±0.04</td>
<td>6.11±0.46</td>
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</table>

F value  <0.05, P<0.01, P<0.05, and P<0.01, respectively; N, Sv, Nv and Rsv increased by 32%, P<0.01, P<0.05 and P<0.01, respectively; for large-dose NaHS, (N), Sv, Nv and Rsv increased by 23%, 14%, 30% and 40%, P<0.05, P<0.01, P<0.05 and P<0.01, respectively; for combined ADR and small-dose NaHS group, (N), Sv, Nv and Rsv increased by 25%, 14%, 30% and 40%, P<0.05, P<0.01, P<0.05 and P<0.01, respectively; for combined ADR and large-dose NaHS group, (N), Sv, Nv and Rsv increased by 25%, 14%, 30% and 40%, P<0.05, P<0.01, P<0.05 and P<0.01, respectively; compared with ADR-treated rats, NaHS treatment alone did not alter the mitochondrial ultrastructure (Table 3).

Table 4. Comparison of TBARs Content, and SOD and GSH-Px Activities in Plasma and Myocardium Among the 5 Groups of Rats (±SD)

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>Plasma TBARs (μmol/L)</th>
<th>Myocardial TBARs (μmol/g)</th>
<th>Plasma SOD activity (U/ml)</th>
<th>Myocardial SOD activity (U/mg)</th>
<th>Plasma GSH-Px activity (U/ml)</th>
<th>Myocardial GSH-Px activity (U/mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>9</td>
<td>3.97±0.546</td>
<td>4.34±0.640</td>
<td>123.68±0.968</td>
<td>139.15±1.919</td>
<td>137.21±3.237</td>
<td>38.66±5.182</td>
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<tr>
<td>ADR</td>
<td>8</td>
<td>5.29±1.062</td>
<td>6.27±0.942</td>
<td>103.17±4.434</td>
<td>105.07±2.951</td>
<td>104.67±5.230</td>
<td>25.53±8.975</td>
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<tr>
<td>Combined ADR+small-dose NaHS</td>
<td>12</td>
<td>5.13±0.965</td>
<td>6.14±0.697</td>
<td>103.68±1.721</td>
<td>107.88±3.515</td>
<td>116.03±2.479</td>
<td>26.17±1.920</td>
</tr>
<tr>
<td>Combined ADR+large-dose NaHS</td>
<td>12</td>
<td>4.72±0.731</td>
<td>5.08±0.996</td>
<td>117.32±10.64</td>
<td>116.07±2.757</td>
<td>126.74±3.172</td>
<td>35.00±6.648</td>
</tr>
<tr>
<td>NaHS</td>
<td>9</td>
<td>4.26±0.557</td>
<td>5.28±1.214</td>
<td>107.32±10.951</td>
<td>118.87±5.153</td>
<td>121.49±2.639</td>
<td>30.05±3.943</td>
</tr>
</tbody>
</table>

F value  <0.01, P<0.05 vs control group; P<0.05, P<0.01, P<0.05 vs ADR group; P<0.05 vs combined ADR and small-dose NaHS group; P<0.05 vs NaHS group.

ADR + NaHS treated rats did not appear to have significant cardiac damage, although occasional slightly swollen chondromes with focal loss of cristae were observed; the sarcoplasmic reticulum and transverse tubular system were slightly flattened and a few lipid droplets were seen. The cardiac ultrastructural changes in both groups of ADR + NaHS treatment were similar. In the control group, the myofibrils were compact, regularly aligned and interspersed by rows of mitochondria that had a normal appearance; the sarcoplasmic reticulum and transverse tubular system were well preserved. The myocardium of the rats having NaHS treatment alone showed no abnormalities (Figure 3).

Stereological analysis of cardiomyocytic mitochondria from the 5 groups of rats revealed a 34% increase in \(D\) and 25% in \(V_v\) of ADR-treated rats compared with the control values (both, P<0.01). Mitochondrial numbers per unit area (N), Sv, Nv and Rsv of the rats in the ADR group were reduced by 27%, 16%, 30% and 33%, respectively (all, P<0.01), as compared with control rats. Administration of NaHS (2.8 and 14.0 μmol·kg\(^{-1}\)·day\(^{-1}\), respectively, for 10 weeks) markedly attenuated ADR-induced mitochondrial swelling and deformation as exhibited by a significant reduction in \(D\) and \(V_v\), and marked increases in N, Sv, Nv and Rsv of mitochondria from the combined ADR+NaHS groups (for small-dose NaHS treatment, \(D\) and \(V_v\) reduced by 20% and 20%, respectively, both P<0.01; N, Sv, Nv and Rsv increased by 25%, 14%, 30% and 40%, P<0.05, P<0.01, P<0.05 and P<0.01, respectively; for large-dose NaHS group, \(D\) and \(V_v\) decreased by 31% and 25%, respectively, both P<0.01; N, Sv, Nv and Rsv increased by 32%, 18%, 39% and 55%, P<0.05, P<0.01 and P<0.01, respectively) compared with ADR-treated rats. NaHS treatment alone did not alter the mitochondrial ultrastructure (Table 3).

**Effect of NaHS Treatment on ADR-Stimulated TBARs Formation**

ADR administration notably reduced the plasma and myocardial TBARs levels by approximately 33% and 44% (P<0.01), respectively, compared with control rats. When combined with NaHS treatment the plasma TBARs content was lowered slightly (~3% and ~12% for small- and large-dose groups, respectively), but had no statistical significance (all, P>0.05) compared with ADR group. Co-treatment with large-dose NaHS (14 μmol·kg\(^{-1}\)·day\(^{-1}\)) significantly reduced the myocardium TBARs content by approximately 23% (P<0.01) compared with the rats that received ADR only. The plasma and myocardium TBARs levels in the NaHS alone group were virtually the same as in the control group (Table 4).

**Effect of NaHS Treatment on ADR-Induced Decrease in SOD Activity**

ADR treatment resulted in approximately 20% reduction in plasma activity and 23% in myocardial SOD activity compared with control rats (all P<0.01). Administration of NaHS (14 μmol·kg\(^{-1}\)·day\(^{-1}\), large-dose) markedly increased the plasma SOD activity by approximately 14% compared with rats treated with ADR only (P<0.01). Surprisingly, the plasma and myocardial SOD activities in the NaHS alone group were decreased significantly as compared with those in the control group (P<0.01 and P<0.05, respectively) (Table 4).

**Effect of NaHS Treatment on ADR-Induced Reduction of GSH-Px Activity**

When compared with the control group, GSH-Px activities in the plasma and myocardium were reduced markedly (P<0.05 and P<0.01) in ADR rats. A notable elevation in myocardial GSH-Px content, amounting to approximately...
37%, was observed in the combined ADR+large-dose NaHS group as compared with the ADR group (P<0.05). The myocardial GSH-Px activity in the NaHS alone group was, however, significantly lower than the control level (P<0.05) (Table 4).

**Discussion**

Ventricular remodeling is the pathologic basis of cardiomyopathy. It induces increased cardiac muscle mass, increased ventricular cavity volume, and changes in ventricular diameter (increased transverse diameter), which result in progressive cardiac dysfunction. ADR can cause cumulative dose-dependent cardiotoxicity, including ADR-CM, and even CHF. Most studies support the view that both an increase in oxidative stress and a decrease in antioxidants plays an important role in the pathogenesis of ADR-CM. However, the precise mechanisms of ADR-induced myocardial injury have not been defined.

In this study, we successfully established ADR-CM in rats. After continuous ADR administration for 10 weeks, rats showed inanite behavior, decreased body temperature, physical activity and food intake, increased rate of breathing, and significant weight loss. Cardiac function was significantly inhibited, as demonstrated by significantly decreased LVSP, ΔLVP, +/−LVdp/dtmax and elevated LVDP and LVEP. The ratios of HW/BW and LVW/BW increased remarkably. Between weeks 8 and 10, 4 of the 12 rats in the ADR group died of CHF before the predetermined endpoint of the dissection day. No deaths occurred in the other groups. The micro- and ultrastructure of the myocardium in the ADR-treated rats changed markedly. Quantitative analysis of the mitochondrial morphology indicated that D and Vv were markedly increased and N, Sv, Nv and Rsv were significantly decreased as compared with the control rats. These results revealed that the myocardial mitochondria of ADR-treated rats were greater in size and smaller in number than in the control group, which correlated with the alterations of the mitochondria observed under electron microscopy. Thus, we successfully created a rat model of cardiomyopathy induced by ADR.

In recent years, growing evidence has suggested that H,S is an endogenous gasotransmitter, characterized by continuous generation, fast transmission, expansive action, and rapid inactivation. In our previous studies, we found that the endogenous CSE/H,S pathway was involved in the pathophysiological processes of many cardiovascular diseases. However, the role of endogenous H,S in the pathogenesis of cardiomyopathy has not been reported. Endogenous H,S is the final metabolite of sulfur-containing amino acids in the body and, in vivo, it exists in both the gaseous form and the NaHS dissolved form. NaHS can be hydrolyzed into Na+ and H,S, the latter being able to bind H+ in the body to produce H,S, which forms a dynamic equilibrium with NaHS. The H,S concentration in a NaHS solution is stable, and NaHS solution has been widely used in various experiments as a H,S donor.

In the rats with ADR-induced cardiomyopathy, the H,S contents of plasma and myocardium were significantly lower than those in the control group. Furthermore, to examine the possible role of reduced production of endogenous H,S in the pathogenesis of ADR-CM, supplying the exogenous H,S donor NaHS (14 μmol·kg⁻¹·day⁻¹) elevated the plasma H,S level by 46%. With combined ADR and a small- or large-dose of NaHS, the H,S myocardial content was increased by 72% and 81%, respectively, compared with ADR alone. The fact that the T1/2 of H,S in plasma is much shorter than it is in myocardium probably makes the difference between the plasma and myocardial tissue content of H,S in small-dose NaHS-treated rats. Administration of NaHS markedly prevented ADR-induced cardiac dysfunction, as demonstrated by the significant higher +/−LV dp/dtmax with combined ADR and small- or large-dose treatment than with ADR alone. Thus, after NaHS treatment, cardiac pathologic changes of the ultra- and microstructures were obviously alleviated. Stereological analysis of cardiac myocytic mitochondria showed notable reduction in D and Vv and remarkable increases in N, Sv, Nv and Rsv compared with ADR-treated rats. These results are consistent with increased morphological mitochondrial injury. Administration of NaHS alone (14 μmol·kg⁻¹·day⁻¹) did not cause any damage to either cardiac function or structure (including the ultrastructure of mitochondria). Taken together, these findings suggest that decreased production of endogenous H,S could be involved in the pathogenesis of ADR-CM.

Cardiac function is highly dependent on oxidative energy generated in the mitochondria and mitochondria are susceptible to oxidative damage. The sensitivity of myocardial tissue to ADR may be partially related to its high energy demand and thus it is likely to be more dependent on the energy-generating function of intact mitochondria than other organs. In our study, the significantly increased size and decreased number of mitochondria in the heart of rats treated with ADR may be linked to its vulnerability, because mitochondria are a major target of ADR toxicity. The onset and severity of myocardial injury induced by ADR correlates with mitochondrial ROS production. Therefore, in the development of ADR-CM, mitochondrial damage is thought to be the critical and early event that subsequently results in other subcellular changes. In the rats with our combined ADR+NaHS treatment, mitochondria were markedly decreased in size and the mean number of mitochondria per unit area was increased compared with the ADR group rats, which may show a link between H,S-mediated cardioprotection and ADR-induced mitochondrial injury. The exact mechanisms need to be further investigated.

Overproduction and/or ineffective scavenging of ROS may play a crucial role in the pathogenesis of ADR-CM. LPO is a consequence of oxidative stress injury and has been considered a major mechanism of ROS attack. An increased level of ROS may stimulate LPO, thereby causing oxidative damage to the cell membrane and other subcellular components, which ultimately leads to myocardial degeneration, necrosis, and interstitial changes. TBARs are the main products of LPO, thus the TBARs level is commonly used as an indicator of LPO formation and as a biomarker of oxidative stress. An elevated level of TBARs can reflect enhanced membrane LPO. In addition, cells are protected against oxidative insult by diverse antioxidant enzymes, such as SOD and GSH-Px. H,S could decrease ROS generation, improve cell viability, activate the ATP-dependent K⁺ (KATP) and Cl⁻ channels, and increase the levels of glutathione participating in the regulation of oxidative stress. Moreover, H,S is a highly reactive molecule and may easily react with other compounds, especially ROS and reactive nitrogen species (RNS). Indeed, H,S reacts with at least 4 different ROS [superoxide radical anion, H₂O₂, peroxynitrite and hypochlorite]. All these compounds are highly reactive and their interaction with H,S results in the protection of proteins and lipids against...
ROS/RNS-mediated damage. In our previous work, we reported that H₂S could directly scavenge oxygen-free radicals and reduce the accumulation of lipid peroxides. Besides, as in the oxygen-free radical-induced in vitro myocardial injury of our previous experiment, we discovered that the H₃S donor, NaHS, could inhibit LPO injury in the myocardium induced by oxygen-free radical generation. In the present study, LPO was estimated by the appearance of TBARs, using the modified thiobarbituric acid (TBA) method that quantifies a chromogen produced by the reaction of TBA with malondialdehyde (MDA) under acidic conditions, and the pink chromogen (MDA-TBA₂ adduct) is measured by spectrophotometry at a wavelength of 535 nm. The TBARs level is determined from the MDA equivalence standard. Although the specificity of the TBA assay toward compounds other than MDA is controversial, it continues to be the most widely used method of screening and monitoring LPO. Our study demonstrated a remarkable increase in TBARs content and a distinct decrease in the SOD and GSH-Px activities in the plasma and myocardium of rats exposed to ADR. With co-administration of large-dose NaHS, the myocardial TBARs level was significantly reduced by 17% and the plasma SOD activity was higher by 14% compared with ADR alone. Surprisingly, the plasma and myocardial SOD activities were significantly lower in the NaHS-alone group than in the control group. The myocardial GSH-Px activity was higher by 37% in the combined ADR + large-dose NaHS group than in the ADR-alone group, but was lower by 21% in the NaHS-alone group compared with the control group. Studies have shown cytotoxicity with H₂S overdosing, which could deplete GSH and activate oxygen to form ROS. Therefore, we suggest that in ADR-treated rats, the endogenous H₂S level decreased, and exogenous administration of NaHS exerted a beneficial effect by increasing the activity of antioxidant enzymes and inhibiting oxidative stress injury, but in normal rats with NaHS-alone treatment, long-term administration of exogenous H₂S could have the reverse effect. The exact mechanisms of this process deserve further study.

In light of our results, we suggest that the endogenous H₂S level was decreased in rats with ADR-CM, and that the downregulated H₂S enhanced LPO and decreased the capability of scavenging oxygen-free radicals. Accumulated peroxide levels could contribute to cardiac dysfunction and myocardial injury. Therefore, reduced endogenous H₂S production plays an important role in the pathogenesis of ADR-CM. Combined with previous research findings, we propose that H₂S treatment may have beneficial effects in modulating myocardial structure and cardiac functions, attenuating ADR-induced ultrastructural mitochondrial injury, at least in part, by reducing the accumulation of lipid peroxides and improving the activity of endogenous antioxidant enzyme systems. However, the precise mechanisms still need to be investigated.

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