Cellular Physiology and Biochemistry

Cell Physiol Biochem 2017;42:308-319
DOI: 10.1159/000480411
Published online: August 31, 2017
Accepted: June 15, 2017

Original Paper

Hydrogen Sulfide-Preconditioning of Human Endothelial Progenitor Cells Transplantation Improves Re-Endothelialization in Nude Mice with Carotid Artery Injury

Xiao Ke[a,b] Jun Zou[c] Qingsong Hu[d] Xiaoqing Wang[a] Chengheng Hu[e] Rongfeng Yang[e] Jiawen Liang[e] Xiaorong Shu[d] Ruqiong Nie[d] Changnong Peng[a]

[a]Department of Cardiology, Shenzhen Sun Yat-sen Cardiovascular Hospital, Shenzhen, [b]Department of Intensive Unit, Shenzhen Sun Yat-sen Cardiovascular Hospital, Shenzhen, [c]Department of Cardiology, Affiliated NanHai Hospital of Southern Medical University, Nanhai, [d]Department of Cardiology, Sun Yat-sen Memorial Hospital of Sun Yat-sen University, Guangzhou, [e]Department of Cardiology, The First Affiliated Hospital of Sun Yat-sen University, Guangzhou, China

Key Words
Endothelial progenitor cells • Preconditioning • Re-endothelialization

Abstract
Background/Aims: The aim of present study was to test the hypothesis that preconditioning with sodium hydrosulfide (NaHS) could enhance the capacity of migration, adhesion and proliferation of endothelial progenitor cells (EPCs) *in vitro*, and also could improve the efficacy of EPCs transplantation for re-endothelialization in nude mice with carotid artery injury. The paper further addressed the underlying mechanisms. ***Methods:*** EPCs were isolated from peripheral blood mononuclear cells of healthy male volunteers and the markers of EPCs were analyzed by flow cytometry. Thereafter, different concentrations of NaHS (25, 50, 100, 200 and 500 uM) were used for preconditioning EPCs. *In vitro* and *in vivo* migration, adhesion and proliferation as well as nitric oxide (NO) production of EPCs were evaluated. Carotid artery injury model was produced in nude mice and thereafter, NaHS-preconditioned EPCs were transplanted in order to evaluate their capacity of re-endothelialization. ***Results:*** Cellular immuno-staining showed that isolated cells expressed the key markers of EPCs. *In vitro*, EPCs proliferation rates and NO production were gradually increased in a NaHS-concentration dependent manner, while these benefits were blocked at a concentration of 500 uM NaHS. Similarly, the migration and adhesion rates of EPCs were also increased the most prominently at a concentration of 200

X. Ke, J. Zou and Q. Hu are contributed equally to this work.

Ruqiong Nie,
and Changnong Peng

Department of Cardiology, Sun Yat-sen Memorial Hospital, Sun Yat-sen University, Guangzhou, and Shenzhen Sun Yat-sen Cardiovascular Hospital, Shenzhen, (China)
E-Mail nieruqiong@163.com and pengchangnong1965@163.com

μM NaHS. *In vivo*, compared to the control group, treatment with NaHS-preconditioned EPCs significantly enhanced the capacity of re-endothelialization of EPCs. Fluorescent microscope revealed that there were more EPCs homing to the injury vessels in the NaHS-preconditioned EPCs group than the non-preconditioned group. With the administration of AMPK or eNOS inhibitors respectively, the above benefits of NaHS-preconditioning were abrogated. ***Conclusion:*** These results suggested that NaHS-preconditioning enhanced the biological function and re-endothelialization of EPCs through the AMPK/eNOS signaling pathway.

Introduction

Cardiovascular diseases including coronary heart disease (CHD) and peripheral arterial disease remain a major cause of morbidity and mortality worldwide. Percutaneous coronary intervention (PCI) is an important treatment of CHD. Compared to bare-metal stents (BMSs) implantation, drug-eluting stents (DESs) significantly reduces targeted vessels restenosis as well as improves long-term outcomes. However, late and very late in-stent thrombosis remain the key issues of stent implantation [1, 2]. Several studies have shown that endothelial dysfunction is involved in atherosclerotic cardiovascular disease development [3-5].The balance between endothelial injury and repair plays an important role in atherogenesis [5]. Notably, the favorable effects of DESs are due to its effect on preventing vascular smooth muscle cells (VSMCs) proliferation. However, vascular endothelial repair would also be compromised which in turn causes delayed re-endothelialization and late stent thrombosis [6] .Thus, promoting re-endothelialization may play a role in preventing DES thrombosis and sustaining vascular homeostasis [7, 8].

Cell-based therapy is a promising strategy for endothelial repair in vascular diseases [9]. Endothelial progenitor cells (EPCs), mobilizing from bone marrow into peripheral blood, have been showed to play an important role in vascular repair in response to injury [10, 11]. However, owing to the low retention and survival rates in the injured vascular wall, the clinical benefits of EPCs transplantation for vascular diseases are limited [12, 13].Therefore, to promote transplanted EPCs homing to and retaining in the injured vascular walls are the key prerequisites for improving the efficiency and efficacy of re-endothelialization and vascular repair.

Hydrogen sulfide (H_2S), the third member of the gaso-transmitter family, is a key signaling molecule which has a variety of biological benefits for vascular repair [14, 15]. Endogenous H_2S is produced from the main substrate of L-cysteine through catalyzing cystathionine beta-synthase (CBS) and cystathionine-gamma-lyase (CSE). Exogenous H_2S stimulates endothelial cells proliferation and migration, which in turn contribute to angiogenesis, coronary collateral vessel growth, and protection of ischemia-reperfusion (IR) injury [16]. In diabetic rats, H_2S can accelerate diabetic wound repair by promoting angiogenesis [17]. It has also been demonstrated that preconditioning with H_2S could promote mesenchymal stem cells survival and improve myocardial repair in rats with myocardial infarction [18]. Nevertheless, questions regarding the efficacy and associated mechanisms of H_2S preconditioning for vascular repair are unresolved. Therefore, the aim of present study was to test the hypothesis that preconditioning with sodium hydrosulfide (NaHS), a H_2S donor, could enhance the migration, adhesion and proliferation rates of EPCs *in vitro*, and also could improve the efficacies of EPCs transplantation for re-endothelialization in rats with carotid artery injury, and the underlying mechanisms were also be explored.

Materials and Methods

EPCs culture and characterization

EPCs were isolated and cultured in accordance to previous described methods [19-21]. Briefly, peripheral blood mononuclear cells (PBMNCs) were isolated from healthy volunteers (20 to 40 years old

male after informed consent was obtained) and cultured on fibronectin-coated plates using endothelial basal medium-2 (Lonza, Basel, CC-4176) supplemented with EGM-2 Bullte Kit (Lonza) and 20 % Fetal bovine serum (FBS, GIBCO). After 4 days' culture, the non-adherent cells were discarded and adherent cells were cultured for another 7 days and then used for the following experiments. According to previous described [22, 23], early EPCs were defined as positive staining for both 1, 1'-dioctadecyl-3, 3,3',3'-tetramethylindocarbocyanine (DiI)-acetylated low density lipoprotein (ac-LDL) (20 μg/ml; Invitrogen, Carlsbad, CA, USA) and FITC-labeled BS-1 lectin (10 μg/ml; Sigma-Aldrich, St. Louis, MO, USA). The expressions of endothelial markers of cultured EPCs were examined using flow cytometry analysis with the following antibodies: phycoerythrin (PE)-labeled monoclonal mouse anti-human antibodies recognizing CD31 (BD Pharmingen), von Willebrand factor (vWF) (BD Pharmingen), kinase-insert domain receptor (KDR) (R&D Systems), and CD14 (BD Pharmingen). Based on the flow cytometry findings, the adherent mononuclear cells were identified as early EPCs which were consistent with previous reports [24, 25].

Tube formation assay

In vitro capillary-like structure formation of EPCs was evaluated using growth factor reduced Matrigel (BD Biosciences). Briefly, Matrigel (50ul/well) was added to 96-well plates and incubated at 37°C for 30 min. The cells (2 * 10^4 cells per well) were seeded on Matrigel and cultured for 8h. Tube formation was quantified using an inverted microscope (Olympus BX51).

Cells proliferation assay

H_2S was administrated by the form of sodium hydrosulfide (NaHS; Sigma-Aldrich). The concentration and incubation time of NaHS were determined based on our pilot study. The effects of NaHS on EPCs viability was assessed by CCK8 (Dojindo Molecular Technologies, Kumamoto, Japan). The cells were treated with a concentration of NaHS from 25 to 500 uM for 24h. To explore whether the AMPK-eNOS pathway contributed to the cyto-protection of NaHS, EPCs were treated with 10 uM compound C (an inhibitor of AMPK, purchased from Sigma-Aldrich) or 100 uM L-NAME (an inhibitor of eNOS, Calbiochem) for 30min before exposure to NaHS for 24h.

Western blot analysis

EPCs were lysed with cell lysis buffer (Cell Signaling Technology, Boston, MA, USA) according to the manufacturer's instructions. Cell lysates were quantified by BCA methods according to the manufacturer's instructions (Sangon Biotechnology, Shanghai, China). Totally, 50 μg proteins were subjected to SDS-PAGE and then transferred to polyvinylidene fluoride membranes. The following antibodies were used: rabbit anti-AMPKα antibody (1:1000; Cell Signaling Technology), Phospho-AMPKα (Thr 172) rabbit mAb (1:1000;Cell Signaling Technology), Phospho-eNOS (Ser1177) rabbit mAb (1:1000;Cell Signaling Technology), eNOS (49G3) rabbit mAb (1:1000;Cell Signaling Technology), and GAPDH (14C10) rabbit mAb (1:1000;Cell Signaling Technology). Proteins were visualized with HRP-conjugated anti-rabbit IgG (1:5000; Cell Signaling Technology).To detect the effect of stimulation of PB-derived EPCs with H_2S on the phosphorylation of AMPK and eNOS, EPCs were incubated with 200 uM NaHS for 12h before proteins were obtained.

Nitric oxide (NO) production

Measurement of NO production. NO production of EPCs was measured as the generation of nitrite. The cells were cultured with EBM-2 (growth factor-free) for 24h after NaHS stimulation. The supernatants were used to determine NO level using a NO assay kit by the nitrate reductase method (Nanjing Jiancheng Institute of Biological Engineering, Nanjing, China).

Intracellular NO production of EPCs. Intracellular NO production of EPCs was determined by staining with 4-amino-5methylamino-2, 7,-difluroflurescein (DAF-FM; Molecular Probes, Carlsbad, CA,USA) and assessed by laser scanning confocal microscope as previous described [26].

In vitro EPCs migration assays

EPCs migration assays were performed using a Transwell system (Corning Costar, Tewksbury, MA, USA) with 8uM polycarbonate filter inserts in 24-well plates as described in our previous study [21]. Briefly, a total of 2 * 10^4 EPCs were suspended in 250ul EBM-2 medium in the upper chamber, and the lower chamber was placed in a 24-well culture dish containing 500ul EBM-2 supplemented with 50ng/ml VEGF.

After incubation at 37°C for 24h, cells were fixed with 4 % paraformaldehyde and stained by DAPI. The migrated cells were counted by independent investigators who were blinded to the treatment groups.

In vitro EPCs adhesion assays

A monolayer of human umbilical vein endothelial cells (HUVECs) was prepared for 48h before placing $2 * 10^5$ cells in each well of a four-well plate. The HUVECs were pre-treated with 1 ng/ml tumor necrosis factor-α (TNF-α, Peprotech) for 12h. Thereafter, $1 * 10^5$ CM-DiI-labeled EPCs were added to each well, and the cells were incubated at 37°C for 3h. The non-attached cells were gently removed with PBS, and the adherent EPCs were fixed in 4 % of PFA and randomly counted by an independent investigator who was blinded to the treatments.

Animal model production and in vivo re-endothelialization assay

The carotid artery injuries model and EPCs transplantation were performed in accordance to previous described methods [20, 21, 27]. Six to eight weeks old male NRMI nu/nu athymic nude mice (obtained from the Laboratory Animal Center of Sun Yat-sen University, Guangzhou, China) were used in current study. All animals were raised in the Experimental Animal Center of Sun Yat-sen University. All experimental procedures and protocols were complied with the Animal Care and Use Ethics Committees of Sun Yat-Sen University. The animals were anaesthetized with Ketamine (100mg/kg IP) and xylazine (5mg/kg IP). The surgeries were performed under a stereoscopic microscope. The left carotid artery was exposed via a midline incision on the ventral side of the neck. The bifurcation of the carotid artery was located, and two ligatures were placed around the external carotid artery, which was then tied off with the distal ligature. An incision hole was made between the ligatures to introduce the denudation device. A curved flexible wire (0.35-mm diameter) was introduced into the common carotid artery and passed over the lining of artery for three times so as to denude the endothelium. The wire was then removed, and the external carotid artery was tied off proximal to the incision hole with the proximal ligature. EPCs ($1 * 10^6$) that had been cultured for 7 days were re-suspended in 100 μl of pre-warmed PBS (37°C) and were transplanted 3h after carotid artery injury via tail vein injection with a 27 G needle. The same volume of PBS was injected into sham-operation mice as a control. Three days after carotid artery injury, endothelial regeneration was evaluated by staining denuded areas with 50 μl of 5 % Evans Blue dye via tail vein injection. To examine the homing of transplanted EPCs to the site of the injured carotid vessel, labeled EPCs ($1 * 10^6$) were incubated with CM-DiI (Cell TrackerTM CM-DiI, Invitrogen) in accordance to the manufacturer's instructions. CM-DiI-labeled EPCs incorporated in the injured vessels were quantitatively analyzed under a fluorescence microscope (Olympus BX51).

Statistical analysis

All of the data are reported as the mean ± SD. Statistical significance was evaluated by Student's t-test. A value of $P < 0.05$ was considered to statistical significance. All statistical analyses used SPSS statistical software (SPSS version 13.0).

Results

Early EPCs characterization

EPCs were isolated from fresh human PBMNCs by density gradient centrifugation and grown in EGM-2 media for 7 days. As presented in the Figure1A, cellular immuo-staining showed that the adherent cells were able to uptake of DiI-ac-LDL, bind FITC-lectin and form capillary-like structures on Matrigel (Fig.1C). Meanwhile, early EPCs expressed endothelial markers (CD31, vWF and KDR) and a monocyte marker CD14 at a comparable level (Fig.1B). These results were consistent with previous studies [20, 28] and confirmed that EPCs had been successfully isolated using this approach.

Effect of preconditioning on EPCs proliferation and NO production

The effect of NaHS on EPCs proliferation was assessed by CCK8. After 7 days, cultured EPCs from healthy subjects were pre-incubated with 25, 50, 100, 200 and 500uM of

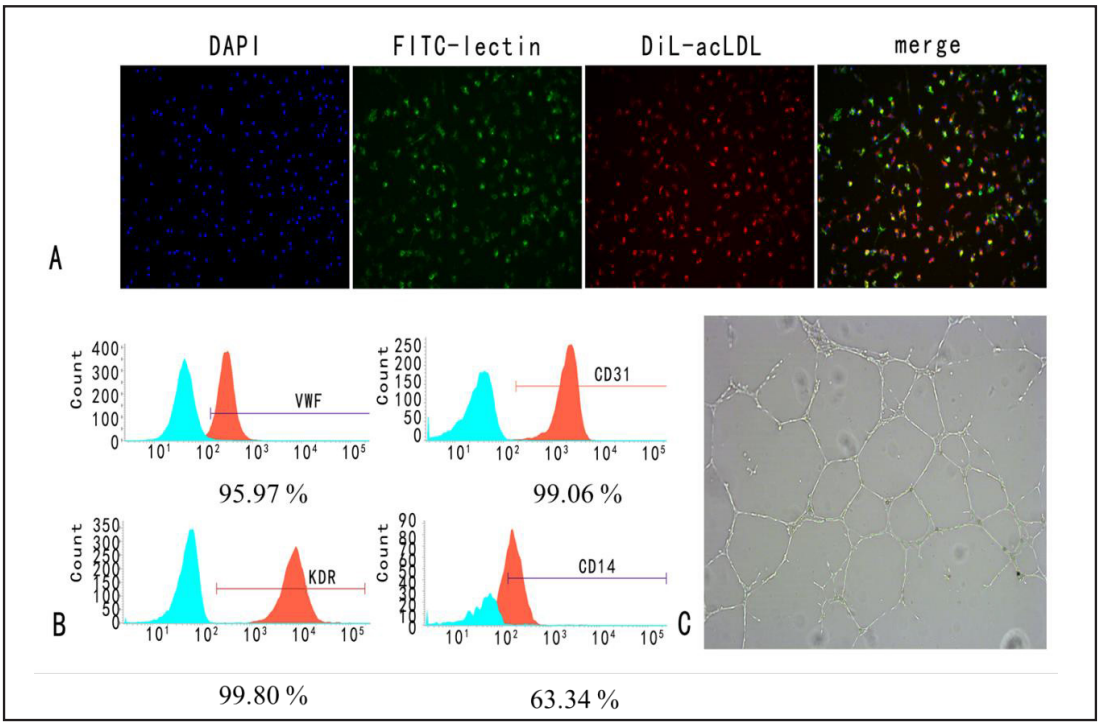

Fig. 1. Characterization of EPCs derived from human PBMNCs. (A) Representative photographs of EPCs took up DiI-ac-LDL and bound FITC lectin. Scale bar: 100 um. (B) Markers of EPCs detected by flow cytometry analysis using CD31, vWF, KDR and CD14 (IgG isotype control was shown in blue, n = 4 per group). (C) EPCs formed capillary-like networks when seeded onto Matrigel surfaces. Scale bar: 100 um.

NaHS, respectively. As shown in the Fig. 2A, cells proliferation gradually increased from a concentration of 25uM to 200 uM. Nevertheless, cells proliferation was inhibited at 500uM.

It was speculated that NO was imperative for H_2S to exert protective effects [29, 30] , therefore, we evaluated NO level in the conditioned media at 48h of NaHS stimulation. As shown in the Figure2B, NaHS promoted NO production in a dose-dependent manner. Nonetheless, at a concentration of 500uM, NO production was reduced which was further confirmed by DAF-FM fluorescence dye. We also found that the intracellular NO level in NaHS-preconditioning EPCs (200uM NaHS) was up-regulated most significantly compared to the control group (Fig. 2C). Based on these findings, NaHS concentration of 200 uM was defined as the maximum dose for the following study.

NaHS-preconditioning enhanced EPCs migration, adhesion and repair capacity

The transwell was used to evaluate EPCs migration and the results showed that the numbers of migrating cells after NaHS-preconditioned were significantly increased in a dose-dependent manner (Fig.3A and B). Similarly, after 7 days of culture, the adhesion was also significantly promoted in a dose-dependent manner (Fig. 3C and D). The ability of endothelial repair by EPCs was assessed in a carotid artery injury mode in nude mice (Fig. 4A). To investigate whether NaHS-preconditioning EPCs transplantation could accelerate re-endothelialization, Evans Blue staining was performed. Notably, compared to PBS infusion, treatment with non-preconditioned EPCs and NaHS-preconditioned EPCs (*in vitro* 200 uM of NaHS treatment for 24h) significantly enhanced re-endothelialization capacity of EPCs *in vivo* ($P < 0.01$, Fig. 4B).To determine whether the transplanted EPCs were incorporated at sites of injury vessels, each nude mouse was transplanted with $1 * 10^6$ CM-DiI-labeled EPCs after carotid artery injury. Fluorescent microscope revealed that the transplanted EPCs were incorporated at the sites of injury. Data showed that there were more EPCs homing to the

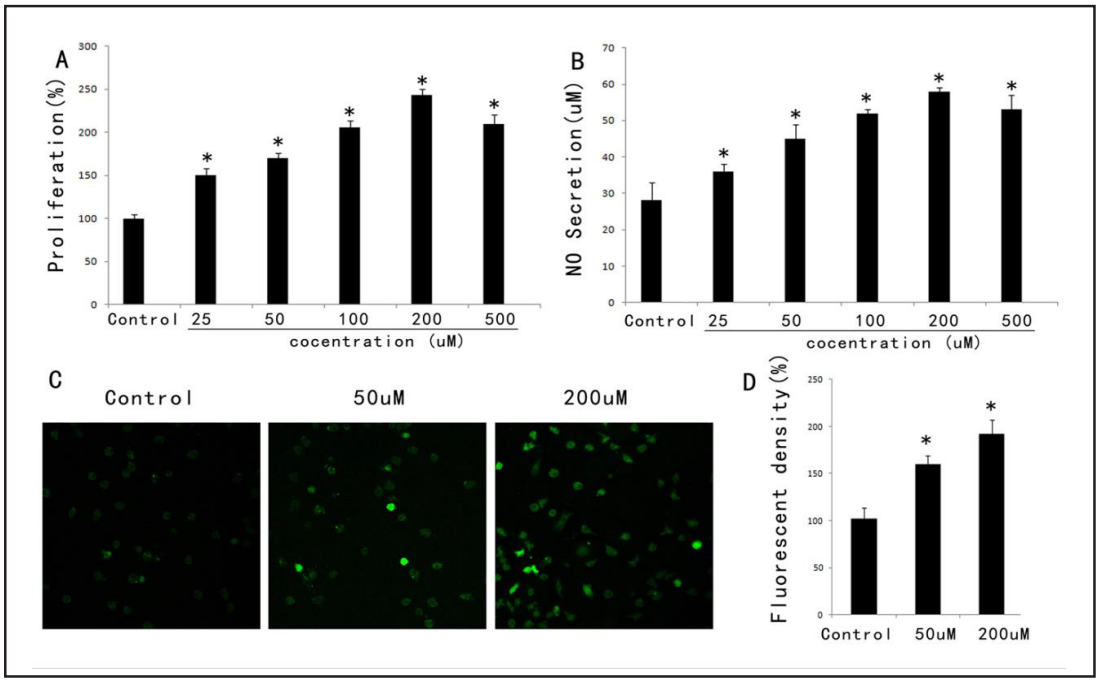

Fig. 2. Effects of NaHS on EPCs proliferation and NO production. (A) EPCs proliferation was measured using CCK8. EPCs proliferation after 24h treatment with various NaHS concentrations (0-500uM) (n = 5 per group, *P<0.05 vs control). (B) NO production was assayed using the nitrate reductase method with NaHS-preconditioning for 24h. NO production of EPCs with various NaHS concentrations (0-500uM) (n = 5 per group, *P<0.05 vs control). (C and D) Intracellular NO level was measured by DAF-FM. (C), Intracellular NO production in EPCs. (D), Relative quantification of NO level.

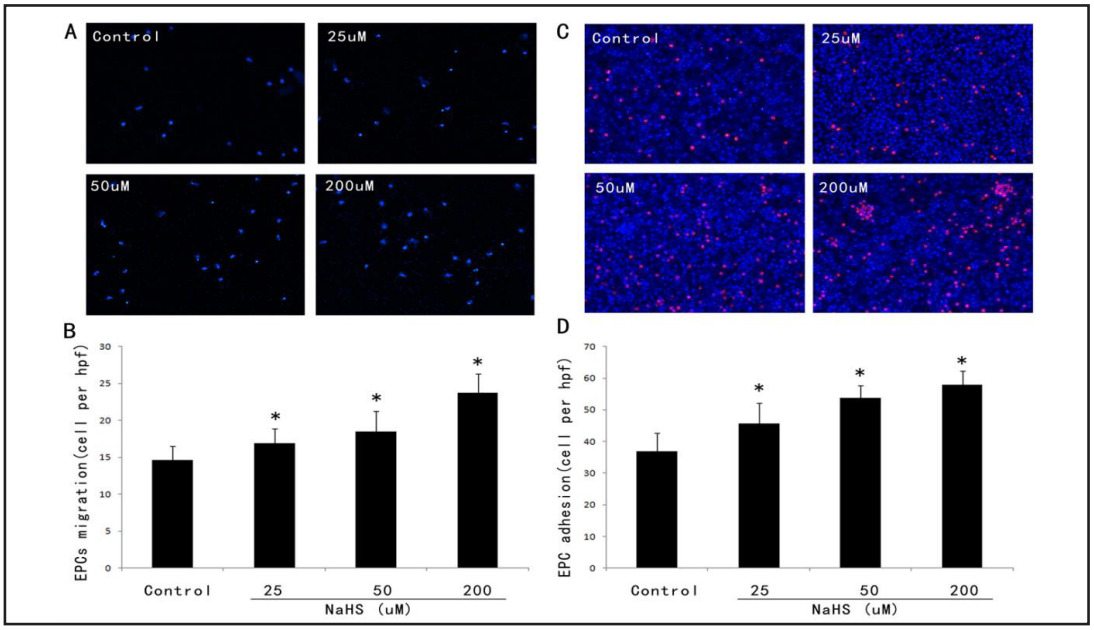

Fig. 3. Effect of NaHS on EPCs migration and adhesion. (A and B) Representative photographs (A) and Quantitative analyses (B) showed EPCs migration after pre-treated with different levels of NaHS in vitro (*P<0.05 vs control, n = 5 per group). (C and D) Representative photographs (C) and Quantitative analyses (D) showed the DiI-labeled EPCs adhesion to HUVECs (*P<0.05 vs control, n = 5 per group).

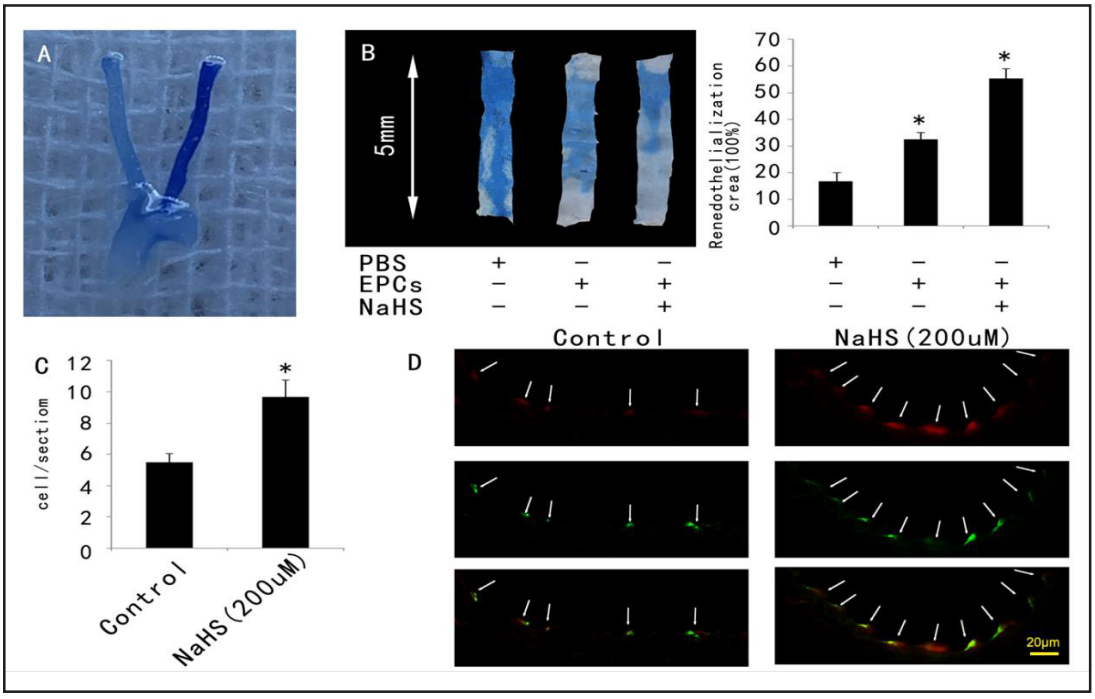

Fig. 4. Effect of NaHS of EPCs for carotid artery repair. (A) Evans blue staining was used to identify injured carotid artery. Representative photograph showed injured and contralateral uninjured carotid artery. DiI-labeled EPCs were identified as red fluorescent cells, and green fluorescence represented the FITC-lectin positive endothelial layer. (B) EPCs transplantation after pre-treatment with 200uM of NaHS for 24h resulted in improvement of re-endothelialization (*P<0.01 vs. the PBS group, # P<0.01 vs. EPCs group, n = 5 per group). (C) Larger number of NaHS-preconditioned EPCs was detected (*P<0.01 vs EPCs group, n = 5 per group). (D) EPCs tracing in vivo: an enface view of the injured carotid arteries Showed the CM-DiI-labeled EPCs attached to the vascular injured site on day 3.

injury vessels in the NaHS-preconditioned EPCs group than the non-conditioned EPCs group (Fig. 4C and D).

AMPK/eNOS signaling pathway involved in in vitro and in vivo repair capacity of EPCs

The activations of AMPK and eNOS are essential to EPCs proliferation and functioning. We therefore examined whether the important roles of NaHS in the proliferation, NO production, migration and adhesion of EPCs were mediated by AMPK/eNOS signaling pathway. We used inhibitors including compound C (an AMPK inhibitor) and L-NAME (an eNOS inhibitor) to inhibit the AMPK/eNOS signaling pathway, respectively. Following stimulation with NaHS (200 uM) for 24h, phosphorylation levels of AMPK and eNOS were enhanced in the NaHS-preconditioned EPCs. However, the increases in AMPK and eNOS phosphorylation were inhibited by pre-incubation with compound C and L-NAME (Fig. 5A and B). In addition, the migration and adhesion activity of EPCs were also suppressed with pre-incubation of compound C and L-NAME (Fig. 5C and D). We also investigated whether the AMPK/eNOS signaling pathway was associated with the re-endothelialization capacity of NaHS-preconditioned EPCs *in vivo*. Consist with the *in vitro* results, NaHS treatment significantly increased the re-endothelialization capacity of EPCs, but Compound C and L-NAME abolished the efficacy (Fig. 5E and F). Collectively, these results suggested that NaHS -preconditioning enhanced the re-endothelialization capacity of EPCs through the AMPK/eNOS signaling pathway.

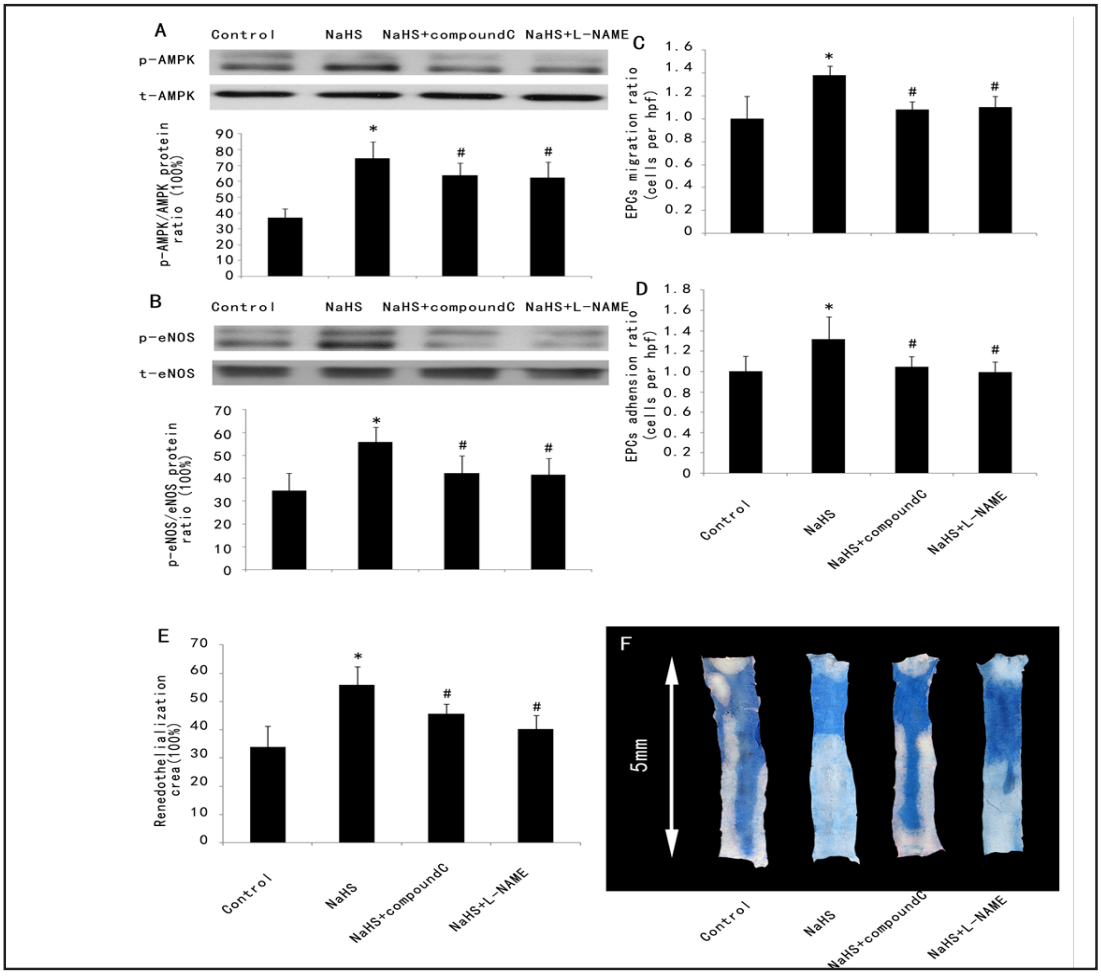

Fig. 5. NaHS improved EPCs function in vitro and re-endothelializationin vivo by activating AMPK/eNOS pathway. (A and B) Representative photographs and quantitative analyses of protein. NaHS significantly increased AMPK and eNOS phosphorylation in EPCs (*P<0.01 vs. control group, #P<0.01 vs. NaHS group, n = 5 per group). (C and D) Quantification analysis of migration and adhesion to HUVECs with NaHS pre-treatment (*P<0.01 vs the PBS group, #P<0.01 vs EPCs group, n = 5 per group). (E and F) Representative photographs and quantitative analyses of re-endothelialization with 200uM NaHS-preconditioning of EPCs in response to AMPK or eNOS inhibitors (*P<0.01 vs the control group, # P<0.01 vs. NaHS group, n = 5 per group).

Discussion

Re-endothelialization plays an important role in tissue regeneration and repair. Implantation of DES impairs endothelial function which in turn leads to delayed re-endothelialization and increased risk of late or very late in-stent thrombosis. Therefore, accelerating re-endothelialization is a significant therapeutic means for vascular repair, thrombosis prevention, and excessive neo-intimal hyperplasia suppression [31, 32].

Cell-based therapy has become a promising therapeutic strategy for vascular diseases, and re-endothelialization with stem cells transplantation has also become a new method to prevent vessel re-stenosis after stent placement. However, majority of transplanted cells were necrotic and apoptotic due to poor environment. We used a novel pharmacological preconditioning strategy to increase transplanted EPCs survival rate. Notably, H_2S has been consider as the third gaso-transmitter next to NO and carbon monoxide [33] .The principal findings of the present study are as follows: preconditioning of EPCs with NaHS significantly

promotes the migration, adhesion and proliferation of EPCs as well as increases NO production *in vitro*; and *in vivo* study, NaHS treatment markedly up-regulates the AMPK/eNOS signaling pathway as well as enhances re-endothelialization capacity of EPCs. Our findings for the first time demonstrate the potential effects of NaHS on improving the efficacy of EPCs for repair of endothelial injury.

Previous studies have showed that accelerating re-endothelialization is an important therapeutic means for repairing vascular injury and maintaining vascular homeostasis [34, 35]. EPCs transplantation represents a promising therapeutic strategy for restoration of the integrity of vascular endothelium. However, previous strategies of EPCs transplantation yielded modest effects on vascular repair due to acute-phase of transplanted cells necrosis and apoptosis [36]. The lower survival rate significantly influenced the efficacy of stem cells therapy [37-39]. The reasons for low survival rate were multifactorial including ischemic injury and associated inflammation and oxidation [40-44].

In recent years, researchers focused on strategies to improve cells survival rate after transplantation. Numerous studies suggested that genetically modified EPCs with protective genes such as Akt, Bcl-2, β2AR, CXCR4, CXCR7, BH4, FOX2, eNOS could improve survival rate which in turn could contribute to promote therapeutic efficacies [20-21, 25, 27]. Medications, including statins and angiotensin converting enzyme inhibitors, have also been used to evaluate their effects on EPCs mobilization, and compared to gene-engineering, preconditioning of EPCs with chemical agents were more clinically plausible.

H_2S is an important signaling molecule with multiple biological properties. In mammalian cells, endogenous H_2S is predominantly synthesized by CBS, CSE, cysteine aminotransferase (CAT) and 3-mercaptopyruvate sulfur-transferase (MST). Recently, evidence suggests that H_2S plays important roles on vascular homeostasis, stem/progenitor cells proliferation and differentiation, and inflammation and oxidation amelioration [39, 45, 46]. Our current study indicated that 500 um NaSH has no additional benefit for EPCs, and based on previous report, we considered that the potential mechanisms might be related to the absence of any detectable Ca^{2+} signal with a higher concentration of NaHS as reported by prior study [4]. In addition, high concentration of NaHS might also result in cytotoxicity which in turn also leaded to NO production and proliferation decrease.

The main purpose of present study was to investigate the efficacy of NaHS-preconditioning of EPCs for vascular repair. We firstly investigated the cyto-protective effect of NaHS on EPCs isolated from peripheral blood of healthy donors *in vitro*, and it showed that 500uM NaHS caused a reduction in cell proliferation, which was consistent with the results of Potenza et al. [15]. Therefore, we used NaHS with a concentration of 25-200uM in subsequent studies. Based on previous report [15], we postulated that the potential mechanism associated with inhibitory effect of 500 uM NaHS might be related to dys-regulation of calcium dependent signaling pathway and cellular toxicity. Importantly, we found that NaHS-preconditioning significantly improved EPCs function with a maximal concentration of 200 uM. Specifically, our results showed that NaHS-preconditioning promoted EPCs migration, adhesion, proliferation and NO production compared to the control group.

With the favorable findings from *in vitro* study, we further investigated the effects of NaHS-preconditioning for improving the efficacies of EPCs for re-endothelialization in a nude mouse carotid artery injury model. As shown in the Fig. 4 B and D, more infused DiI-Labeled EPCs in NaHS-preconditioned group were found incorporated into the FITC-lectin positive endothelial layer compared to the non-preconditioned EPCs group. A significantly larger area of re-endothelialization was also observed which might be attributed to increasing NO production with NaHS stimulation [47]. Furthermore, NaHS stimulation increased AMPK/eNOS phosphorylation, which also contributed to improvement of proliferation, migration, and adhesion of EPCs *in vitro* and accelerated re-endothelialization *in vivo*. In contrast, suppressing AMPK/eNOS signaling pathway by compound C and L-NAME abrogated the vascular repair effect of NaHS *in vivo*. The migration, adhesion and NO production *in vitro* were also reduced. Collectively, these findings confirmed that NaHS/AMPK/eNOS signaling pathway involved in the improvement of efficacies of EPCs transplantation.

Conclusion

In summary, our present study shows that transplantation of NaHS-preconditioned EPCs accelerates EPCs homing and re-endothelialization thorough the AMPK/eNOS signaling pathway. NaHS-preconditioned EPCs transplantation may be a novel and promising cell-based therapeutic approach for vascular diseases.

Acknowledgements

We appreciated very much for the help Dr. Fang Yang provided to us. The work was supported by the National Nature Science Foundation of China (NO.81370309), the Natural Science Foundation of Guangdong Province of China (2016A030313248), the Science and Technology project of Guangzhou city of China (201604020129), the Science and Technology project of Shenzhen city of China (JCYJ20150402094341897and JCYJ20170307161535847), and the Sanming Project of Medicine in Shenzhen and The science and project of Shenzhen city of China (20170413114916687).

Disclosure Statement

None.

References

1 Inoue T, Croce K, Morooka T, Sakuma M, Node K, Simon DI: Vascular inflammation and repair: implications for re-endothelialization, restenosis, and stent thrombosis. JACC Cardiovasc Interv 2011;4:1057-1066.
2 Wessely R. New drug-eluting stent concepts: Nat Rev Cardiol 2010;7:194-203.
3 Siasos G, Tousoulis D, Antoniades C, Stefanadi E, Stefanadis C: L-Arginine, the substrate for NO synthesis: an alternative treatment for premature atherosclerosis? Int J Cardiol 2007;116:300-308.
4 Sen T, Aksu T: Endothelial progenitor cell and adhesion molecules determine the quality of the coronary collateral circulation/Endothelial progenitor cells (CD34+KDR+) and monocytes may provide the development of good coronary collaterals despite the vascular risk factors and extensive atherosclerosis. Anadolu Kardiyol Derg 2012;12:447-448.
5 Mannarino E, Pirro M: Endothelial injury and repair: a novel theory for atherosclerosis. Angiology 2008;59:69S-72S.
6 Hutter R, Carrick FE, Valdiviezo C, Wolinsky C, Rudge JS, Wiegand SJ, Fuster V, Badimon JJ, Sauter BV:Vascular endothelial growth factor regulates reendothelialization and neointima formation in a mouse model of arterial injury. Circulation 2004;110:2430-2435.
7 Finn AV, Nakazawa G, Joner M, Kolodgie FD, Mont EK, Gold HK, Virmani R: Vascular responses to drug eluting stents: importance of delayed healing. Arterioscler Thromb Vasc Biol 2007;27:1500-1510.
8 Caixeta A, Leon MB, Lansky AJ, Nikolsky E, Aoki J, Moses JW, Schofer J, Morice MC, Schampaert E, Kirtane AJ, Popma JJ, Parise H, Fahy M, Mehran R: 5-year clinical outcomes after sirolimus-eluting stent implantation insights from a patient-level pooled analysis of 4 randomized trials comparing sirolimus-eluting stents with bare-metal stents. J Am Coll Cardiol 2009;54:894-902.
9 Zou T, Fan J, Fartash A, Liu H, Fan Y:Cell-based strategies for vascular regeneration. J Biomed Mater Res A 2016;104:1297-1314.
10 Zhu S, Malhotra A, Zhang L, Deng S, Zhang T, Freedman NJ, Storms R, Peppel K, Goldschmidt-Clermont PJ, Dong C:Human umbilical cord blood endothelial progenitor cells decrease vein graft neointimal hyperplasia in SCID mice. Atherosclerosis 2010;212:63-69.
11 Liu Y, Yang R, Liu X, Zhou Y, Qu C, Kikuiri T, Wang S, Zandi E, Du J, Ambudkar IS, Shi S:Hydrogen sulfide maintains mesenchymal stem cell function and bone homeostasis via regulation of Ca(2+) channel sulfhydration. Cell Stem Cell 2014;15:66-78.

12 Tang YL, Tang Y, Zhang YC, Qian K, Shen L, Phillips MI:Improved graft mesenchymal stem cell survival in ischemic heart with a hypoxia-regulated heme oxygenase-1 vector. J Am Coll Cardiol 2005;46:1339-1350.
13 Alexandru N, Popov D, Dragan E, Andrei E, Georgescu A:Circulating endothelial progenitor cell and platelet microparticle impact on platelet activation in hypertension associated with hypercholesterolemia. PLoS One 2013;8:e52058.
14 Mani S, Li H, Untereiner A, Wu L, Yang G, Austin RC, Dickhout JG, Lhoták Š, Meng QH, Wang R:Decreased endogenous production of hydrogen sulfide accelerates atherosclerosis. Circulation2013;127:2523-2534.
15 Zhang HX, Liu SJ, Tang XL, Duan GL, Ni X, Zhu XY, Liu YJ, Wang CN:H2S Attenuates LPS-Induced Acute Lung Injury by Reducing Oxidative/Nitrative Stress and Inflammation. Cell Physiol Biochem 2016;40:1603-1612.
16 Cai WJ, Wang MJ, Moore PK, Jin HM, Yao T, Zhu YC:The novel proangiogenic effect of hydrogen sulfide is dependent on Akt phosphorylation. Cardiovasc Res 2007;76:29-40.
17 Wang G, Li W, Chen Q, Jiang Y, Lu X, Zhao X: Hydrogen sulfide accelerates wound healing in diabetic rats. Int J Clin Exp Pathol 2015;8:5097-5104.
18 Xie X, Sun A, Zhu W, Huang Z, Hu X, Jia J, Zou Y, Ge J: Transplantation of mesenchymal stem cells preconditioned with hydrogen sulfide enhances repair of myocardial infarction in rats. Tohoku J Exp Med 2012;226:29-36.
19 Asahara T, Murohara T, Sullivan A, Silver M, van der Zee R, Li T, Witzenbichler B, Schatteman G, Isner JM: Isolation of putative progenitor endothelial cells for angiogenesis. Science 1997;275:964-967.
20 Chen L, Wu F, Xia WH, Zhang YY, Xu SY, Cheng F, Liu X, Zhang XY, Wang SM, Tao J:CXCR4 gene transfer contributes to *in vivo* reendothelialization capacity of endothelial progenitor cells. Cardiovasc Res 2010;88:462-470.
21 Ke X, Shu XR, Wu F, Hu QS, Deng BQ, Wang JF, Nie RQ:Overexpression of the beta2AR gene improves function and re-endothelialization capacity of EPCs after arterial injury in nude mice. Stem Cell Res Ther 2016;7:73.
22 Basile DP, Yoder MC:Circulating and tissue resident endothelial progenitor cells. J Cell Physiol 2014;229:10-16.
23 Yang Z, Xia WH, Su C, Wu F, Zhang YY, Xu SY, Liu X, Zhang XY, Ou ZJ, Lai GH, Liao XX, Jin YF, Tao J:Regular exercise-induced increased number and activity of circulating endothelial progenitor cells attenuates age-related decline in arterial elasticity in healthy men. Int J Cardiol 2013;165:247-254.
24 Zhang J, Chen C, Hu B, Niu X, Liu X, Zhang G, Zhang C, Li Q, Wang Y: Exosomes Derived from Human Endothelial Progenitor Cells Accelerate Cutaneous Wound Healing by Promoting Angiogenesis Through Erk1/2 Signaling. Int J Biol Sci 2016;12:1472-1487.
25 Xia WH, Chen L, Liang JW, Zhang XY, Su C, Tong X, He J, Li Y, Cao Z, Lin XF, Tao J:BMP4/Id2 signaling pathway is a novel therapeutic target for late outgrowth endothelial progenitor cell-mediated endothelial injury repair. Int J Cardiol 2017;228:796-804.
26 Cheng Z, Jiang X, Pansuria M, Fang P, Mai J, Mallilankaraman K, Gandhirajan RK, Eguchi S, Scalia R, Madesh M, Yang X, Wang H:Hyperhomocysteinemia and hyperglycemia induce and potentiate endothelial dysfunction via mu-calpain activation. Diabetes 2015;64:947-959.
27 Zhang XY, Su C, Cao Z, Xu SY, Xia WH, Xie WL, Chen L, Yu BB, Zhang B, Wang Y, Tao J:CXCR7 upregulation is required for early endothelial progenitor cell-mediated endothelial repair in patients with hypertension. Hypertension 2014;63: 383-389.
28 Critser PJ, Yoder MC:Endothelial colony-forming cell role in neoangiogenesis and tissue repair. Curr Opin Organ Transplant 2010;15:68-72.
29 Yuan S, Kevil CG:Nitric Oxide and Hydrogen Sulfide Regulation of Ischemic Vascular Remodeling. Microcirculation 2016;23:134-145.
30 Cheng Z, Garikipati VN, Nickoloff E, Wang C, Polhemus DJ, Zhou J, Benedict C, Khan M, Verma SK, Rabinowitz JE, Lefer D, Kishore R. Restoration of Hydrogen Sulfide Production in Diabetic Mice Improves Reparative Function of Bone Marrow Cells. Circulation 2016;134:1467-1483.
31 Gulati R, Jevremovic D, Peterson TE, Witt TA, Kleppe LS, Mueske CS, Lerman A, Vile RG, Simari RD:Autologous culture-modified mononuclear cells confer vascular protection after arterial injury. Circulation 2003;108:1520-1526.
32 Werner N, Priller J, Laufs U, Endres M, Böhm M, Dirnagl U, Nickenig G:Bone marrow-derived progenitor cells modulate vascular reendothelialization and neointimal formation: effect of 3-hydroxy-3-methylglutaryl coenzyme a reductase inhibition. Arterioscler Thromb Vasc Biol 2002;22:1567-1572.

33 Yang G, Wu L, Jiang B, Yang W, Qi J, Cao K, Meng Q, Mustafa AK, Mu W, Zhang S, Snyder SH, Wang R:H2S as a physiologic vasorelaxant: hypertension in mice with deletion of cystathionine gamma-lyase. Science 2008;322:587-590.
34 Wassmann S, Werner N, Czech T, Nickenig G:Improvement of endothelial function by systemic transfusion of vascular progenitor cells. Circ Res 2006;99: e74-e83.
35 Werner N, Kosiol S, Schiegl T, Ahlers P, Walenta K, Link A, Böhm M, Nickenig G:Circulating endothelial progenitor cells and cardiovascular outcomes. N Engl J Med 2005;353:999-1007.
36 Yumoto H, Hirao K, Tominaga T, Bando N, Takahashi K, Matsuo T: Electromagnetic wave irradiation promotes osteoblastic cell proliferation and up-regulates growth factors via activation of the ERK1/2 and p38 MAPK pathways. Cell Physiol Biochem 2015;35:601-615.
37 Zhang M, Methot D, Poppa V, Fujio Y, Walsh K, Murry CE:Cardiomyocyte grafting for cardiac repair: graft cell death and anti-death strategies. J Mol Cell Cardiol 2001;33:907-921.
38 Toma C, Pittenger MF, Cahill KS, Byrne BJ, Kessler PD:Human mesenchymal stem cells differentiate to a cardiomyocyte phenotype in the adult murine heart. Circulation 2002;105:93-98.
39 Zheng D, Dong S, Li T, Yang F, Yu X, Wu J, Zhong X, Zhao Y, Wang L, Xu C, Lu F, Zhang W:Exogenous Hydrogen Sulfide Attenuates Cardiac Fibrosis Through Reactive Oxygen Species Signal Pathways in Experimental Diabetes Mellitus Models. Cell Physiol Biochem 2015;36:917-929.
40 Lev EI, Leshem-Lev D, Mager A, Vaknin-Assa H, Harel N, Zimra Y, Bental T, Greenberg G, Dvir D, Solodky A, Assali A, Battler A, Kornowski R:Circulating endothelial progenitor cell levels and function in patients who experienced late coronary stent thrombosis. Eur Heart J 2010;31:2625-2632.
41 Khurana R, Mayr M, Hill JM: Endothelial progenitor cells, late stent thrombosis and delayed re-endothelialisation. EuroIntervention 2008;3:518-525.
42 De Winter RJ, Klomp M:Understanding the role of endothelial progenitor cells in cardiovascular disease, coronary artery lesion progression, and in-stent restenosis. JACC Cardiovasc Interv 2010;3:87-89.
43 Padfield GJ, Newby DE, Mills NL:Understanding the role of endothelial progenitor cells in percutaneous coronary intervention. J Am Coll Cardiol 2010;55: 1553-1565.
44 Pelliccia F, Cianfrocca C, Rosano G, Mercuro G, Speciale G, Pasceri V:Role of endothelial progenitor cells in restenosis and progression of coronary atherosclerosis after percutaneous coronary intervention: a prospective study. JACC Cardiovasc Interv 2010;3:78-86.
45 Yu Q, Lu Z, Tao L, Yang L, Guo Y, Yang Y, Sun X, Ding Q:ROS-Dependent Neuroprotective Effects of NaHS in Ischemia Brain Injury Involves the PARP/AIF Pathway. Cell Physiol Biochem 2015;36:1539-1551.
46 Xiao J, Zhu X, Kang B, Xu J, Wu L, Hong J, Zhang Y, Ni X, Wang Z: Hydrogen Sulfide Attenuates Myocardial Hypoxia-Reoxygenation Injury by Inhibiting Autophagy via mTOR Activation. Cell Physiol Biochem 2015; 37:2444-2453.
47 Altaany Z, Moccia F, Munaron L, Mancardi D, Wang R:Hydrogen sulfide and endothelial dysfunction: relationship with nitric oxide. Curr Med Chem 2014;21: 3646-3661.