Hydrogen Sulfide Improves Wound Healing via Restoration of Endothelial Progenitor Cell Functions and Activation of Angiopoietin-1 in Type 2 Diabetes

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Impaired angiogenesis and its induced refractory wound lesions are common complications of diabetes. It has been reported that hydrogen sulfide (H$_2$S) has pro-angiogenic effects. We hypothesize that H$_2$S improves diabetic wound healing via restoring endothelial progenitor cell (EPC) function in type 2 diabetes. The Db/db mice were treated with sodium hydrosulfide (NaHS), 4-hydro-xythiobenzamide group (HTB), or saline for 18 days, respectively. Db/+ mice were treated with either DL-propargylglycine (PAG) or saline for 18 days. Plasma H$_2$S levels were significantly decreased in db/db mice and restored in the NaHS and HTB mice compared to the diabetic control group. Wound closure rates in the NaHS and HTB groups were significantly faster than those of the db/db group, in which the PAG group had slower wound closure rates. Wound skin capillary densities were enhanced in the NaHS and HTB groups. The EPC functions were significantly preserved in the NaHS and HTB groups, but decreased in the PAG group. Meanwhile, EPC functions of the db/+ mice were significantly reduced after in vitro PAG treatment or cystathionine-$\gamma$-lyase (CSE) silencing; EPC functions db/db mice were significantly improved after in vitro NaHS treatment. The expressions of Ang-1 in wound skin tissue and in EPCs were up-regulated in the NaHS and HTB groups compared to db/db controls, but down-regulated by in vivo PAG and in vitro si-CSE treatment compared to normal controls. Diabetic EPC tube formation capacity was significantly inhibited by Ang-1 siRNA before NaHS treatment compared to db/db EPCs treated with NaHS only. Taken together, these results show that H$_2$S improves wound healing via restoration of EPC functions and activation of Ang-1 in type 2 diabetic mice.

**Key words:** hydrogen sulfide, wound healing, endothelial progenitor cells, angiopoietin-1, type 2 diabetes mellitus
Diabetes mellitus and its chronic complications such as foot problems are prevalent in the worldwide. In fact, diabetic foot ulcer is the leading cause of non-traumatic amputation (1). It is well known that angiogenesis is crucial for wound healing (2, 3). Endothelial progenitor cells (EPCs) play an important role in angiogenesis and neovascularization (4). However, much evidence shows that some aspects of EPC angiogenic ability as measured by tube formation, adhesion, and migration, decrease in diabetes (5, 6). EPC-related angiogenic deficiency induced by diabetes causes chronic vascular complications, refractory wounds, and foot ulcers (3, 7, 8). Therefore, amendment of EPC function is a critical way to improve wound healing.

Mammalian tissues synthesize hydrogen sulfide (H$_2$S) via two pyridoxal-5’-phosphate-dependent enzymes catalyzing the metabolism of L-cysteine: cystathionine beta-synthase (CBS) and cystathionine-γ-lyase (CSE), as well as by a recently identified third pathway via the combined action of 3-mercaptop- pyruvate sulfurtransferase and cysteine aminotransferase(9, 10). H$_2$S has been considered a poisonous gas for a long time. However, evidence accumulating over the last decade shows that H$_2$S could act as a novel gasotransmitter and take part in many physiological and pathological processes such as angiogenesis (11), vasodilation (12-14), and inhibition of apoptosis in vascular endothelial cells (15) and cardiomyocytes (16). In the cardiovascular system, the principal enzyme involved in the formation of H$_2$S is CSE, which converts cystathionine to L-cysteine, yielding pyruvate, ammonia, and H$_2$S (17). CSE is expressed in vascular endothelial cells, smooth muscle cells, and myocardial cells(18), but to date, whether CSE is expressed in the endothelial progenitor cells (EPCs) is unclear.

Regarding the relationship between H$_2$S and diabetes, current evidence shows that the synthesis and circulating level of H$_2$S decreases in non-obese diabetic (NOD) mice (19),
streptozotocin (STZ) induced type 1 diabetic mice (20), and type 2 diabetic patients (20). H$_2$S has been reported to accelerate gastric ulcer healing (21) and burn wound skin healing (22, 23). Papapetropoulos et al. found that topical administration of H$_2$S improved the recovery from burn wounds in wild type rats, whereas genetic ablation of CSE delayed it in mice (22). However, little is known about H$_2$S’s role in chronic skin wound healing in diabetes, especially in type 2 diabetes mellitus. It has also not been elucidated whether H$_2$S stimulates angiogenesis by influencing the functions of EPCs.

Angiopoietin (Ang) plays a major role in endothelial survival and vascular maturation. As a paracrine agonist, Ang-1 can induce its receptor Tie 2’s phosphorylation and promote endothelial survival and vessel stabilization (24). Ang-1/Tie 2 signal has been reported to contribute to diabetes-induced angiogenesis impairment (25, 26), and reduced Ang-1 expression in high-glucose concentration down-regulated the PI3K/Akt signaling pathway, and phosphorylated eNOS, which results in the impairment of EPC vessel-forming capacity (25, 27). Vascular endothelial growth factor (VEGF) has been reported as a proverbial factor to stimulate angiogenesis and mediate the improving course of wound healing (28, 29).

The present study aimed to test our hypothesis that H$_2$S might improve angiogenesis and restore EPC function in an in vivo wound healing db/db diabetic mouse model. We also investigated the expression of Ang-1 in wound skin tissue and EPCs since it is a possible important factor that mediates the angiogenic effects of H$_2$S donor. Showing that controlling H$_2$S can improve wound healing effectively and become a potential strategy for chronic lower extremity ulcerations in diabetic populations would be of great clinical significance.
RESEARCH DESIGN AND METHODS

Experimental animals and drugs. All animal procedures were approved by and conducted in accordance with the Central South University Institutional Animal Care and Use Committee established guidelines. The B6.Cg- m+/+Leprdb/J (leptin receptor-deficient diabetes, db/db, eight-12 weeks, purchased from Jackson Laboratories) mouse is a well-established type 2 diabetic animal model with continuous hyperinsulinemia and high plasma glucose levels (30). Their matched non-diabetic control littermates (db/+) were used as controls.

Hydrogen sulfide (H$_2$S) was administered in the form of sodium hydrosulfide (NaHS) (Sigma-Aldrich) and 4-hydroxylthio- benzamide (4-HTB) (Alfa Aesar), which is well-established as a reliable H$_2$S donor (22, 31). The former is a rapid releasing H$_2$S donor widely used in recent in vivo/in vitro H$_2$S studies (22, 23, 32), and the latter HTB is a slow-releasing H$_2$S donor which is effective in improving gastric ulcer healing in rats (21). DL-propargyl-glycine (PAG) (Sigma-Aldrich), an irreversible competitive CSE inhibitor, was used to confirm the role of H$_2$S in db/+ mice subjected to skin wounding (33, 34). Blood glucose from tail veins was measured with a rapid glucose meter (Lifescan One-touch Ultra 2) and results were expressed as mg/dl. Diabetes mellitus was diagnosed when blood glucose was higher than 300mg/dl.

Full-thickness excisional dorsal skin wounds. Full-thickness punch biopsy wounds were created on male db/db mice and their age- and gender- matched db/+ mice as we previously described (35, 36). Described briefly, a 6mm-diameter full-thickness skin wounds were made on the dorsal skin with a biopsy punch (Acuderm Inc, Florida). Wounds were dressed with
Tegaderm (Nexcare, 3M) and changed every other day, and wound closure rates were measured by tracing the wound area every other day onto an acetate paper. Gross wound closure was quantified by Image-pro Plus 5.1 (Media Cybernetics) and wound healing was expressed as the percentage of the original wound area that had healed, calculated as (1-[wound area day X/wound area day 0]) × 100.

**Animal grouping and treatment.** Mice were randomly assigned to five groups: db/db + NaHS (50 μmol·kg⁻¹·day⁻¹, n=9), db/db + HTB (30 μmol·kg⁻¹·day⁻¹, n=9), db/db control (saline, n=9), db/+ control (saline, n=10), and db/+ + DL-PAG (50mg·kg⁻¹·day⁻¹, n=5); these drugs were injected intraperitoneally (i.p.) once a day from the day of wounding until 16 days later(22, 23, 37). At the end of 16 days, or at day eight after wound creation, mice were sacrificed under isoflurane anesthesia after the blood drawing. Blood samples were drawn from the right ventricles by using syringes containing ethylenediaminetetraacetic acid (ETDA) and then were centrifuged (10000g, 4°C) for 10 minutes. Thereafter, plasma was aspirated and stored at −80°C for H₂S concentration measurement. The skin wound tissues were carefully dissected, and granulation tissues within the wound as well as a 10 mm margin of wound were collected.

**EPC therapy for diabetic wounds.** To determine whether H2S improves EPC-mediated wound healing in diabetes, 1×10⁶ diabetic EPCs were transplanted on db/db mouse wounds immediately following skin punch biopsies. Described briefly, one group of db/db mice (n=8) received i.p. injection of NaHS (50 μmol·kg⁻¹·day⁻¹) every other day until 16 days later; One group of db/db mice (n=8) mice received i.p. injection of 0.9% saline every other day until 16 days later; these above two groups of mice received topical transplants of 1×10⁶ diabetic EPCs
on wounds as we described previously (38). Another group of db/db (n=8) mice used as a control only underwent skin punch biopsies.

**Measurement of H$_2$S concentration in plasma and CSE activity in skin tissue.** H$_2$S in the plasma was determined using previously described methods (39-41). Plasma (120 µl) was mixed with 100µl water and 120 µl trichloroacetic acid (TCA, 10% v/v), and reacted for 10 minutes at room temperature, then centrifuged at 4°C, 14,000g for 10 minutes. The clear supernatant was transferred to an eppendorf tube, then Zn acetate (1%) 60 µl, N, N-dimethyl-p-phenylenediamine sulfate 40 µl (20 mM in 7.2 M HCl) and FeCl$_3$ 40 µl (30 mM in 1.2 M HCl) were added in proper sequence and reacted for 20 minutes at room temperature.

For CSE activity assay, the skin tissue was homogenized in an ice-cold 100 mM potassium phosphate buffer (pH 7.4) with an electric homogenizer (PowerGen 125, Fisher Scientific). Then the homogenate was centrifuged at 4°C, 10,000 g for 10 minutes, and the supernatant (430 µl in phosphate buffered saline [PBS]) was mixed with 20 µl L-cysteine (20mM), 20 µl pyridoxal-5’-phosphate (2mM), and 30µl 0.9% sodium chloride, then reacted for 30 minutes in a 37°C water bath. Then, 250 µl Zn acetate (1%) and 250µL TCA (10%) were added to terminate the reaction. After that, N, N-dimethyl-p-phenylenediamine sulfate 133 µl (20 mM in 7.2 M HCl) and FeCl$_3$ 133 µL (30 mM in 1.2 M HCl) were added. After 20 minutes, the absorbance of the standards and samples at 670 nm was measured on a spectrophotometer (Spectra Max 190, Molecular Devices). H$_2$S concentration was calculated against a calibration curve of NaHS (3.125– 200 µM) (41). All samples were assayed in duplicate. Results were expressed as plasma H$_2$S concentration in micromole/liter. The optimal density value of skin samples obtained at 670 nm was adjusted by protein concentration and extrapolated from the
standard curve obtained from the same plate. Results of CSE activity were expressed as H₂S concentration in micromole/µg protein.

**Isolation and culture of bone marrow-derived EPCs.** EPCs were isolated from the bone marrow of db/+ and db/db mice and cultured afterwards according to our established methods with minor modifications (35, 42). Described briefly, bone marrow mononuclear cells were isolated from the femurs and tibias of mice by density gradient centrifugation. After two washing steps, mononuclear cells were plated onto vitronectin-(Sigma-Aldrich) coated six-well plates and maintained in endothelial basal medium-2 (Lonza) supplemented with Endothelial Growth Medium 2 (EGM-2, Lonza) with 5% fetal bovine serum. Cells were cultured at 37°C with 5% CO₂ in a humidified atmosphere. EPCs after seven days of culture were used for further analysis. To characterize bone marrow isolated EPCs, stem cell markers (CD34, Sca-1), endothelial cell markers (Flk-1, CD144), and monocyte marker (CD11b) were examined on cultured EPCs by flow cytometry based on our established methods (38, 43).

**Cytotoxicity assay** The CellTiter 96® AQueous One Solution Cell Proliferation assay kit (Promega) was used to determine the cytotoxicity of NaSH and PAG in the *in vitro* studies. Described briefly, 2 × 10⁴ EPCs in 100 µl media were dispensed into each well of 96-well culture plates and cultured overnight. Plates were subsequently incubated for 30 minutes with NaHS (100 µM) or PAG (100 nM). A total of 20 µl of The CellTiter 96® AQueous One Solution Reagent containing tetrazolium compound (3-[4,5-dimethylthiazol-2-yl]-5-[3-carboxymethoxyphenyl]-2-[4-sulfophenyl]-2H-tetrazolium, MTS) and electron coupling reagent (phenazine ethosulfate; PES) was added to each well. Two hours later, the absorbance was recorded at 490nm using a 96-well plate reader.
H$_2$S treatment by NaHS in diabetic EPCs. EPCs isolated from db/db mice were reseeded into six-well plates at 5× 10$^5$ cells/well, and incubated at 37°C for 24 hours, and serum starved for eight hours. Then they were treated with NaHS at concentrations of 10, 50, and 100 µM in EGM-2 medium for 30 minutes in a 37°C incubator before function evaluation.

CSE knockdown by siRNA in normal EPCs. After seven days in culture, EPCs from db/+ mice were reseeded into six-well plates at 2× 10$^5$ cells/well and incubated at 37°C until the cells were 60%-80% confluent. After that, EPCs were serum starved for six hours before siRNA transfection. Then the EPC-conditioned media on CSE-siRNA (sc-142618, Santa Cruz) was diluted to a 10 µM working solution and delivered to cells at a 5 nM final concentration through a siRNA transfection reagent (sc-29528, Santa Cruz). After five to seven hours of transfection, the transfection mixture was removed and replaced with fresh EGM-2 and incubated for an additional 48 hours. A non-related scramble siRNA (Control A, sc-37007, Santa Cruz) was used as a transfection control.

CSE inhibition by DL-PAG in normal EPCs. EPCs isolated from db/+ mice and cultured for seven days were reseeded into six-well plates at 5× 10$^5$ cells/well and incubated at 37°C for 24 hours, then serum starved for six to eight hours and treated with DL-PAG at concentrations of 3-10-30 mM in medium for 30 minutes; the medium containing PAG was then removed and washed twice with PBS for function assay.

Ang-1 knockdown by siRNA in H$_2$S treated diabetic EPCs. EPCs isolated from db/db mice were reseeded into six-well plates at 5× 10$^5$ cells/well, and incubated at 37°C for 24 hours, and serum starved for eight hours. Then the EPC-conditioned media on Ang-1 siRNA (Dharmacon)
was diluted and delivered to cells at a 5 nM concentration through a siRNA transfection reagent (Dharmacon). After six to seven hours, the transfection mixture was removed and replaced with fresh EGM-2 and incubated for an additional 48 hours before 30 minutes NaHS treatment at concentration of 50 µM. Of the non-related scramble siRNA treated db/db EPCs, only NaHS 50µM treated db/db EPCs and the untreated db/+ EPCs were used as controls. After the above treatment, EPC tube formation was assessed.

**EPC tube formation and adhesion assays.** For tube formation assay, 48-well plates were coated with growth factor-reduced Matrigel (150 µl/well, Becton Dickinson). EPCs (5×10^4), prepared as described above were plated in 200 µl EGM2 medium and incubated at 37°C with 5% CO_2 for 12 hours overnight to form the tube. Images of tube morphology in a random five fields per well were taken by inverted microscope (Nikon Eclipse TE2000-U) with 4× magnifications(23); tube lengths were drawn with Adobe Photoshop CS4 software and analyzed with Image-Pro plus 5.1 software. For adhesion assay, EPCs were plated at 2.5 × 10^4 /well in triplicate to a vitronectin pre-coated 96-well plate. After two hours of incubation at 37°C with 5% CO_2 in EGM2, non-adherent cells were removed by washing twice with PBS. Then cells were fixed with 2% paraformaldehyde for 20 minutes and the nuclei were stained with Hoechst (5µg/ml, Sigma-Aldrich) for 20 minutes. The adherent cells were counted in five random fields in each well with an inverted fluorescence microscope (Nikon) with 10× magnifications, and the mean value of three wells was determined for each sample.

**Immunohistochemistry.** Capillary density was quantified by histological analysis as previously described (38). At day 8 after wounding, round shape 6mm skin samples including the healed or unhealed wound area were fixed with 2% paraformaldehyde (PFA, Sigma), then
embedded in a cross-section position in paraffin and sectioned at 4-µm intervals. Slides were
de-paraffinized and hydrated, blocked with normal rabbit serum (Vector Laboratories) for 30
minutes, incubated for 60 minutes at room temperature with an anti-CD31 antibody (1:50; Santa Cruz), and further incubated with Vectastain Elite ABC Reagent (Vector Laboratories)
for 30 minutes and Nova Red (Vector Laboratories) for 15 minutes. Slides were counterstained
with Gill (Lerner) 2 Hematoxylin (VWR Scientific) for 10 seconds, differentiated in 1% glacial
acetic acid, and rinsed in running tap water. Pictures were taken under a Nikon Eclipse
TE2000-U microscope using Metamorph software. Five random microscopic fields (20× magnification) were counted to determine the number of capillaries per wound, which was
expressed as capillary/high power field (capillary/hpf).

**Immunofluorescence and confocal microscopy.** EPCs were isolated as described above and
cultured in EGM-2. BrdU staining was performed on EPCs as we previously described (38).
Cultured EPCs were labeled with 5-bromo-2’-deoxyuridine and 5-fluro-2’ddeoxygen (BrdU
labeling reagent, Invitrogen). 1× 10⁶ EPCs were then transplanted to db/db wounds (6 mm
punch biopsy) as described above. On day 8 of wound healing, the mouse was euthanized
and the wound and surrounding skin was recovered and fixed in 2% paraformaldehyde for 18
hours. Samples were then switched to 70% EtOH. The samples were embedded in paraffin
wax and 4 mm sections were used for analysis. For staining with rabbit CD31 (1:50, Santa
Cruz) and chicken BrdU (1:500, Abcam), primary antibodies were incubated overnight at 4°C.
Then, slides were incubated with Alexis-488 (1:500, Invitrogen Molecular Probes) and Cy3
(1:1,000, Jackson ImmunoResearch Lab), followed by 30 seconds of incubation with Hoeschst
nuclear stain. Positively-stained cells in five random fields were imaged at 10× magnification
on a Nikon fluorescence microscope (Olympus, Melville). Imaging conditions were maintained at identical settings with original gating performed within each antibody-labeling experiment with the negative control (no primary antibody).

**Studies on type 2 diabetic patients.** Eight non-diabetic and seven diabetic human subjects underwent full-skin excisional biopsy in their lower extremities. The non-diabetic subjects were recruited from patients admitted to the Division of Vascular Surgery of Shanghai Clinical Center because of traumatic wounds. None of the non-diabetic subjects had cardiovascular, renal, or hepatic disease. The diabetic subjects were recruited from Shanghai Clinical Center of Diabetes; subjects who had foot or leg ulceration and underwent the debridement operation in our Vascular Surgery Division were included in the study. The clinical characteristics of participating subjects are outlined in **Table 1**. All lower limb skin biopsies were performed during the limb operation procedure by surgeons and the skin beside wound (margin within 1cm) was taken. Protocols were approved by the Institutional Review Board of Shanghai Jiaotong University and Shanghai Clinical Center of Diabetes. Written informed consent was obtained from every subject. The skin tissues were used to examine the expressions of Ang-1, CSE, and VEGF by Western Blot.

**Western blot analysis.** Total protein was extracted from cultured endothelial progenitor cells (EPCs) after *in vitro* culture and treatment as described above, or from harvested wound skin tissue including a 6mm region of wound-located skin and 10mm skin tissue around the wound bed by homogenization of wounds skins in tissue lysis buffer (Sigma-Aldrich). Protein concentration was determined by BCA protein assay kit (Pierce). Equal amounts of proteins (30-50µg) were boiled and separated by 6%-12% sodium dodecyl sulfate-polyacryl- amide gel
electrophoresis (SDS-PAGE) and transferred onto nitrocellulose (NC) membrane (Amersham Hybond ECL, GE healthcare). The CSE (mouse monoclonal anti-CSE, Santa Cruz, USA), CBS (mouse monoclonal anti-CBS, Santa Cruz), angiopoietin-1 (rabbit polyclonal anti-angiopoietin-1, Abcam) were determined with its specific first antibodies. The dilutions of primary antibody were 1:500 for angiopoietin-1, 1:10000 for β-actin (Santa Cruz), and 1:250 for all other antibodies. Luciferin conjugated secondary antibody was used at 1:5000. The bands were scanned and analyzed by Odyssey system.

**Statistical analysis.** Results are expressed as mean ± S.E.M. Differences between the two groups were compared with paired or unpaired student’s t test. One-way ANOVA with Tukey multiple comparison tests were used to compare each parameter when there were two or more independent groups. Two-way ANOVA of grouped analyses was used to compare the wound recovery rate at different time points, and then Bonferroni post hoc t tests were performed to compare replicate means by row when ANOVA was statistically significant. Values of $P<0.05$ were considered statistically significant.

**RESULTS**

The plasma concentration of H$_2$S and CSE activity in skin tissue was decreased in diabetic mice, which was corrected by H$_2$S donor therapy. At eight-ten weeks of age, the body weights of the db/db mice were significantly greater than that of the db/+ mice (40.06±3.37g vs. 25.99±1.22g, $p<0.01$), and the blood glucose levels of the db/db mice was significantly higher than that of the db/+ mice (475.89±42.27mg/dL vs. 139.9±33.49 mg/dl, $p<0.01$). The baseline plasma H2S concentration of the db/+ mice was significantly higher than
that of the db/db mice (Fig. 1A). The plasma H$_2$S concentrations of the db/db mice were further decreased than that of db/+ mice after wound creation, and were reversed by NaHS and HTB at day 8 (Fig. 1B) and day 18 (Fig. 1C). In contrast, PAG down regulated the H$_2$S plasma concentrations in the db/+ mice (Fig. 1B, and 1C). Though the baseline level of H$_2$S synthase CSE activity in the skin tissue of db/db mice was higher than that of the db/+ mice (Fig. 1D), the CSE activity of the db/db mice was significantly lower compared with that of the db/+ mice after wound creation at day 8 (Fig. 1E).

**H$_2$S therapy accelerated wound healing in diabetic mice.** The baselines of wound healing in both db/db mice and db/+ mice were determined. The data demonstrated that the wound areas of the db/db mice closed significantly slower than those of the db/+ mice from day 2 to day 16 (Fig. 2A and 2B). The administration of H$_2$S donor, either rapid-releasing NaHS or slow-releasing HTB, improved wound recovery rates compared with saline-treated db/db controls (Fig. 2B and 2C) starting from Day 2 to Day 16. The overall wound-healing rate in the two H$_2$S donor groups was still slower than that of the db/+ group (Fig. 2B and 2C). In contrast, the administration of H$_2$S synthase CSE inhibitor, DL-PAG, significantly delayed the wound closure rate compared to the db/+ group (Fig. 2B and 2C). These data suggest that H$_2$S treatment improved wound healing in type 2 diabetic mice, while the inhibition of H$_2$S synthesis *in vivo* delayed the wound-healing course.

**Slow-releasing H$_2$S donor stimulates angiogenesis of wound skin tissue** At day 8 after treatments, capillary numbers in the diabetic group were significantly lower than those in the normal control group (Fig. 3A and 3B). The slow-releasing H$_2$S donor HTB significantly increased the newly formed capillary densities compared to that of diabetic controls (Fig. 3A...
and 3B), whereas the vessel densities in skin tissue treated by NaHS augmented the newly formed capillary densities to some extent, but without reaching statistical significance. On the contrary, wound skin taken from mice treated with PAG had lower new vessel densities compared to wound skin taken from normal db/+ controls (Fig. 3A and 3B). There was no significant difference in the baselines of capillary number of unwounded skin between db/+ mice and db/db mice (Fig. 3C).

**H₂S donor accelerated the rate of EPC-mediated wound healing in diabetic mice.** Wound healing improved significantly in the diabetic mice that received transplantation of EPCs. Furthermore, NaSH significantly augmented the efficiency of EPC cell therapy in diabetic mice (Fig. 4A and 4B, P<0.05 vs. db/db mice). These data suggest that H₂S significantly accelerated EPC-mediated wound healing in diabetic mice. On day 8 of wound beds, some BrdU-labeled EPCs integrated into vascular-like structures (CD31 positive) and the other BrdU-labeled EPCs integrated in the dermis; On day 16 of wound beds, more BrdU-labeled cells integrated into the epithelial layers (Fig. 4C).

**H₂S donor improved the tube formation and adhesive function of diabetic EPCs.** To investigate the functions of EPCs, EPC tube formation on Matrigel and EPC adhesion in vitronectin pre-coated plates were observed in EPCs isolated from mice after 16 days of treatment of H₂S or PAG. The cell population profiles of cultured EPCs (Day 7) were as follows: Sca-1 (60.8±7.52%), CD34 (47.6±4.37%), Flk-1 (22.6±3.17%), CD144 (8.7±1.19%), and CD11b (37.5±2.54%), indicating that seven-day cultured EPCs are heterogeneous populations containing both higher percentages of stem cells and endothelial cells. The db/db EPCs formed significantly fewer tube networks than db/+ EPCs (Fig. 5A). *In vivo* H₂S therapy
of NaHS and HTB significantly improved the ability of diabetic EPCs to form tube networks compared with db/db control EPCs (Fig. 5A). On the contrary, PAG treatment significantly reduced the tube length compared with normal controls (Fig. 5A). Similar to tube formation, the adhesive numbers of EPCs in the db/db group was markedly less than that in the normal group (Fig. 4B), and administration of NaHS and HTB significantly increased the adhesion function of EPCs than that of untreated db/db EPCs (Fig. 5B). Contrarily, EPC adhesion in the PAG group was significantly attenuated compared with that of the db/+ control group (Fig. 4B).

To observe the influence of H$_2$S on the functions of diabetic EPCs in vitro, EPCs isolated from the bone marrow of db/db mice were treated with NaHS at concentrations of 10-50-100 µM in EGM-2 for 30 minutes before tube formation and adhesion determination. NaHS significantly augmented the tube networks of diabetic EPCs at all three concentrations compared with non-modified db/db EPCs (Fig. 5C). The deficient adhesion of diabetic EPCs was also reversed by NaHS with concentrations of above 10 µM in vitro (Fig. 5D). These aforementioned data suggest that both in vivo and in vitro H$_2$S donor treatment restore deficient EPC tube formation and adhesive functions in db/db mice.

**H$_2$S inhibition with DL-PAG and CSE siRNA reduced the function of normal EPCs in vitro.** In order to verify whether H$_2$S inhibition could impair EPC function, H$_2$S synthesis of EPCs was inhibited with different concentrations of DL-PAG (3, 10, 30mM), which was added to EGM-2 medium for 30 minutes. The results indicated that the tube length of EPCs treated with PAG 10 mM and 30 mM decreased significantly compared with that of normal controls (Fig. 5E). Similarly, the adhesive EPC numbers were reduced markedly in the PAG 10mM, and 30mM intervention groups compared with control EPCs (Fig. 5F). These results suggest that
the inhibition of H\textsubscript{2}S synthesis in EPCs reduces their functions. The tube formation results from the CSE silencing experiment in EPCs further demonstrated the critical effect of H\textsubscript{2}S on EPC angiogenesis. The CSE expression of EPCs after si-CSE treatment was reduced nearly by 70\%, as shown by Western blotting (Fig. 5G), while the expression of CBS wasn’t influenced by the silencing of CSE in EPCs (Fig. 5H). The average tube length of EPCs after CSE knockdown decreased to approximately 40\% compared to that of controls (Fig. 5I), and EPC adhesion also was markedly attenuated compared to controls (Fig. 5J). These data identified that H\textsubscript{2}S is critical for EPC functions, including tube formation and adhesion abilities. In addition, cytotoxicity assay showed that treatment with NaHS or PAG did not affect the viability of EPCs (Fig. 5K).

**H\textsubscript{2}S donor up-regulated the expressions of Ang-1 in wound skin tissues and EPCs of diabetic mice, while CSE suppression down-regulated the Ang-1 expression in normal EPCs.** To understand the possible mechanism through which H\textsubscript{2}S improves EPC function, the expressions of Ang-1 and VEGF in wound skin tissue and EPCs were measured by Western blotting. The results indicated that the *in vivo* H\textsubscript{2}S donor treatment significantly increased the expressions of Ang-1 and VEGF not only in EPCs from these mice but also in wound skin tissues (Fig. 6A-6C). On the contrary, CSE inhibitor PAG inhibited the expression of Ang-1 in the wound skin tissue (Fig. 6A) and in EPCs (Fig. 6B). Similarly, the silencing of CSE in cultured normal EPCs with CSE siRNA *in vitro* treatment significantly decreased their angiopoietin-1 expression (Fig. 6D).

**H\textsubscript{2}S promoted the angiogenesis of EPCs via restoration of Ang-1 expression.** To identify whether H2S stimulates the angiogenic function of EPCs through Ang-1 signaling, EPCs were
pretreated with Ang-1 si-RNA before *in vitro* NaHS treatment. EPCs were isolated from the bone marrow of db/db and normal mice, and cultured in EGM-2 for seven days; then diabetic EPCs \((5 \times 10^5)\) were then reseeded and treated *in vitro* with NaHS 50 μM only in EGM-2 for 30 minutes, or Ang-1 siRNA at 5 nM in medium for six-seven hours before NaHS treatment. The results revealed that the improved tube length with NaHS was significantly inhibited by prior Ang-1 siRNA treatment and was significantly lower than that of normal EPCs (Fig. 7A and 7B, all \(p<0.05\)). These results suggest that, H₂S exert its role of promoting the EPC angiogenesis, at least partially, via restoration of Ang-1.

**The expressions of Ang-1, CSE, and VEGF were decreased in the human skin tissue of diabetic foot ulcers.** To investigate whether the dysregulations of Ang-1, CSE, and VEGF also applied to the skin tissue from foot ulcers of diabetic subjects, we compared their expression between non-diabetic and type 2 diabetic subjects. The data showed that the expressions levels of Ang-1, CSE, and VEGF were 36%, 41%, and 46% lower, respectively, in the diabetic subjects compared to the non-diabetic subjects (Fig. 8).

**DISCUSSION**

H₂S is the “third” gasotransmitter next to nitric oxide (NO) and carbon monoxide (CO). Its biological effects have not yet been completely understood. The present study demonstrates that 1) plasma H₂S levels and CSE activities in skin wounds decreased in type 2 diabetic mice; 2) Both *in vivo* and *in vitro* H₂S treatment amended the tube formation and
adhesive functions of diabetic EPCs; 3) H$_2$S donor therapy improved EPC-mediated wound healing in diabetic mice via elevating angiogenesis; 4) H$_2$S treatment increased the expression of Ang-1 and VEGF in EPCs from diabetic mice and Ang-1 siRNA inhibited the ameliorative effect of NaHS on EPC angiogenic functions; 5) The expressions of Ang-1, CSE, and VEGF were decreased in diabetic ulcers in diabetic patients.

The baselines of plasma H$_2$S in diabetic mice were significantly lower than those of the non-diabetic mice, which is consistent with the plasma H$_2$S measurements in NOD mice and STZ-induced type 1 diabetic mice. Day 8 or day 16 after skin wounds, the plasma H$_2$S levels were further decreased in the diabetic mice, whereas the plasma H$_2$S levels in the non-diabetic mice were maintained at baseline level. Interestingly, the baseline activity of CSE in skin tissues was ~1.3 fold higher in the diabetic mice than in the non-diabetic mice, which might be a compensative response to decreased plasma H$_2$S levels. However, on day 8 after wounds, the CSE activity in skin wounds of the diabetic mice was significantly attenuated and failed to produce enough H$_2$S for wound healing, whereas the CSE activity in the non-diabetic skin wounds was higher than the baseline, which might be a mechanism for the unchanged H$_2$S levels. Since the expression of another H$_2$S synthase, CBS, was not changed in skin tissue and the EPCs of diabetic mice, it is more likely that the delayed wound healing in diabetic mice is due to the deficiency of H$_2$S synthesis in the vascular system, just as Brancaleone et al. reported in NOD mice.

H$_2$S had been found to improve the burn skin wound and gastric ulcers induced by acetate acid in wild type mice or rats. It was reported that H$_2$S improves endothelial wound healing in an in vitro model. Wallace J.L et al. revealed an enhancing effect of H$_2$S in
acetic acid-induced gastric ulcers in a Wistar rat model (21) and in a skin burn wound model in CSE-knockout mice (22). This evidence suggests that H₂S contributes to wound healing. However, no evidence of its efficacy on diabetic wounds has been reported so far. In the present study, two H₂S donors, NaHS and 4-HTB, were administrated in vivo; one is the widely used rapid-releasing donor, the latter one is the slow-releasing H₂S donor. The usual dosage range is 10-100 µmol/Kg, as reported in recent in vivo studies (22, 23, 37). Based on this evidence and considering that the physiological H₂S levels in the plasma of mammals were 10-100 µmol/L (44), we chose 50 µmol/Kg as the treatment dosage of NaHS in the present study. As for 4-HTB, only one experience reported by Wallace et al. could be referenced; they used HTB 10-30 µmol/Kg twice a day via intragastric administration in male Wistar rats (21), so we administered the dose of 30 µmol/Kg to diabetic mice and proved its efficacy on diabetic wound healing. The results of this study demonstrate that i.p. injection of H₂S donors, both NaHS and 4-HTB, improved the recovery rate of wound healing in type 2 diabetic mice. The results show they had similar effects on wound healing in diabetic mice. Since NaHS and 4-HTB are easily available H₂S donors, they may be potential remedies to ameliorate foot ulcerations in diabetic patients.

Insufficient angiogenesis is one of most important reasons for delayed diabetic wound healing. EPCs’ prominent functions in angiogenesis have been widely demonstrated (38, 45, 46). In the present study, the tube formation and adhesive function of diabetic EPCs was impaired compared to normal mice with a matched genetic background, suggesting that their angiogenic effect was attenuated. The pro-angiogenic effect of H₂S has been recently recognized. Cai WJ et al. reported a novel Akt-mediated proangiogenic role of H₂S in C57 BL/6 mice (23), Topical
administration of H$_2$S enhanced wound healing in CSE knockout mice, and the researchers concluded that endogenous H$_2$S stimulates endothelial cell-related angiogenic properties through a KATP channel/MAPK pathway (22). H$_2$S also enhances tube formation and migration in cultured vascular endothelial cells (37). The present study demonstrated that H$_2$S donors facilitated diabetic wound healing via stimulating capillary formation at the site of injury. Furthermore, we demonstrated that H$_2$S donor accelerated EPC-mediated wound healing in diabetic mice. To date, it is not elucidated whether H$_2$S ameliorates diabetic wound healing via influencing EPC functions. In the present study, we first investigated the angiogenic effects of H$_2$S donor on EPCs isolated from diabetic mice in vitro. Our results revealed that the tube formation and adhesion of EPCs increased significantly by chronic treatments with both rapid-releasing and slow-releasing H$_2$S donor in diabetic mice. Furthermore, the angiogenic function of diabetic EPCs also was restored by NaHS in vitro. These results support that H$_2$S improves the angiogenic function of diabetic EPCs. On the contrary, after the normal mice and their EPCs were treated with H$_2$S synthetase (CSE) inhibitor DL-PAG in vivo and in vitro, or si-CSE in vitro, the tube network formation and adhesive ability of normal EPCs declined markedly at the same time the wound closure rate was delayed. We have demonstrated that topical EPCs transplantation significantly improves wound healing in the diabetic mice (38, 47). In the present study, we found that NaSH significantly augmented the efficiency of EPC cell therapy in diabetic mice. Consistent with our previous studies, some EPCs integrated into vascular-like structures and the other EPCs integrated in the dermis at the early stage of wound beds (on day 8) indicating that H$_2$S accelerate wound healing via EPC-mediated angiogenesis. Interestingly, we also found that
more cells integrated into the epithelial layers at the later stage of wound beds (on day 16), which may be investigated in the future studies. Both in vitro and in vivo evidence suggest that H$_2$S is vital for preserving normal EPC functions and wound repair in type 2 diabetic mice.

Angiogenic signals are mainly activated by endothelium-specific receptor tyrosine kinase (RTKs). VEGF, fibroblast growth factor (FGF), and Ang-1 are ligands of RTKs. Ang-1 promotes endothelial differentiation from embryonic stem cells and induced pluripotent stem cells (48). Ang-1 gene expression was down-regulated in EPCs cultured in a high glucose environment, and recombinant Ang-1 could increase the vessel-forming capacity of EPCs in vitro (49). Ang-1 gene expression was down-regulated in EPCs cultured in a high glucose environment, and recombinant Ang-1 could increase the vessel-forming capacity of EPCs in vitro (49). The present study showed that in vivo treatment with H$_2$S donors significantly increased the expressions of Ang-1 and VEGF in EPCs from diabetic mice. Most importantly, we also found attenuated expression of Ang-1 and VEGF in diabetic wounds in patients. Although the current study cannot exclude the effects of gender, medications, and comorbidities on the expressions of Ang-1, CSE, and VEGF, our results provided evidence that angiogenic factors and H$_2$S were significantly attenuated in local diabetic wounds, which may contribute to refractory wounds in diabetic patients. Ang-1 was reported to potentiate VEGF-induced angiogenesis (50). Ang-1 signaling was known to regulate both vascular quiescence and angiogenesis through its cognate receptor Tie-2 (51). However, we did not observe altered expression of the Ang-1 receptor Tie-2 between diabetic EPC or diabetic wounds. In parallel, the tube formation and adhesion ability of H$_2$S amended EPCs increased
significantly, which was blocked by pretreatment by Ang-1 si-RNA before H₂S. These results suggest that the restoration of EPC angiogenic functions by H₂S may occur, at least partly, through the mediation of Ang-1. H₂S activates phosphatidylinositol-3-kinase (PI3K) and mitogen activated protein kinases (MAPK) to regulate angiogenic factors in endothelial cells (22, 52, 53). However, the mechanisms by which H₂S regulates Ang-1 in EPCs are still unknown. Further research should be carried out to clarify how and to what degree H₂S improves diabetic wound healing and EPC function via Ang-1.

In summary, the present study, for the first time, demonstrates that H₂S is vital in maintaining normal EPC angiogenic function, and H₂S donor could accelerate the wound healing in type 2 diabetic mice. These effects of H₂S are related to restoring the angiogenic functions of EPCs via up-regulating Ang-1 expression. The findings that H₂S donor can amend EPC function and improve skin wound recovery may lead to novel therapeutic strategies for diabetic vascular complications and diabetic skin ulcers.

**AUTHOR CONTRIBUTIONS:** Drs. F.L and D.D C designed the experiments, conducted the in vitro cell functional tests, *in vivo* cell therapy, and wrote the manuscript. Drs. X.S and H.H. X performed the Western blot on patient skin samples. Dr. H.Y. conducted the in vitro cell biochemical tests and contributed to the discussion. Drs. A.F.C. and W.P.J designed the study, contributed to the discussion, and reviewed and edited the manuscript. Drs. F.L, D.D.C, and A.F.C are the guarantors of this work and, as such, had full access to all the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis.
ACKNOWLEDGEMENTS

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CONFLICTS OF INTEREST: None
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Figure Legends

Figure 1. The plasma level of \( \text{H}_2\text{S} \) and CSE activity in wound skin tissue was decreased in diabetic mice, which was reversed by it donors. 1A, Baseline of plasma \( \text{H}_2\text{S} \) level in db/+ and db/db mice (n=5 each group; *\( p < 0.05 \) vs. db/+). 1B, Plasma \( \text{H}_2\text{S} \) level at day 8 after wound establishment and \( \text{H}_2\text{S} \) donor or PAG treatment (n=5 each group; *\( p < 0.01 \) vs. db/+ control; # \( p < 0.01 \) vs. db/db control). 1C, Plasma \( \text{H}_2\text{S} \) concentrations at day 16 after wound creation and \( \text{H}_2\text{S} \) donor or PAG treatment (n=5 each group; *\( p < 0.05 \) vs. db/+ control; # \( p < 0.05 \) vs. db/db control). 1D, Baseline of the activity of \( \text{H}_2\text{S} \) synthase CSE in the skin tissue of db/+ and db/db mice (n=5 each group; *\( p < 0.05 \) vs. db/+). 1E, There was less CSE activity in the skin tissue of db/db mice than in db/+ mice. (n=5 each group; *\( p < 0.01 \) vs. db/+ control).

Figure 2. Both \( \text{H}_2\text{S} \) rapid-releasing (NaHS) and slow-releasing donor (HTB) accelerated the wound healing in diabetic mice, but CSE inhibitor PAG delayed the wound closure rate in matched db/+ controls. Mice were randomly assigned to five groups: db/db + NaHS (50 \( \text{µmol} \cdot \text{kg}^{-1} \cdot \text{day}^{-1} \), n=9), db/db + HTB (30 \( \text{µmol} \cdot \text{kg}^{-1} \cdot \text{day}^{-1} \), n=9), db/db control (saline, n=9), db/+ control (saline, n=10), and db/+ +DL-PAG (50 mg·kg\(^{-1}\)·day\(^{-1}\), n=5). Six mm-diameter full-thickness punch biopsy wounds were created on the above described five groups of mice. Wound closure rates were measured by tracing the wound area every other day and gross wound closure was quantified by Image-pro Plus 5.1. 2A, Representative wound pictures with time after wound performance in five groups; 2B and 2C, Comparisons of wound closure rates in five groups were expressed by percentage of closed wound area (*\( p < 0.05 \), db/db vs. db/+ or PAG group; #\( p < 0.05 \), #\( p < 0.05 \), db/+ vs. PAG group; &\( p < 0.05 \), db/db vs. HTB or NaHS group).
Figure 3. H₂S donor-stimulated angiogenesis in wound skin from db/db mice. The granule skin tissue within the wound area was collected at day 8 after NaHS, HTB or PAG treatment, and stained with CD31. 3A, Representative new capillary pictures of five groups under microscope with 4× magnifications and 20× magnifications. Black dashed lines indicate the original wound edges. Black boxes indicate the areas enlarged correspondingly in the 20× magnification panels. Black arrows point to CD31 positive capillaries. 3B, Comparison of capillary numbers in five groups (n=5 each group, *p <0.05 vs. db/+ control, # p <0.05 vs. db/db control). 3C: Comparison of capillary number in unwounded skin in db/+ mice and db/db mice (n=5 each group, p=0.1326). Hpf: high power field.

Figure 4. H₂S donor accelerated the rate of EPC-mediated wound healing in diabetic mice. One group of db/db mice (n=8) received intraperitoneal injection of NaHS (50 µmol·kg⁻¹·day⁻¹) every other day for 16 days (db/db + NaSH Tx EPCs); One group of db/db mice (n=8) mice received intraperitoneal injection of 0.9% saline every other day for 16 days (db/db + saline Tx EPCs); These above described two groups of mice received topically transplanted 1 ×10⁶ diabetic EPCs on wounds immediately following skin punch biopsies. Another group of db/db (n=8) mice used as control only underwent skin punch biopsies (db/db only). 4A, Representative wound pictures with time after wound performance in three groups. 4B, Comparisons of wound closure rate in three groups were expressed by percentage of closed wound area (*p <0.05, db/db vs. db/db+NaSH Tx EPCs or db/db+saline Tx EPCs; #p <0.05 db/db+NaSH Tx EPCs vs. b/db+saline Tx EPCs). 4C, Representative photographs demonstrated EPC integration into the dermis and vascular structure. On day 8 of wound beds, some BrdU-labeled EPCs (green fluorescence) integrated into vascular-like structures.
(CD31 positive, red fluorescence) and the other BrdU-labeled EPCs integrated in the dermis. On day 16 of wound beds, more BrdU-labeled EPCs integrated into the epithelial layers. The nuclei were counter-stained with DAPI (blue fluorescence). The merged image is shown in 4C. The skin sample was imaged at 10× magnifications using a Nikon 90i upright microscope. Scale for 10 × = 200 μm.

Figure 5. H₂S donor restored the endothelial progenitor cell (EPCs) functions of diabetic mice both in vivo and in vitro, whereas H₂S inhibition suppressed the angiogenic functions of normal EPCs. EPCs were isolated from the bone marrow of five groups after 16-days of in vivo treatment, and cultured in EGM2 for seven days before assay, or collected directly from db/db or db/+ mice and the reseeded EPCs (5×10⁵) were treated in vitro with NaHS 10-50-100 μM in EGM2 for 30 minutes, PAG 3-10-30 mM in EGM2 for 30 minutes or CSE-siRNA at 50nM in medium for six-seven hours after six-eight hours of serum starvation before tube formation on Matrigel and adhesion assay. 5A, Representative tube network pictures under microscope with 4× magnification and comparison of tube length among five groups after 16 days of in vivo treatment of H2S donor (n=4-5; *p<0.01 PAG vs. db/+ control, db/db vs. db/+; # p<0.01 NaHS vs. db/db, HTB vs. db/db control). 5B, Representative adhesive EPCs pictures under fluorescence microscope with 10× magnification and comparison of adhesive cell numbers of five groups (n=3-5; *P<0.01 db/db vs. db/+, PAG vs. db/+ control;  # P<0.01, NaHS vs. db/db, HTB vs. db/db control). 5C, Representative tube network pictures under microscope with 4× magnification and comparison of tube length in four groups treated with different NaHS concentrations and controls (n=3-4; *p<0.01 vs. db/db control); 5D. Representative adhesive EPC pictures under fluorescence microscope with magnification (10×)
and comparison of the adhesive EPC numbers of four groups treated with different NaHS concentrations and controls (n=5; \(^*p<0.01\) vs. db/db control). **5E**, Representative pictures of tube networks under microscope with magnification (4×) and comparison of mean tube length in four groups with in vitro PAG 3-10-30 mM treatment (n=5 each group, \(^*p<0.01\) vs. control).

**5F**, Typical pictures of adhesive EPCs under fluorescence microscope with 10× magnification and comparison of adhesive cells numbers in four groups with different concentrations of PAG treatment (n=4, \(^*p<0.01\), vs. db/+ control). **5G**, CSE silencing with CSE-siRNA in normal EPCs inhibited the CSE expression by 70% in si-CSE transfected EPCs (n=4-5, \(^*p<0.01\) vs. control) **5H**, CSE silencing didn’t alter the CBS expression of normal EPCs (\(p>0.05\) vs. control); **5I**, The tube length of EPCs after CSE silencing decreased by nearly 40% (n=7 each group, \(^**p<0.01\) vs. control). **5J**, The adhesive EPC numbers decreased by 55% in CSE-silenced EPCs (n=4, each group, \(^**p<0.01\) vs. control). **5K**, Cell viability was not affected by NaSH (100 \(\mu\)M) or PAG (30 mM) (n=4-5, \(p>0.05\)).

**Figure 6.** \(H_2S\) donor up-regulated the expressions of Ang-1 in both wound skin tissues and EPCs in type 2 diabetes, while CSE suppression down-regulated Ang-1 expression in normal EPCs. Protein was extracted from 6 mm OF wound skin and 0.5 cm of skin outside of the wound, and seven-day cultured EPCs after 16 days of in vivo treatment by two \(H_2S\) donors and PAG, or normal EPCs with in vitro CSE siRNA 50 nM treatment and its controls. **6A-6B**, Protein bands and comparison of EPCs and skin tissue Ang-1 expression in five groups (n=4-5; \(*p<0.01\) PAG vs. db/+ control, db/db vs. db/+ control; \#\(p<0.01\), NaHS vs. db/db control, HTB vs. db/db control ). **6C**, Protein bands and comparison of VEGF expression of EPCs in five groups (n=4-5; \(*\ p<0.01\) db/db vs. db/+; \#\( p<0.05\) NaHS vs. db/db control, HTB vs. db/db
control). 5D, Protein bands and comparison of Ang-1 expression in EPCs with and without CSE si-RNA treatment (n=4-5; *p<0.01 vs. control).

**Figure 7. H$_2$S promoted EPC angiogenesis via restoration of Ang-1 expression.** EPCs were isolated from bone marrow of db/db and normal mice and cultured in EGM-2 for seven days, then db/db EPCs ($5 \times 10^5$) were reseeded and treated *in vitro* with NaHS 50 nM only in EGM-2 for 30 minutes, or Ang-1siRNA at 5 nM in medium for six-seven hours before NaHS 50 nM treatment. Then, tube formation on Matrigel and adhesion on vitronectin assay were performed in all EPCs. 7A, Representative pictures of tube networks under microscope with 4× magnification. 7B. Comparison of tube length in four groups of db/+ EPCs and db/db EPCs treated with and without NaHS, NaHS and Ang-1 siRNA. (n=4 each group, *p<0.05 vs. db/+, # p <0.05 vs. db/db only, & p <0.05 vs. db/db treated with NaHS only).

**Figure 8. The expressions of Ang-1, CSE, and VEGF were decreased in human skin tissue of diabetic foot ulcers.** 8A. Representative protein bands of Ang-1, CSE, and VEGF of skin tissue. 8B. The expression of Ang-1, CSE, and VEGF were lower in the foot ulcer skin tissue of diabetic patients than in non-diabetes controls (n=7-8 each group; *p <0.05 vs. control).
### Table 1 Clinical characteristic of participated subjects

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<th>Non-diabetic subject (n=8)</th>
<th>Diabetic subject (n=7)</th>
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<tr>
<td><strong>Age (y)</strong></td>
<td>40.6 ± 4.3</td>
<td>56.5 ± 7.5</td>
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<tr>
<td><strong>Gender</strong></td>
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<tr>
<td>Female</td>
<td>3 (37.5%)</td>
<td>4 (57.1%)</td>
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<tr>
<td>Male</td>
<td>5 (62.5%)</td>
<td>3 (43.9%)</td>
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<td><strong>Fast plasma glucose</strong></td>
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<td>9.1 ± 1.1</td>
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<td><strong>Medications</strong></td>
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<td><strong>Co-morbidities</strong></td>
<td>Low extremity traumatic injury</td>
<td>Foot ulcer, peripheral neuropathy, microalbuminuria</td>
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H₂S donor improves wound healing through restoring endothelial progenitor cell function and activating angiopoietin-1 in type 2 diabetes

Figure 1.
Figure 2.

2A

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<tr>
<th>Day</th>
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<th>db/db</th>
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<th>HTB</th>
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2B

![Graph](image46)

2C

![Graph](image47)
Figure 3

3A

- db/+ PAG
- db/db
- db/db HTB
- db/db NaSH
**Day 8**

**3B**

![Bar Chart](image)

- **Capillary/hpf**
- **db/+**
- **PAG**
- **db/db**
- **HTB**
- **NaSH**

**db/+**

- **Statistical Significance:**
  - * (p-value not specified)
  - # (p-value not specified)

**3C**

![Bar Chart](image)

- **Baseline**
- **Capillary/hpf**
- **db/+**
- **db/db**

- **Statistical Significance:**
  - p=0.1326

**Diabetes**
Figure 4

4A

Day 0

Day 8

Day 16

4B

Wound closure rates (%)

Day

4C

Day 8

Day 16
Figure 5.

5A

5B

5C

5D

5E

5F
Figure 6.

6A Ang-1

6B Ang-1

6C VEGF

6D Ang-1
Figure 7.

7A

7B

![Graph showing tube length fold over different conditions](image)

**Diabetes**
Figure 8.

8A. Ang-1
     CSE
     VEGF
     GAPDH
     Control  Diabetes

8B.  

- Ang-1/GAPDH: Control > Diabetes
- CSE/GAPDH: Control = Diabetes
- VEGF/GAPDH: Control < Diabetes

** indicates statistical significance.