

Effects of hydrogen sulfide on inducible nitric oxide synthase activity and expression of cardiomyocytes in diabetic rats

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Abstract. The aim of the present study was to investigate the effects of hydrogen sulfide (H₂S) on the activity and expression of inducible nitric oxide synthase (iNOS) in the myocardial tissue of type 1 diabetic rats. Rats were divided randomly into four groups: Normal control (NC), diabetes mellitus (DM), DM+DL-Propargylglycine (DM+PAG) and DM+sodium hydrosulfide (DM+NaHS) groups. Type 1 diabetes was induced in the respective groups by a single intraperitoneal (i.p.) injection of streptozotocin. Rats in the DM+PAG and DM+NaHS groups were injected with PAG and NaHS (i.p.) once a day, respectively. The level of fasting blood glucose (FBG), the heart-weight to body-weight (HW/BW) ratio and the ventricular hemodynamic parameters were measured. The activities of serum total NOS (tNOS), iNOS, lactate dehydrogenase (LDH), creatine kinase (CK) and creatine kinase MB isozyme (CK-MB), and the content of nitric oxide (NO) were detected. The contents of myocardial malondialdehyde (MDA) and NO, and the activities of superoxide dismutase (SOD), tNOS and iNOS were determined. The myocardial tissue was examined for histological and ultrastructural alterations. The expression level of iNOS at the transcriptional and translational levels in the myocardial tissue was estimated. The level of FBG was increased in the DM group compared with the NC group, verifying the diabetic condition of the rats. The function of the left ventricle, the myocardial histological alterations and ultrastructures were damaged in the DM group. The DM group additionally demonstrated an increase in the serum NO content and tNOS, iNOS, LDH, CK and CK-MB activities. The myocardial MDA, NO content and tNOS levels were additionally increased in this group. The iNOS activity was increased significantly whereas the

myocardial SOD activity was decreased. The increase in the iNOS activity was supported by an enhanced expression level of myocardial iNOS mRNA and protein in the DM group. In the DM+PAG group, in the absence of H₂S, the dysfunction of the left ventricle and the oxidative stress injury were increased compared with the DM group. The activity and the expression of tNOS and iNOS were increased significantly. However, the rats in the DM+PAG group demonstrated the opposite effects. In conclusion, H₂S exhibits a protective effect on the myocardium in type 1 diabetic rats, which may be associated with the suppression of iNOS activity and expression, a decrease in the NO content and the inhibition of oxidative stress injury.

Introduction

Diabetes mellitus (DM) is a metabolic disease that causes hyperglycemia and a number of serious complications, including cardiovascular disease. Diabetes coupled with cardiovascular disease is a leading cause of morbidity and mortality (1,2). Diabetic cardiomyopathy (DCM) is characterized by a decreased diastolic and systolic myocardial function, independent of coronary artery disease and hypertension, with a risk of heart failure (3). Previous studies have shown that hyperglycemia, lipid accumulation, oxidative stress, calcium overload, mitochondrial dysfunction, myocardial inflammation and apoptosis may be involved in the pathogenesis of DCM (4,5).

Nitric oxide (NO), carbon monoxide (CO) and hydrogen sulfide (H₂S) are members of the modern family of gaso-transmitters, which play important regulatory roles in the body (6). NO, the first identified gaseous signaling molecule that can be produced from L-arginine, is catalyzed by the NO synthase (NOS). There are various isoforms of NOS (i.e., nNOS, iNOS, eNOS) in different types of mammalian cells. As an endothelium-derived relaxing factor, physiologically low concentrations of NO can cause the opening of sarco-plasmic reticular ryanodine receptor Ca²⁺ channels to enhance myocardial contractility. However, high concentrations of NO induce the production of low amounts of cGMP, which reduce myocardial contractile function (7,8). NO is thereby considered to be involved in the regulation of cardiac function.

In the past, H₂S was considered as a toxic gas with the odour of rotten eggs. Now H₂S, the third gas signaling molecule

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identified after NO and CO, can be generated endogenously by pyridoxal-5'-phosphate-dependent enzymes in the mammalian body, and has a variety of biological functions in tissues and organs (9). In recent years, much evidence has demonstrated that H₂S plays a critical role in the physiology and pathophysiology of the cardiovascular system (10,11). DL-Propargylglycine (PAG) is an irreversible inhibitor of the H₂S synthesizing enzyme-cystathionine- γ -lyase (CSE). PAG can block H₂S synthesis activity, thereby decreasing the level of endogenous H₂S *in vivo*, while sodium hydrosulfide (NaHS) serves as a donor of exogenous H₂S. In a previous study (12), we reported that exogenous H₂S could alleviate myocardial injury in diabetic rats; however, the mechanism remains unclear. Several studies have reported that H₂S can inhibit the activity of NOS, hence reducing the NO content (13). In the present study, we investigated the protective effects of H₂S on the myocardial tissue in diabetic rats, and whether it is associated with the inducible NOS (iNOS)/NO system in the myocardium.

Materials and methods

Animals. Male Sprague-Dawley rats (160-200 g) were purchased from the Animal Center of Bengbu Medical College (Bengbu, Anhui, China). The rats were fed normal diet and had free access to water. All rats were housed in cages at room temperature (22±1°C) with a fixed 12 h light/dark cycle. All animal procedures were conducted in accordance with the United States National Institutes of Health Guide and were approved by the Animal Use and Care Committee of Bengbu Medical College.

Materials and reagents. Streptozotocin, PAG and NaHS were purchased from Sigma-Aldrich (St. Louis, MO, USA). Malondialdehyde (MDA), superoxide dismutase (SOD), lactate dehydrogenase (LDH), creatine kinase (CK), creatine kinase MB isozyme (CK-MB), NO and NOS typed assay kits were purchased from Nanjing Jiancheng Bioengineering Institute (Nanjing, Jiangsu, China). TRIzol (total RNA extraction reagent) was purchased from Invitrogen (Carlsbad, CA, USA). iNOS and β -actin primers were synthesized by Shanghai Sangon Biological Engineering Co., Ltd., (Shanghai, China). The primers for iNOS were 5'-ATCCCCGAAACGCTACACT T-3' and 5'-TCTGGCGAAGAACAATCC-3', the amplified fragment length was 314 bp; the primers for β -actin were 5'-GATGGTGGGTATGGGTTCAGAAGGAGG-3' and 5'-GCT CATTGCCGATAGTGATGACC-3', the amplified fragment length was 632 bp. Rabbit iNOS antibody was purchased from Abcam (Cambridge, MA, USA). Rabbit β -actin antibody was purchased from Proteintech Group, Inc. (Wuhan, Hubei, China). Goat anti-rabbit secondary antibody was purchased from Biosharp Company (Hefei, China).

Experimental methods

Induction of diabetes and experimental protocol. Animals were divided randomly into four groups: Normal control (NC) group, diabetes mellitus (DM) group, DM+DL-Propargylglycine (DM+PAG) group and DM+sodium hydrosulfide (DM+NaHS) group ($n=8$). Diabetes was induced in overnight fasted rats by administering a single intraperitoneal (i.p.) injection

of 55 mg/kg streptozotocin freshly dissolved in 0.1 mol/l sodium citrate buffer (pH 4.5). The rats in the NC group were injected with a similar volume of sodium citrate buffer alone. The rats with plasma fasting blood glucose (FBG) level over than 16.7 mmol/l (72 h after injection) were considered as diabetic rats. After successful building models, the rats in the DM+PAG and DM+NaHS groups were injected i.p. with PAG (40 mg/kg/day) and NaHS (56 μ mol/kg/day) for 8 weeks, respectively. The rats in the NC and DM groups were injected with a similar volume of normal saline.

Detection of FBG level, body-weight (BW), heart-weight (HW) and HW/BW. The plasma FBG level and BW were measured at the end of the 8th week. HW was measured after the rat was sacrificed. The HW/BW ratio was calculated.

Hemodynamic measurements. After treatment for 8 weeks, all rats were anesthetized with i.p. 4% chloral hydrate (1 ml/100 g), then fixed in the supine position. A tracheal cannula was used to connect the rats to a breathing machine (tidal volume 2-3 ml/100 g, respiratory rate 70-80 times/min). Meanwhile, the right carotid artery was intubated to the left ventricle, connecting and opening the Med-Lab Biological Recording system (Nanjing MedEase Technology Co., Ltd., Nanjing, China). Left ventricular systolic pressure (LVSP), left ventricular end-diastolic pressure (LVEDP) and maximal rise/fall rate of left ventricular pressure ($\pm dp/dt_{max}$) were recorded.

Serum NO content and tNOS, iNOS, LDH, CK, CK-MB activities measurement. At the end of hemodynamic measurements, arterial blood was collected and placed in centrifuge tubes and centrifuged at 3,000 rpm/min for 20 min. The supernatant fluid was collected. NO content and total NOS (tNOS), iNOS, LDH, CK, CK-MB activities were measured by commercially available kits according to the manufacturer's instructions.

Myocardial NO, MDA contents and tNOS, iNOS, SOD activities measurement. At the end of the experimental period, 0.1 g myocardial tissue was homogenized in 0.9 ml ice-cold normal saline. The supernatant was collected after centrifugation for 20 min (3,000 rpm/min). The protein concentration was measured by the bicinchoninic acid (BCA) Protein Assay kit. NO, MDA contents and tNOS, iNOS, SOD activities were estimated in the myocardial tissue according to the instruction manual, separately.

Histological observation by hematoxylin and eosin (H&E) staining. For histological analysis by light microscope, the myocardial tissue was harvested and fixed in 4% paraformaldehyde for 24 h, embedded in paraffin and cut into 5 μ m thick serial sections, and then stained with H&E for light microscope observation.

Ultrastructure observation under transmission electron microscope. Hearts were dissected and small pieces were fixed with 2.5% glutaraldehyde in 0.1 mol/l cacodylate buffer for 2 h, post-fixed in 1% osmium tetroxide in 0.1 mol/l cacodylate buffer for 1 h. Ultrathin sections were cut and contrasted with uranyl acetate followed by lead citrate and

Table I. Levels of FBG, BW, HW and HW/BW in the different groups.

Group	FBG (mmol/l)	BW (g)	HW (mg)	HW/BW (mg/g)
NC	5.67±0.91	437.19±27.13	1,266.87±110.22	2.96±0.25
DM	25.17±3.79 ^a	217.50±20.81 ^a	810.68±50.98 ^a	3.78±0.31 ^a
DM+PAG	26.23±3.82	189.73±18.42 ^b	779.21±42.43	4.15±0.35 ^b
DM+NaHS	22.04±3.66	270.16±24.73 ^c	1,017.45±82.39 ^c	3.34±0.32 ^c

Data were expressed as the mean ± standard deviation (n=8). ^aP<0.01 vs. NC group; ^bP<0.05, ^cP<0.01 vs. DM group; NC, normal control group; DM, diabetes mellitus group; DM+PAG, DM treatment with PAG group; DM+NaHS, DM treatment with NaHS group; FBG, fasting blood glucose; BW, body-weight; HW, heart-weight; HW/BW, heart-weight to body-weight ratio.

Table II. Alterations of ventricular hemodynamic parameters in the different groups.

Group	LVSP (kPa)	LVEDP (kPa)	+dp/dt _{max} (kPa/s)	-dp/dt _{max} (kPa/s)
NC	10.95±1.08	0.22±0.04	645.29±57.45	456.43±47.37
DM	7.67±0.67 ^a	0.43±0.07 ^a	310.90±43.80 ^a	249.10±28.75 ^a
DM+PAG	6.54±0.62 ^b	0.51±0.06 ^c	259.34±40.71 ^b	207.56±27.08 ^b
DM+NaHS	8.82±0.85 ^c	0.34±0.05 ^c	483.45±52.12 ^c	393.14±40.33 ^c

Data were expressed as the mean ± standard deviation (n=8). ^aP<0.01 vs. NC group; ^bP<0.05, ^cP<0.01 vs. DM group; NC, normal control group; DM, diabetes mellitus group; DM+PAG, DM treatment with PAG group; DM+NaHS, DM treatment with NaHS group; LVSP, left ventricular systolic pressure; LVEDP, left ventricular end diastolic pressure; +dp/dt_{max}, maximal rise rate of left ventricular pressure; -dp/dt_{max}, maximal fall rate of left ventricular pressure.

observed with JEM-1230 transmission electron microscope from Jeol, Ltd. (Tokyo, Japan).

Detection of levels of iNOS mRNA by reverse transcription polymerase chain reaction (RT-PCR). RT-PCR was used to detect the expression of iNOS mRNA in myocardium. Briefly, total RNA was extracted with TRIzol according to the manufacturer's instructions. Executing sequentially RNA detection, reverse transcription reaction, PCR amplification (95°C pre-denaturation for 3 min; 95°C denaturation for 50 sec; β-actin: 59.4°C, iNOS: 53.9°C annealing for 50 sec; 72°C extension 60 sec; 72°C balancing for 10 min after 35 cycles; 4°C heat preservation). The PCR products were analyzed on 1.2% agarose gel. The densitometry results for the iNOS gene were compared with the corresponding β-actin levels to account for loading differences.

Detection of iNOS protein by western blot analysis. After the experiment, 0.1 g myocardial tissue was collected from each group and homogenized in 1 ml protein extraction buffer (10 μl Phenylmethanesulfonyl fluoride/PMSF+990 μl lysis buffer). The supernatant was collected after centrifugation and protein concentration was determined using the BCA Protein Assay kit. The quantity of total protein sample is 35 μg, the protein was diluted by SDS-PAGE loading buffer. Executing sequentially degeneration, adding the sample, electrophoresis, transferred to a polyvinylidene difluoride (PVDF) membrane. The membranes were blocked with 5% nonfat milk in Tris- buffered saline Tween (TBST) at 37°C for 2 h, and then they were incubated at 4°C overnight with rabbit iNOS antibody (1:1,000) and rabbit β-actin

antibody (1:2,000). All membranes were incubated for 1 h with HRP-linked anti-rabbit IgG (1:2,000) secondary antibody. The membranes were developed by the chemiluminescent HRP substrate. Signals were detected with ChemiDoc XRS system (Bio-Rad, Berkeley, CA, USA). Grey values of protein bands were analyzed by Quantity One software.

Statistical analysis. Data were expressed as mean ± SD. Statistical comparisons were performed by one-way analysis of variance (ANOVA) and the *Newman-Keuls test*. P<0.05 was considered to indicate a statistically significant difference.

Results

Changes of FBG, BW, HW and HW/BW. Compared with the NC group, FBG and HW/BW were significantly increased (P<0.01), while BW and HW were decreased (P<0.01) in the DM group. In contrast to DM group, BW was decreased (P<0.05) and HW/BW was increased (P<0.05) in the DM+PAG group; BW and HW were increased (P<0.01), HW/BW was significantly decreased (P<0.01) in the DM+NaHS group (Table I).

Changes of hemodynamic parameters. Compared with the NC group, LVSP and ± dp/dt_{max} were decreased (P<0.01), while LVEDP was increased (P<0.01) in the DM group; In contrast to DM group, LVSP and ± dp/dt_{max} were decreased (P<0.05), while LVEDP was increased (P<0.01) in the DM+PAG group; LVSP and ± dp/dt_{max} were increased (P<0.01), while LVEDP was decreased (P<0.01) in the DM+NaHS group (Table II).

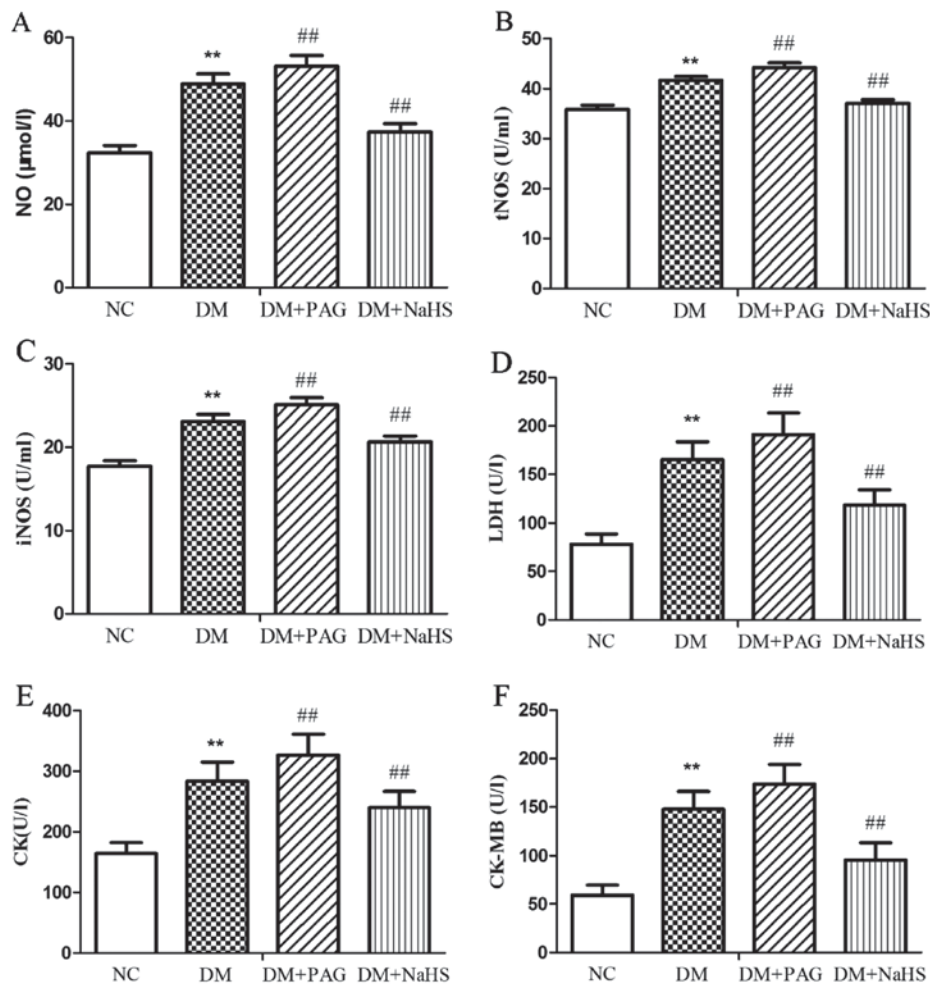


Figure 1. Effects of H₂S on (A) NO content, (B) tNOS activity, (C) iNOS activity, (D) LDH activity, (E) CK activity and (F) CK-MB activity in the serum in the different groups. Data were expressed as the mean \pm SD ($n=8$). ** $P<0.01$ vs. NC group; ## $P<0.01$ vs. DM group; NC, normal control group; DM, diabetes mellitus group; DM+PAG, DM treatment with PAG group; DM+NaHS, DM treatment with NaHS group; NO, nitric oxide; tNOS, total nitric oxide synthase; iNOS, inducible nitric oxide synthase; LDH, lactate dehydrogenase; CK, creatine kinase; CK-MB, creatine kinase MB isozyme.

Changes of NO content and tNOS, iNOS, LDH, CK, CK-MB activities in serum. Compared with the NC group, NO content and tNOS, iNOS, LDH, CK, CK-MB activities in the serum were all increased ($P<0.01$) in the DM group; in contrast to DM group, NO content and tNOS, iNOS, LDH, CK, CK-MB activities in the serum were all increased ($P<0.01$) in the DM+PAG group; NO content and tNOS, iNOS, LDH, CK, CK-MB activities were decreased ($P<0.01$) in the DM+NaHS group (Fig. 1).

Changes of MDA content and SOD activity in myocardial tissue. Compared with the NC group, MDA content was increased ($P<0.01$) and SOD activity was decreased ($P<0.01$) in the DM group; in contrast to DM group, MDA content was increased ($P<0.01$) and SOD activity was decreased ($P<0.01$) in the DM+PAG group; MDA content was decreased ($P<0.01$) and SOD activity was increased ($P<0.01$) in the DM+NaHS group (Fig. 2).

Changes of NO content and tNOS, iNOS activities in myocardial tissue. Compared with the NC group, NO content and tNOS, iNOS activities in the myocardial tissue were all increased ($P<0.01$) in DM group; in contrast to DM group, NO content

and tNOS, iNOS activities were all increased ($P<0.05$, $P<0.01$) in the DM+PAG group; NO content and tNOS, iNOS activities were decreased ($P<0.01$) in the DM+NaHS group (Fig. 3).

Histological changes in myocardial tissue. In the NC group, the myocardial fibers were arranged in neat rows, the myocardial nuclei were clear and the myocardial gap was normal. In the DM group however, the myocardial fibers were disordered, the myocardium was swollen and the myocardial gap was widened. Inflammatory cells had infiltrated and the cytoplasm was uneven in this group. Compared to the DM group, the myocardial damage in the DM+PAG group was more severe. The myocardial damage in the DM+NaHS group was relatively less and most of the cardiomyocytes were arranged neatly. Only a part of the myocardium was swollen, with the dissolution and rupture of a few myocardial fibers and the infiltration of some inflammatory cells (Fig. 4).

Changes in the myocardial ultrastructure. In the NC group, the sarcomeres of the myocardium were arranged in order and maintained homogenous length; the nuclei were normal and the mitochondria were arranged orderly between myofibrils. In the DM group, the integrity of the cardiomyocytes was

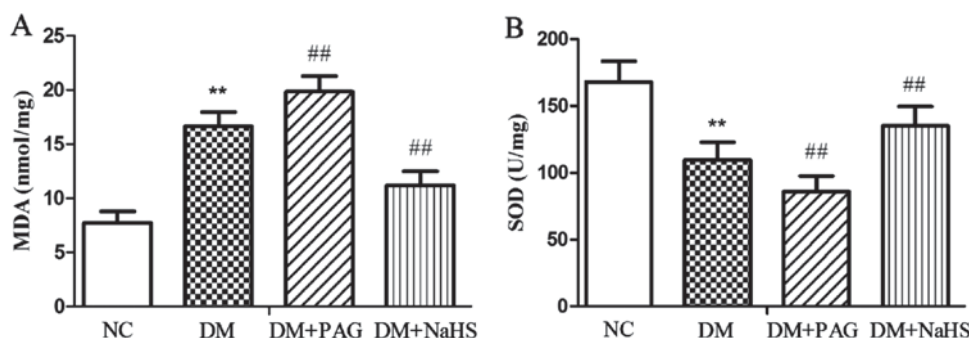


Figure 2. Effects of H₂S on (A) MDA content and (B) SOD activity in the myocardial tissue in the different groups. Data were expressed as the mean \pm SD ($n=8$). ** $P<0.01$ vs. NC group; ## $P<0.01$ vs. DM group; NC, normal control group; DM, diabetes mellitus group; DM+PAG, DM treatment with PAG group; DM+NaHS, DM treatment with NaHS group; MDA, Malondialdehyde; SOD, superoxide dismutase.

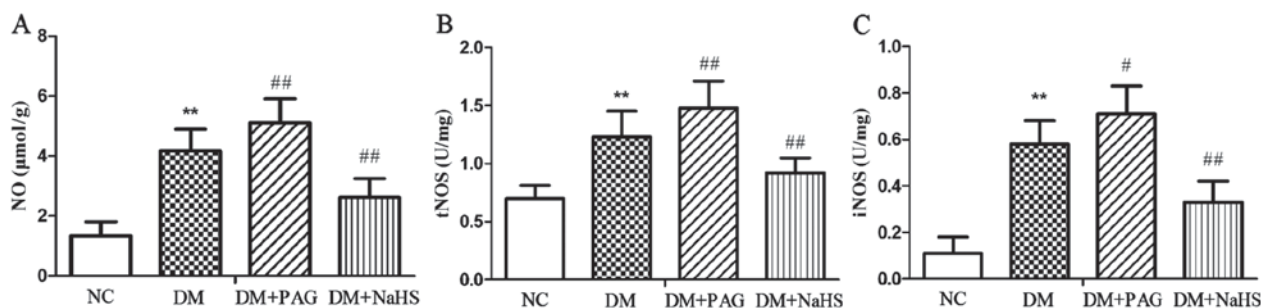


Figure 3. Effects of H₂S on (A) NO content, (B) tNOS activity and (C) iNOS activity in the myocardial tissue in the different groups. Data were expressed as the mean \pm SD ($n=8$). ** $P<0.01$ vs. NC group; * $P<0.05$, ## $P<0.01$ vs. DM group; NC, normal control group; DM, diabetes mellitus group; DM+PAG, DM treatment with PAG group; DM+NaHS, DM treatment with NaHS group; NO, nitric oxide; tNOS, total nitric oxide synthase; iNOS, inducible nitric oxide synthase.

destroyed and mitochondrial matrix was severely damaged. In addition, the mitochondria were swollen and their cristae were vague. In the DM+PAG group, the severity of cardiomyocyte injury was aggravated compared with that in the DM group. Following treatment with NaHS, these injuries were ameliorated in the DM+NaHS group (Fig. 5).

Changes in myocardial iNOS at mRNA and protein level. Compared with the NC group, levels of myocardial iNOS mRNA and protein were significantly increased ($P<0.01$) in the DM group. Compared with the DM group, levels of myocardial iNOS mRNA and protein were increased ($P<0.05$, $P<0.01$) in the DM+PAG group; levels of myocardial iNOS mRNA and protein were significantly decreased ($P<0.01$) in the DM+NaHS group (Figs. 6,7).

Discussion

DM, marked by an absolute or relative deficiency of insulin secretion, is a metabolic disorder syndrome characterized by hyperglycemia. DCM is a specific myocardial disease associated with DM and characterized by cardiac microvascular disease. Our data showed that compared with the NC group, the FBG level, HW/BW ratio, LVEDP, serum LDH, CK and CK-MB activities were increased, while LVSP and $\pm dp/dt_{max}$ were decreased in the DM group. There were histological changes and ultrastructure damage in the DM group. These results demonstrated that sustained high blood glucose may damage the structure and function of cardiomyocytes.

H₂S is considered as the third endogenous signaling gasotransmitter which regulates cardiovascular function. Endogenous H₂S is produced by pyridoxal-5'-phosphate-dependent enzymes, including cystathionine- β -synthase (CBS) and CSE in mammalian tissues during cysteine metabolism (14,15). CBS is the predominant H₂S-generating enzyme in the brain and nervous system, while CSE is mainly expressed in the cardiovascular system (16,17). PAG is an irreversible CSE inhibitor that can decrease endogenous H₂S content, while NaHS is a donor of exogenous H₂S. Our data showed that the administration of NaHS ameliorated cardiac hypertrophy and myocardial injury in DCM, as evidenced by the reduction of the HW/BW ratio and LDH, CK and CK-MB activities, improvement of hemodynamic parameters, and alleviation of myocardial histological changes and ultrastructure damage. In contrast, the rats in the DM+PAG group showed the opposite effect, which further verified that H₂S played a cardioprotective role in rats with DCM. Meanwhile, it raised the question as to the exact mechanism by which H₂S exerted its cardioprotective effect. To answer this, we analyzed the NO content and tNOS, and iNOS activities in the following experiments.

Both NO and H₂S are involved in the regulation of a wide range of physiological processes (18,19). NO, the first short-lived gaseous lipophilic molecule, can be produced in almost all tissues. Several studies have reported the involvement of NO in myocardial contraction and in the regulation of cardiac function. Low concentrations of NO increase the myocardial contractility, while high concentrations of NO

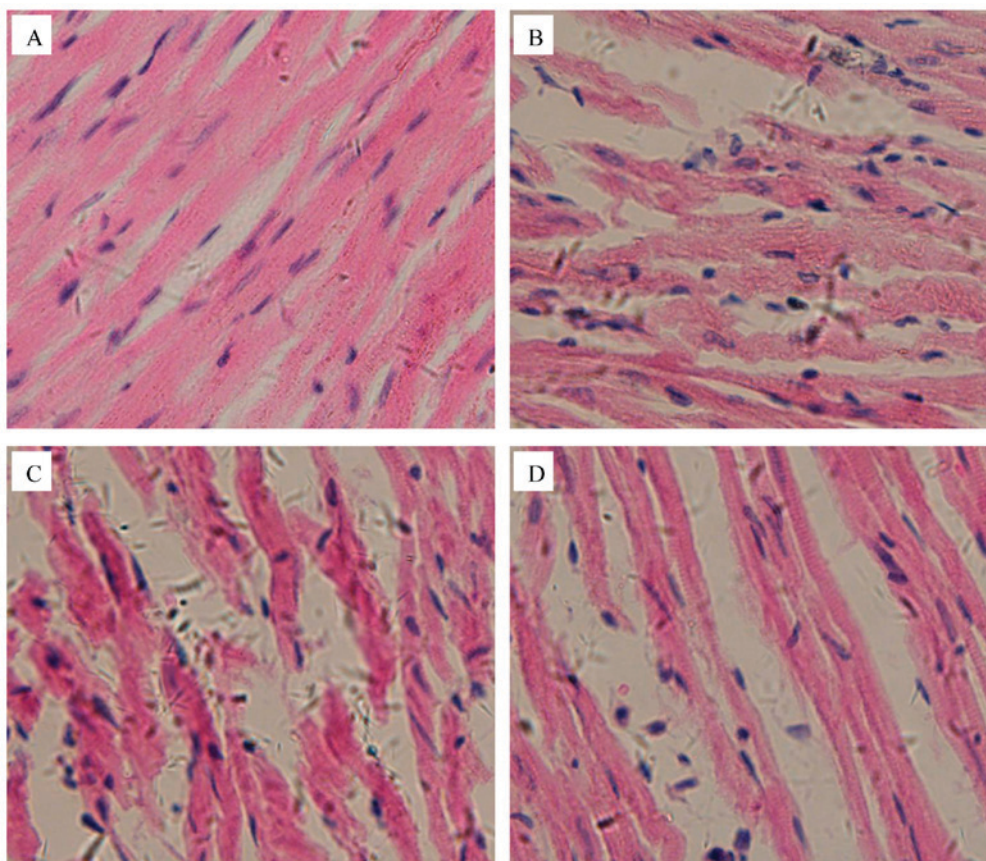


Figure 4. Histological changes of myocardial tissue in the different groups (x400). (A) NC group; (B) DM group; (C) DM+PAG group; (D) DM+NaHS group.

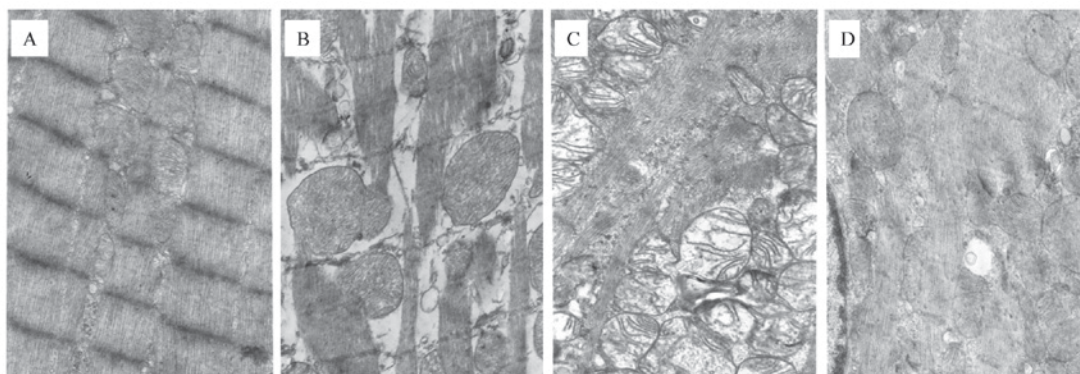


Figure 5. Changes of myocardium ultrastructure under transmission electron microscopy in the different groups (x15,000). (A) NC group; (B) DM group; (C) DM+PAG group; (D) DM+NaHS group.

can inhibit the myocardial contractility (8). In addition, high concentrations of NO can react with superoxide anions to produce cytotoxic peroxynitrite anions. This reaction accelerates the process of lipid peroxidation, and as the body produces excessive oxygen free radicals, oxidative stress injury, aggravated cell damage, and even death may result (20). Our results showed that the myocardial and serum NO contents were significantly increased in the DM group compared with the NC group. *In vivo*, oxygen free radicals can react with polyunsaturated fatty acids to produce lipid peroxides, and therefore the content of MDA can reflect the degree of myocardial oxidative stress injury. SOD is

able to scavenge oxygen free radicals, convert it to oxygen and water, and thus is a detection index of the intracellular reduction power level (21). The experimental results wherein myocardial MDA content increased and SOD activity decreased in diabetic rats, indicated that there was oxidative stress injury in the diabetic myocardium. Therefore, we concluded that high concentrations of NO were harmful to the myocardium. Endogenous NO is constitutively or inductively synthesized in a reaction catalyzed by the NOS family in mammalian cells (22). The NOS family has three distinct NOS isoforms: neuronal (nNOS or NOS-1), inducible (iNOS or NOS-2) and endothelial (eNOS or NOS-3) NOS, which

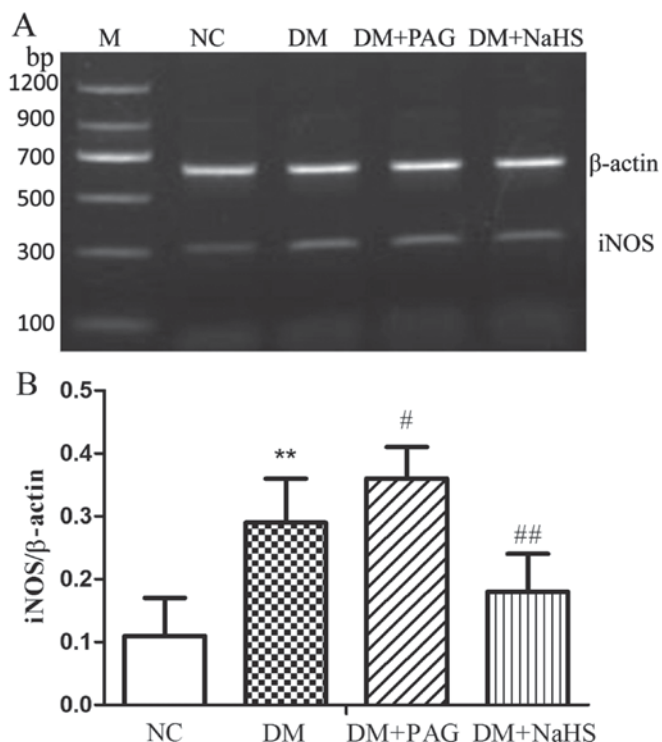


Figure 6. Expression levels of myocardial (A) iNOS mRNA and (B) quantification of the iNOS/ β -actin in the different groups. Data were expressed as the mean \pm SD ($n=8$). ** $P<0.01$ vs. NC group; # $P<0.05$, ## $P<0.01$ vs. DM group; NC, normal control group; DM, diabetes mellitus group; DM+PAG, DM treatment with PAG group; DM+NaHS, DM treatment with NaHS group; iNOS, inducible nitric oxide synthase.

are encoded by three different genes (23). eNOS and nNOS are known as constitutive NOS (cNOS) (24,25), as under normal physiological conditions, cNOS exhibits constitutive expression and is involved in physiological signaling and in cellular maintenance. However, it is noteworthy that iNOS is localized throughout the cytoplasm in mammalian cells, and they can be induced to synthesize much more NO than cNOS (26). In certain pathological conditions such as cellular stress, hyperglycemia and ischemia-reperfusion, iNOS is often induced to produce higher NO (27,28). We therefore detected tNOS and iNOS activities in the serum and myocardium, and the results showed that tNOS and iNOS activities were significantly higher in diabetic rats. The iNOS activities in the serum and in the myocardium were significantly different; however, the difference in cNOS activity was not obvious. Therefore, we deduced that the high concentration of NO in the myocardial tissue was induced mainly by iNOS. The significantly increased expression of iNOS mRNA and protein in diabetic rats compared to normal rats confirmed that iNOS overexpression led to the high concentrations of NO in the diabetic myocardium.

Several studies have discussed the interaction of H_2S with NO in various systems (19,29). Studies have reported that exogenous H_2S could suppress iNOS activity and expression. In RAW 264.7 macrophages, a H_2S donor could inhibit iNOS protein expression and exerted an anti-inflammatory effect (30). In the metabolic syndrome model of rats, exogenous administration of NaHS could inhibit iNOS activity and reduce NO content in the plasma and myocardium to improve

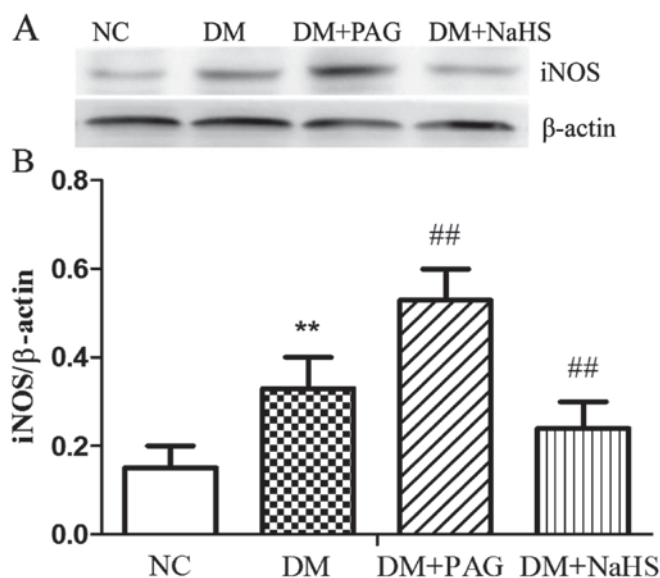


Figure 7. (A) Representative western blot and (B) quantitative analysis of the iNOS/ β -actin protein expression ratio in the myocardial tissue of different groups. Data were expressed as the mean \pm SD ($n=8$). ** $P<0.01$ vs. NC group; ## $P<0.01$ vs. DM group; NC, normal control group; DM, diabetes mellitus group; DM+PAG, DM treatment with PAG group; DM+NaHS, DM treatment with NaHS group; iNOS, inducible nitric oxide synthase.

cardiac function (31). Hua *et al* (32) found that exogenous H_2S could provide protection against Coxsackie virus B3-induced myocardial damage in mice through the suppression of myocardial iNOS mRNA and protein expression. Our data similarly showed that in the DM+NaHS group, iNOS activity and its mRNA and protein expression were significantly decreased, while its activity and expression were significantly increased in the DM+PAG group. Therefore, we inferred that iNOS expression in rats with DCM positively correlated with the severity of myocardial injury. This suggested that the over-production of iNOS induced by H_2S inhibition exacerbated myocardial injury.

In addition, our data also showed that MDA and NO contents were decreased and SOD activity was increased in the DM+NaHS group, while the rats in the DM+PAG group showed the opposite effects. Therefore, without H_2S or with a lower H_2S concentration, myocardial iNOS would be over-expressed, NO would be over-produced, and oxidative stress and myocardial damage would be aggravated in diabetic rats.

In conclusion, H_2S exerted a cardioprotective effect in the rat model of DCM, and its mechanism might be associated with the suppression of iNOS activity and expression, a decreased NO content, a reduction in the formation of lipid peroxides, and thereby a suppression of the oxidative stress injury in order to protect the myocardial tissue.

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