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Hydrogen Sulfide Attenuates High Glucose-induced Myocardial Injury in Rat Cardiomyocytes by Suppressing Wnt/beta-catenin Pathway*

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Summary: Diabetic cardiomyopathy (DCM) is one of the major heart complications of diabetic patients. Hydrogen sulfide (H_2S) is now recognized as an important signaling molecule and has been shown to attenuate the development of diabetic cardiomyopathy. However, the underlying mechanisms linking H_2S and the development of DCM have not been fully elucidated. In the present study, we therefore sought to explore the role and mechanism of H_2S in the pathogenesis of DCM by establishing high glucose-induced injury model in neonatal rat cardiomyocytes (NRCMs) and H9c2 cells. Using cystathionine gamma-lyase (CSE) overexpression and CSE interference vectors transfection, the cell viability, cell apoptosis. and oxidative stress were determined and compared between the treatment of high glucose induction and exgenous NaHS administration. Meanwhile, the relationship between the CSE/H_2S system and Wnt/beta-catenin pathway was analyzed and discussed in the high glucose-induced cardiomyocytes. Our results indicated that H_2S played an important protective role in high glucose-induced apoptosis and oxidative stress in cardiomyocytes, as shown by the decreased reactive oxygen species and malondialdehyde levels, and the increased activities of superoxide dismutase, catalase and glutathione peroxidase. Moreover, H_2S could attenuate the Wnt/β-catenin signalling pathway and up-regulate the expression of haem oxygenase-1 (HO-1) and NAD(P)H:quinone oxidoreductase 1 (NQO1) in the diabetic myocardium cells. Together, these results demonstrated that H_2S could attenuate high glucose-induced myocardial injury in rat cardiomyocytes by suppressing Wnt/β-catenin pathway.
Key words: hydrogen sulfide; Wnt/β-catenin; diabetic cardiomyopathy; high glucose

Diabetes mellitus is a metabolic disorder syndrome characterized by hyperglycemia and hyperlipidemia, which is caused by absolute or relative deficiency of insulin secretion[1]. Epidemiological studies have found that more than 70% of diabetic patients die of cardiovascular disease, and the mortality rate of cardiovascular disease is two to three times higher than that of non-diabetic patients. Among them, diabetic cardiomyopathy (DCM) is one of the main cardiac complications in diabetic patients, with high incidence and serious harm, which is closely related to the high morbidity and mortality of cardiovascular disease in diabetic patients[2].

Hyperglycemia, as an independent risk factor, plays an important role in the occurrence and development of diabetic cardiomyopathy[3]. A large number of studies have shown that this effect may be attributed to the increase of oxidative stress and apoptosis induced by hyperglycemia. In addition, there is still a certain internal relationship between the increase of oxidative stress and apoptosis[2, 4–6], but the specific mechanism is still not clear. In cardiomyocytes, the antioxidant defense system including superoxide dismutase (SOD), reduced glutathione (GSH), catalase (CAT), as well as malondialdehyde (MDA), plays an important role in maintaining the dynamic balance of the production and scavenging of reactive oxygen species (ROS). The imbalance between the production of free radicals and antioxidant defense in pathological conditions such as hyperglycemia or inflammation, has been implicated in the cellular myocardium death process, involving the release of cytochromec into the cytoplasm and initiation of the apoptosis cascade. The mitochondrial dysfunction, decreased ATP production and increased oxidative stress (ROS) were observed in cardiomyocytes in patients with DCM, which ultimately leads to myocardium apoptosis[7]. However, the study of the molecular mechanism of the disease is not very clear, and more efforts are needed.

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Recent studies suggest that hydrogen sulfide (H_2S) is the third gaseous mediator in mammals. H_2S is synthesized from L-cysteine by either cystathionine beta-synthase (CBS) or cystathionine gamma-lyase (CSE), both using pyridoxal 5'-phosphate (vitamin B6) as a cofactor. H_2S/CSE pathway plays an important role in a variety of physiological processes including anti-apoptotic effect[8], relaxation of smooth muscle vasodilation[9], inhibition of myocardial inflammation[10, 11]. *In vivo* experiments showed that exogenous H_2S intervention could protect cardiomyocytes from oxidative stress by increasing the activity of antioxidant enzymes and inhibiting the expression of ROS and lipid peroxidase[12]. In addition, previous studies have shown that Wnt/β-catenin signaling pathway is involved in diabetic myocardial injury[13]. Diabetes can induce myocardial hypertrophy and myocardial fibrosis, which is related to the overexpression of Wnt-induced secreted protein-1 (WISP-1) and its receptor ligand[14]. A large number of experiments have confirmed that H_2S has myocardial protective effect. However, whether H_2S plays a protective effect in the myocardium injury induced by high glucose through Wnt/β-catenin signaling pathway and downstream WISP-1 remains unclear. Therefore, the present study aimed to investigate this hypothesis, which may provide a scientific basis for the development of novel therapeutic strategies based on H_2S/Wnt/β-catenin/WISP-1 signaling pathway for the prevention and treatment of DCM.

1 MATERIALS AND METHODS

1.1 Materials

H9c2 cell line was purchased from the American Type Culture Collection (USA). Alexa Fluor 488 (cat. No. ab150077), anti-α-actin (cat. No. ab68194), anti-CSE (cat. No. EPR15468), anti-Bcl-2 (cat. No. ab59348), anti-caspase-3 (cat. No. ab13847), anti-GAPDH (cat. No. ab70699) and a secondary horseradish peroxidase-conjugated goatanti-rabbit antibody (cat. No. ab7090) were purchased from Abcam (USA). pEGFP-N1 vector that expresses GFP was ordered from Nanjing Kings Rui Biotechnology Co. Ltd. (China). Annexin V-fluorescein isothiocyanate (FITC) and propidium iodide (PI) detection kit was purchased from BD Biosciences (USA). Restriction endonuclease *Xho* Ⅰ and *Bam*HⅠ were purchased from NEB (China). PCR materials and kits, including TRIzol Reagent, TPrime Script RT Master Mix and SYBR Premix Ex Taq kit, were purchased from Takara Biotechnology Co., Ltd. (China). H_2S, MDA, SOD, GSH-Px, HO-1, NQO1 contents and CAT activity were measured using commercial kits from Nanjing Jiancheng Biotechnology Co., Ltd. (China). All the primers, overexpression and interference vectors, negative control, were synthesized by Nanjing Kings Rui Biotechnology Co. Ltd. (China).

1.2 Isolation and Culture of Neonatal Rat Cardiomyocytes

The protocols of isolation and culture of cardiomyocytes in neonatal rat primary myocardial cells (NRCMs) were followed according to the previous publication of Vandergriff's group[15]. In brief, cardiomyocytes of neonatal rats were isolated in trypsin and collagenase. Then the cell viability and integrity were estimated by staining with trypan blue, and the purity of cardiomyocytes was identified by immunofluorescence staining.

1.3 Establishment of Myocardial Injury Cell Model Induced by High Glucose

For the myocardial injury experiments, when the NRCMs and H9c2 cells reached 70%–80% confluence, the culture medium was replaced by fetal bovine serum-free DMEM medium with different concentrations of glucose for 72 h. Different concentrations of glucose (15, 30, 45, and 60 mmol/L) were added into the culture medium, and cell proliferation was measured by MTT assay according to the manufacturer's instructions.

1.4 CSE Overexpression and Interference in NRCMs and H9c2 Cells

The CSE overexpression and interference vectors were designed and constructed by the commercial service. The identification of the vector construction was performed by PCR, using the following primers (CSE-F: CCGCTCGAGATGCAGAAGGACGCCTCC; CSE-R: CGCGGATCCGCGTTAAGGGTGCGCTG). Si-RNA sequence for targeted inhibition of CSE was as follows: GCTCTGGGTGCTGATATTT. NRCMs and H9c2 cells were transfected with the vectors. The cells transfected with empty vector were used as the control. After NRCMs and H9c2 cells were cultured in medium containing 30 mmol/L glucose for 48 h, the cell model of myocardial injury induced by high glucose was established. Then the cells were randomly divided into 6 groups: cells were administrated sodium hydro sulfide (NaHS) at a concentration of 100 μmol/L for 30 min before high-glucose inducement (HG+NaHS group); cells were cultured with high glucose (HG group); cells were transfected with pEGF-N1 vector (EV group); cells were transfected with CSE overexpression vector (OV group); cells were transfected with CSE interference vector (IV group); primary cardiomyocytes or H9c2 cells were cultured in normal medium as the control group.

1.5 Measurement of H_2S Production and Oxidative Stress Levels

The intra-cellular H_2S and MDA content, and SOD, GSH-Px, HO-1, qNQO1 and CAT activity, were measured using commercial kits according to the manufacturers' instructions. In brief, after the indicated treatments, H9c2 cells were lyzed and the supernatants were collected to determine H_2S and MDA contents,

and SOD, CAT and GSH-Px activity. The absorbance (*A*) was measured at 530, 450, 405 and 412 nm using a microplate reader (Bio-Rad Laboratories, Inc., China).

1.6 Detection of Apoptosis by Flow Cytometry

According to the manufacturer's instructions, flow cytometry was used to analyze the apoptosis of NRCMs and H9c2 cells. The apoptosis rate in each stage was detected by flow cytometry, and the data were analyzed by Cytexpert software.

1.7 Western Blot Analysis

After various treatments, NRCMs and H9c2 cells were washed twice with PBS, lysed in RIPA buffer (Beyotime Institute of Biotechnology, China) and centrifuged. The supernatants were collected and quantified for protein concentration using a BCA kit (Beyotime Institute of Biotechnology, China). Equal amounts of protein from each sample were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred onto polyvinylidene difluoride membranes (EMD Millipore, USA). The membranes were blocked with 5% non-fat milk in TBST buffer (50 mmol/L Tris, pH 7.4, 250 mmol/L NaCl, 0.1% Tween 20) for 1 h at room temperature and probed with antibodies including β-catenin, WISP-1, Bcl2, caspase-3 and GADPH (all from Abcam, UK) overnight at 4°C. Following washing with TBST thrice, the membranes were incubated with the appropriate horseradish peroxidase-conjugated secondary antibodies (Cell Signaling Technology, Inc., USA) for 1 h at room temperature and visualized by enhanced chemiluminescence reaction reagents (EMD Millipore, USA).

1.8 Reverse Transcription Polymerase Chain Reaction (RT-PCR)

Total RNA was extracted from H9c2 cells with the indicated treatments using TRIzol Reagent (Thermo Fisher Scientific Inc., Australia). Complementary DNA was synthesized via RT reaction with PrimeScript RT Master Mix [Takara Biotechnology (Dalian) Co., Ltd., China]. RT-qPCR assay was performed using SYBR Premix Ex Taq kit according to the manufacturer's instructions. Reactions were conducted on the ABI Prism 7500 Real-Time PCR System (Thermo Fisher Scientific, Inc., Australia). The mRNA levels were calculated using the $2^{-\Delta\Delta Ct}$ method. Glycerinaldehyde -3-phosphate-dehydrogenase (GAPDH) was used for normalization. The primers used are listed in table 1.

1.9 Statistical Analysis

The data were presented as $\bar{x}\pm s$ from three independent experiments. The results were compared by one-way analysis of variance, followed by least significant difference post-hoc test using SPSS 16.0 software (SPSS, Inc., USA). $P<0.05$ was considered to indicate a statistically significant difference.

2 RESULTS

2.1 Identification of NRCMs

Trypan staining showed that the survival rate of the cardiocytes cultured was more than 80%. Under the inverted phase contrast microscope, it was found that the cells were fusiform or polyhedron and some cells began beat after 12 h. The cell purity was up to 95% by α-actin fluorescent staining assay (fig. 1).

2.2 Identification of Myocardial Injury Induced by High Glucose in NRCMs and H9c2 Cells

MTT assay was used to measure the cells reproductive capacity under high glucose conditions. Results in fig. 2 showed that when the glucose concentration rose to 45 mmol/L, the viability rate of

Table 1 Primers used for RT-PCR

Gene	Forward primer (5′-3′)	Reverse primer (5′-3′)
WISP-1	GACCACACATCAAGGCA	TGGACACTGGAAATCAA
β-Catenin	AGGACTACAAGAAACGG	AGAGTGAAAAGAACGGT
Bcl-2	GGGAGAACAGGGTATGA	AGGCTGGAAGGAGAAGA
Caspase-3	CATGGAGATGAAGGAGTA	CCTGAATGATGAAGAGTT
GAPDH	CAAGTTCAACGGCACAG	CCAGTAGACTCCACGACAT

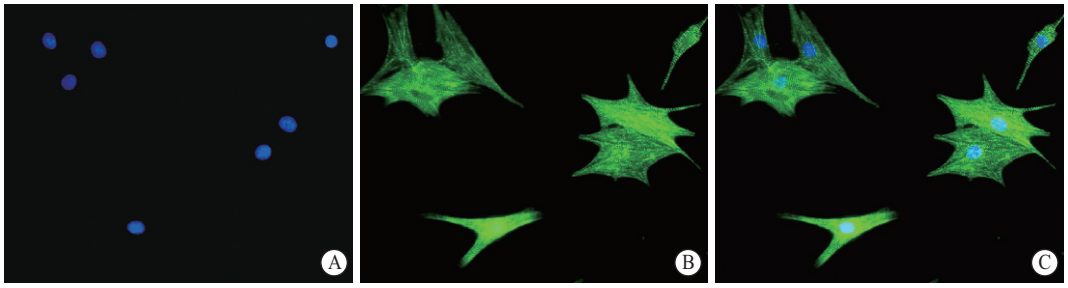

Fig. 1 Identification of neonatal rat cardiomyocytes (NRCMs) by immunofluorescence (×400)
A: NRCMs stained with Dapi (The nucleus is stained with blue fluorescence); B: NRCMs stained with α-actin (Green fluorescence is located in the cytoplasm); C: merged image for NRCMs stained with Alexa and Dapi

cardiac myocytes decreased significantly. Therefore, we used 30 mmol/L glucose in the following experiments.

2.3 Construction of CSE Overexpression Vector and Interference Vector

The protein expression of CSE in blank vector and CSE overexpression groups was determined by Western blot analysis. The results showed that the CSE protein expression was overexpressed or interfered successfully after transfection with corresponding recombinant vectors (fig. 3).

2.4 Effects of Overexpression of CSE on Intracellular H_2S Content and Cell Metabolism

In order to investigate whether overexpression of CSE can increase the content of H_2S in cells, we measured the intracellular H_2S content in each group. The results revealed that OV group had the higher contents of H_2S than in HG and EV groups, and the H_2S content in IV group was decreased compared to HG group (fig. 4A). To understand the role of CSE in cell metabolism, we measured the activity of oxidative stress related enzymes. As shown in fig 4B and 4C, the activity of SOD, GSH-PX, HO-1, NQO1 and CAT increased, but MDA content decreased in OV groups as compared with HG group.

2.5 Effect of Overexpression of CSE on High Glucose-induced Apoptosis

Flow cytometry demonstrated that high glucose increased the percentage of apoptotic cells as compared with the control group, and CSE overexpression reduced the high glucose-induced apoptosis of NRCMs and H9c2 cells (fig. 5A and 5B). To assess the involvement of caspase activation in the blockage of high glucose-induced apoptosis by CSE overexpression and interference, we examined the activities of caspase-3 and Bcl-2 in the mitochondria-mediated apoptosis pathway. As shown in fig. 5C and 5D, the activity of caspase-3 was obviously enhanced while the activity of Bcl-2 was decreased by high glucose exposure, as compared with the control group. However, CSE overexpression significantly reduced the increase in

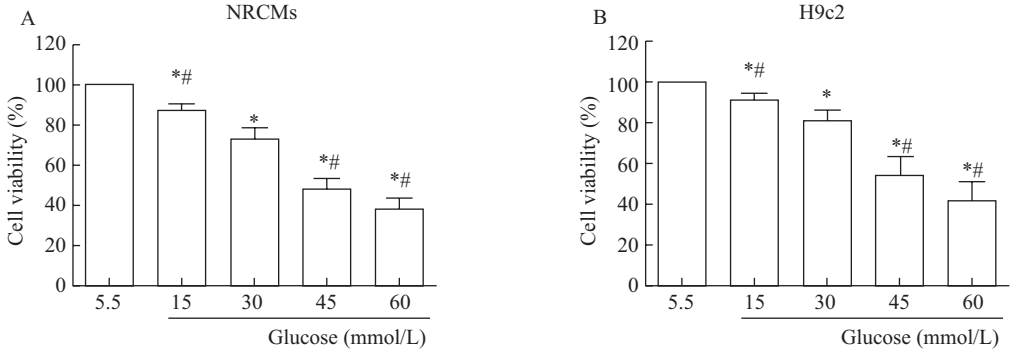

Fig. 2 The cells reproductive capacity measured by MTT

A: effects of different concentrations of glucose on the survival rate of neonatal rat cardiomyocytes (NRCMs); B: effects of different concentrations of glucose on the survival rate of H9c2 cells (n=3; $^{*}P$<0.05 *vs.* 5.5 mmol/L blood glucose group, $^{\#}P$<0.05 *vs.* 30 mmol/L blood glucose group)

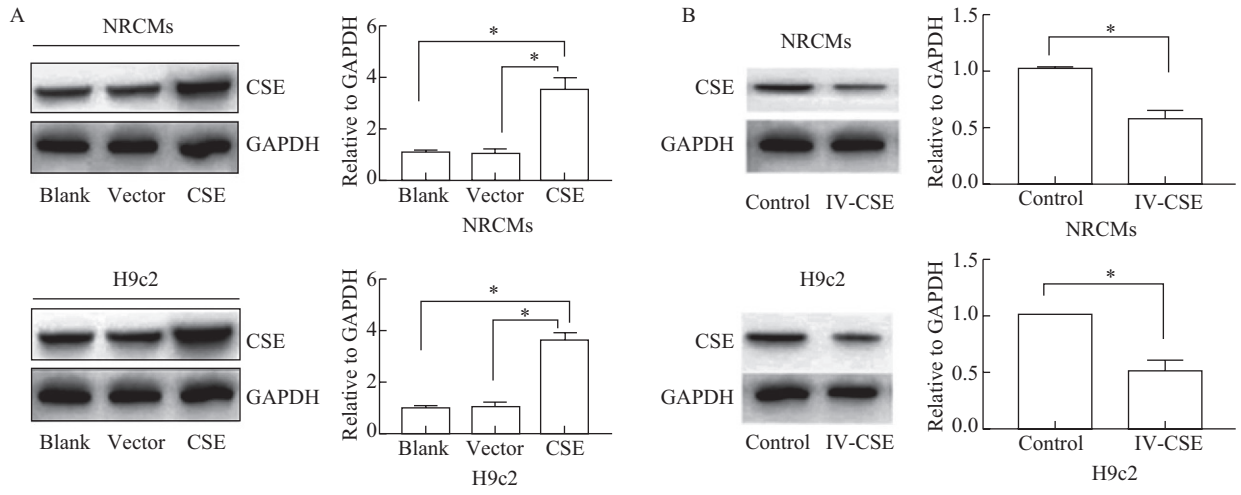

Fig. 3 The CSE protein expression after the overexpression and interference vector transfection

A: The CSE was overexpressed in neonatal rat cardiomyocytes (NRCMs) and H9c2 cells transfected with CSE overexpression vectors; B: The CSE expression was downregulated in NRCMS and H9c2 cells transfected with CSE interference vectors (n=3; $^{*}P$<0.05)

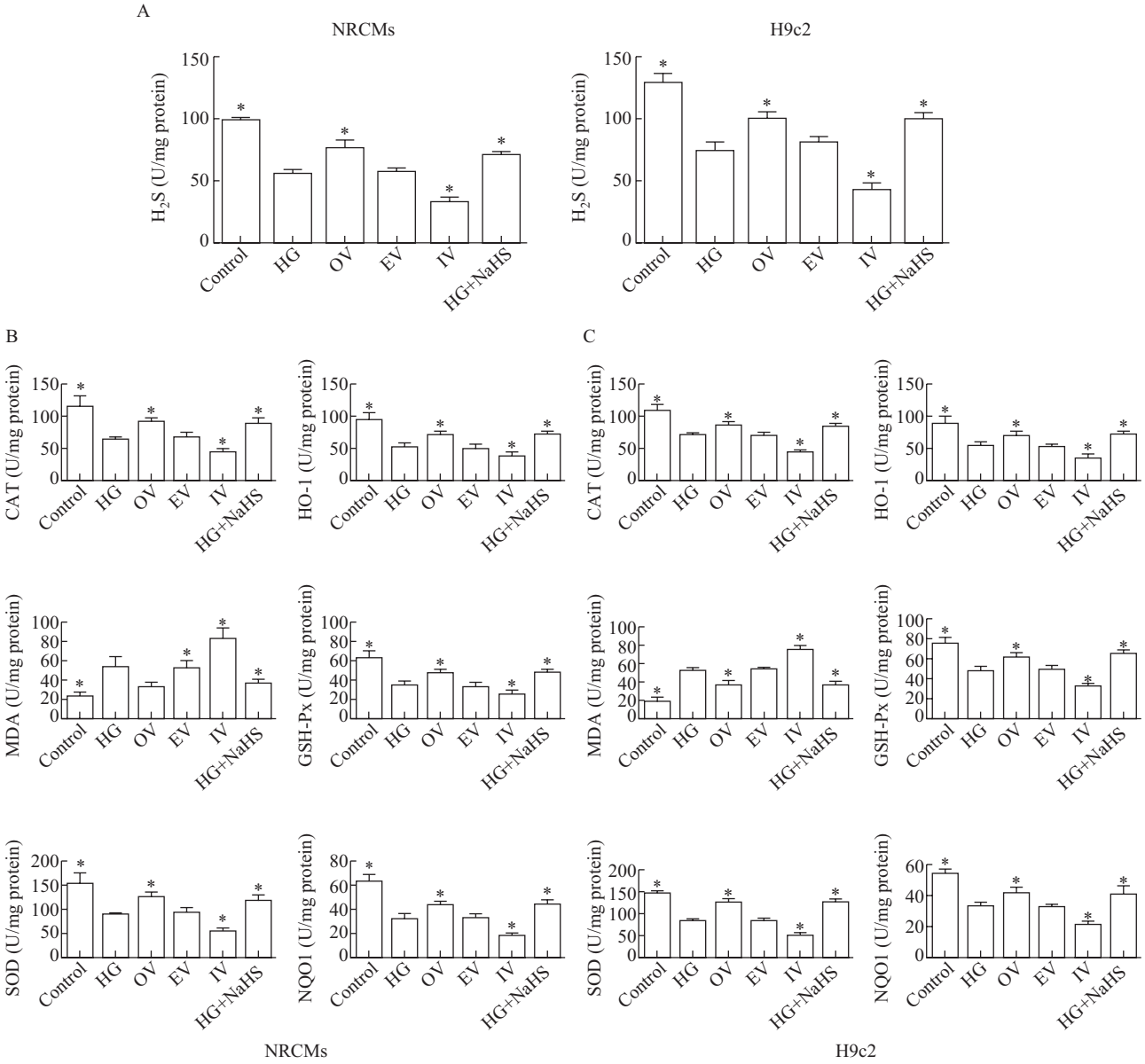

Fig. 4 Effect of CSE overexpression on the intracellular H_2S content and cell metabolism
A: H_2S content in NRCMs and H9c2 cells; B: activity of MDA, SOD, GSH-Px, HO-1, NQO1 and CAT in NRCMs; C: activity of MDA, SOD, GSH-Px, HO-1, NQO1 and CAT in H9c2 cells (Data are expressed as $\bar{x}\pm s$; $n=3$; $^*P<0.05$ *vs.* HG group)

caspase-3 activity.

2.6 Preventive Effects of CSE Overexpression on High Glucose-induced Apoptosis of NRCMs and H9c2 Cells by Regulating Wnt/β-catenin Signaling Pathway of Myocardial Cells

To investigate the influence of CSE overexpression on Wnt/β-catenin signaling of myocardial cells, we measured the protein and mRNA expression levels of WISP-1, β-catenin, caspase-3 and Bcl-2 by Western blotting and RT-PCR respectively. As shown in fig. 6, CSE overexpression significantly decreased WISP-1, β-catenin and caspase-3 expression but increased Bcl-2 expression in high glucose-exposed NRCMs and H9c2 cells. In addition, CSE silencing resulted in a substantial decrease in the Bcl-2 expression. Supplementation with the sodium hydrosulfide, like CSE overexpression treatment, also reversed the high glucose-induced cell apoptosis (fig. 6A and 6B). These results revealed that CSE may exert an anti-apoptotic effect in high glucose-treated NRCMs and H9c2 cells via regulating the Wnt/β-catenin signaling pathway.

3 DISCUSSION

At present, diabetes has become a major global health problem. Long-term chronic hyperglycemia, as one of the basic metabolic disorders of diabetes can cause extensive myocardial metabolic disorders, mainly

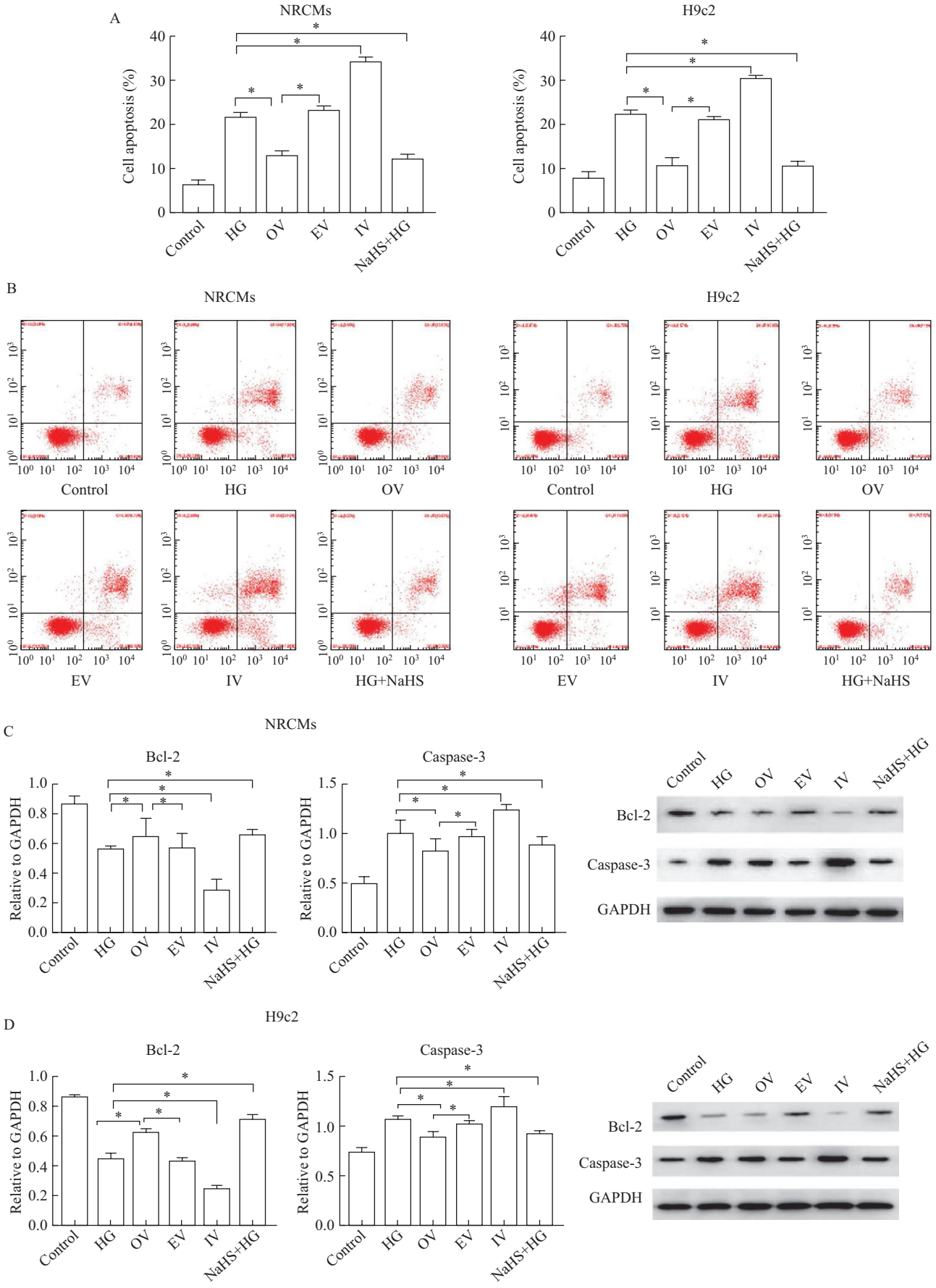

Fig. 5 The effect of CSE overexpression and interference on the cell apoptosis and cell cycle
A and B: the apoptosis in each group of NRCMs and H9c2 cells; C and D: protein expression of Bcl-2, caspase-3 in NRCMs (C) and H9c2 cells (D) (Data are expressed as $\bar{x}\pm s$; $n=3$; $^{*}P<0.05$)

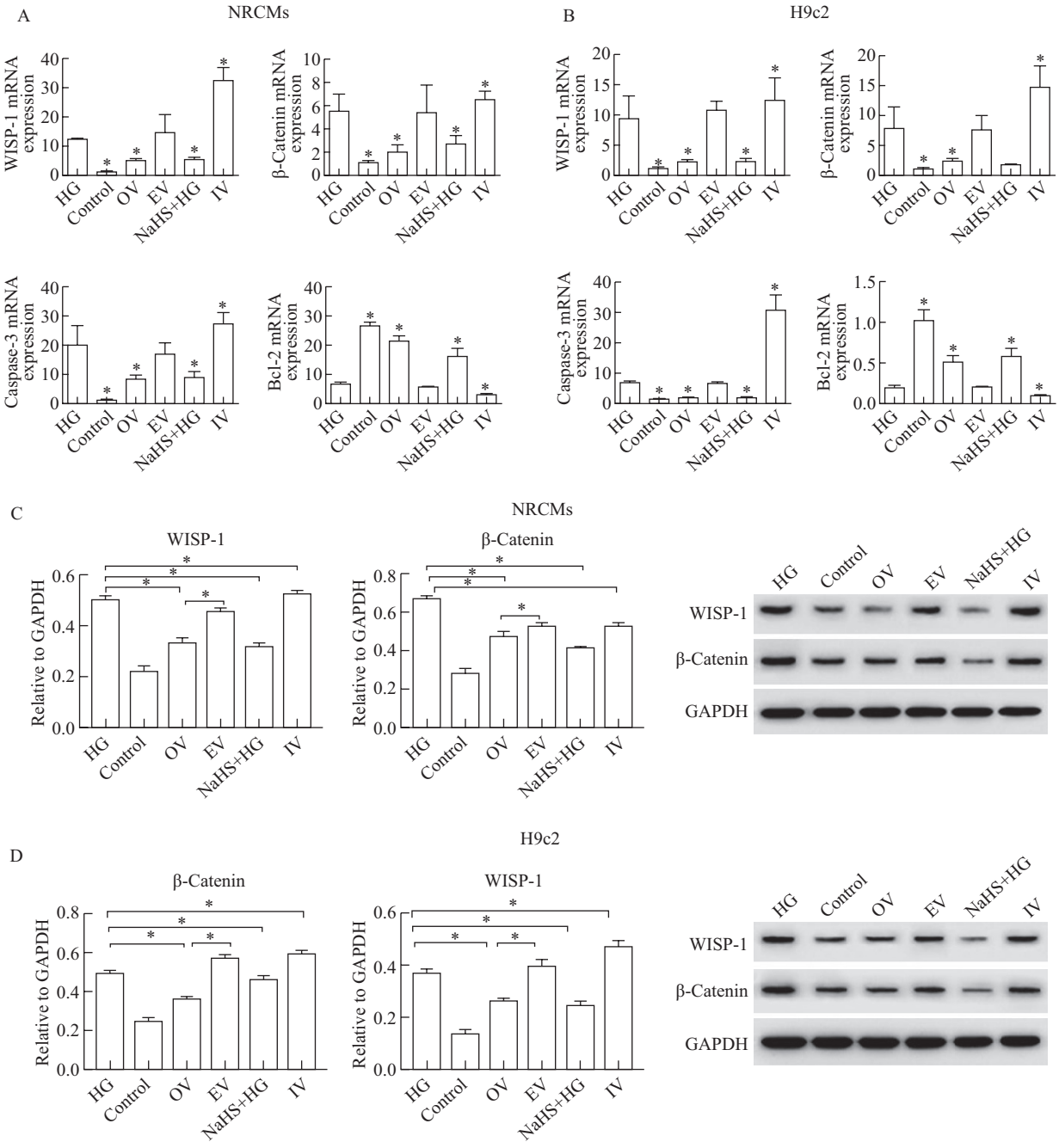

Fig. 6 Effect of CSE on the protein and mRNA expression of Wnt/β-catenin pathway
A: the mRNA expression of WISP-1, β-catenin, Caspase-3 and Bcl-2 in NRCMs; B: the mRNA expression of WISP-1, β-catenin, Caspase-3 and Bcl-2 in H9c2 cells; C: the protein levels of WISP-1and β-catenin in NRCMs; D: the protein levels of WISP-1 and β-catenin in H9c2 cells (Data are expressed as $\bar{x}\pm s$; $n=3$; $^{*}P<0.05$)

manifested as myocardial hypertrophy and fibrosis which are prone to develop into DCM eventually.

H_2S is a highly diffusible gasotransmitter, which influences cellular and organ functions by a number of different mechanisms. CSE is one major H_2S-producing enzyme with L-cysteine as the main substrate in mammalian cells. CSE expression and activity and ultimately the amount of H_2S synthesis are controlled by a complex integration of transcriptional, post-transcriptional and post-translational mechanisms. Reduced H_2S production as a result of CSE inhibition in rats increases blood pressure and administration of H_2S attenuates blood pressure elevation[16]. Tons of studies reported decreased CSE expression and H_2S levels in myocardium with ischemia reperfusion (I/R) injury, and supply of exogenous H_2S provides a cardiac protective effect[11]. CSE/H_2S is also involved in the attenuation of diabetic myocardial injury[11]. High glucose reduced CSE expression in the primary NRCMs, and treatment with NaHS significantly reversed the diabetic rat hearts

function in yang's study[17]. Besides these, many other factors were shown to protect heart via regulating CSE/H_2S system. The results of the current study are consistent with the previous report. Endogenous H_2S content was significantly decreased in high glucose-induced injury in NRCMs and H9c2 cells. To understand the role of CSE in the oxidative stress, we measured the activity of oxidative stress related indicators. The activity of SOD, GSH-PX, HO-1, NQO1 and CAT was increased, and MDA content was decreased in CSE overexpression group as compared with the high glucose-induced group. Inhibition of H_2S-producing enzyme CSE plays a similar effect with high glucose induction.

It is wildly accepted that cardiomyocyte apoptosis plays a primary role in the pathogenesis of myocardial I/R injury. Myocardial apoptosis is a complicated process that is mediated by a series of enzymes and molecules, including the opening of the mitochondrial permeability transition pore, release of cytochromes, and activation of caspases[18]. The Bcl-2 family of proteins has emerged as the key regulatory components of the apoptotic process. The Bcl-2 family consists of anti-apoptotic proteins (such as Bcl-2 and Bcl-xL) and pro-apoptotic molecules (such as Bax and Bak), which function primarily to protect or disrupt the integrity of the mitochondrial membrane and control the release of pro-apoptotic proteins. The activation of caspase-3 triggers the execution of cell apoptosis. CSE/H_2S pathway was also reported to enhance ROS production and mitochondrial disruption, pronounced DNA damage and increased apoptosis[19]. In the present study, high glucose led to a significant increase in the activity of caspase-3, decreased in the activity of Bcl-2, ultimately, apoptosis in NRCMs and H9c2 cells. However, CSE overexpression and exogenous H_2S administration reduced the apoptotic cells and the activities of caspase-3 in high glucose-exposed cells. These results suggested that CSE/H_2S system exerts anti-apoptotic effects in high glucose injured cardiomyocytes.

Wnt/β-catenin, one of the classical Wnt signaling pathways, has the function to determine the fate of cell differentiation during stem cell development. Wnt ligand is a secretory glycoprotein that binds to Frizzled receptors and then forms a larger complex on the cell surface with LRP5/6[19]. Stable β-catenin is transported into the nucleus via Rac1 and other factors, binding LEF/TCF transcription factors in the nucleus, replacing auxiliary inhibiting factors and recruiting additional auxiliary activation factors for Wnt target genes[20]. WISP-1, as one of the downstream target genes of β-catenin, promotes cell proliferation and extracellular matrix synthesis, mediates cell adhesion, and stimulates cell transfer, etc., is considered to be the main cytokine that facilitates fibrosis[17, 20]. Studies have revealed that WISP-1 and its receptor adhesion were overexpressed after myocardial infarction in diabetic rats with hypertrophy and fibrosis. WISP-1 could trigger downstream reactions through adhesion signaling pathway, resulting in increased WISP-1 signal, cardiomyocyte hypertrophy, fibroblast proliferation and myocardial fibrosis[17]. β-catenin signaling has been shown to be implicated in exerting the pro-apoptotic effect in previous studies[21, 22]. However, the relationship between the activity of the Wnt/β-catenin signaling pathway and apoptosis has not been fully elucidated. In the present study, CSE/H_2S up-regulation increased the mRNA and protein expression levels of WISP-1 and β-catenin as compared with the high glucose treatment, suggesting CSE/H_2S may protect cardiomyocytes from the high glucose injury through wnt/β-catenin pathway.

In this preliminary study, performed on high glucose-induced injury of NRCMs and H9c2 cells, we investigated the relationship between CSE/H_2S and Wnt/β-catenin pathway after high glucose exposure. Genetic CSE overexpression and exgenous H_2S administration could lead to a pronounced beneficial effect of cell viability, apoptosis and oxidative stress. Although the exact mechanisms must be further investigated, our study suggests that cardiac CSE/H_2S system may interact through Wnt/β-catenin pathway in cardiovascular dysfunction. Considering the key role that CSE/H_2S system plays in DCM disease, a better understanding of the regulation of CSE/H_2S system will help us to develop novel and more effective strategies to target CSE and alter H_2S production for prevention of DCM.

Conflict of Interest Statement

The authors declare that there is no conflict of interest related to the contents of this article.

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