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Title: A new H2S-releasing peptide hydrogel limits the development of intimal hyperplasia in human vein segments

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Abstract: Currently available interventions for vascular occlusive diseases suffer from high failure rates due to re-occlusive vascular wall adaptations, a process called intimal hyperplasia (IH). Naturally occurring hydrogen sulfide (H2S) works as a vasculoprotective gasotransmitter in vivo. However, given its highly reactive and hazardous nature, H2S is difficult to administer systemically. Here, we developed a novel hydrogel capable of localized slow release of precise amounts of H2S and tested its benefits on IH.
The H2S-releasing hydrogel was prepared from a short peptide attached to an S-aroylthiooxime H2S donor. Upon dissolution in aqueous buffer, the peptide self-assembled into nanofibers, which formed a gel in presence of calcium. This new hydrogel delivered H2S over the course of several hours, in contrast with fast-releasing NaHS.
The H2S-releasing peptide/gel inhibited primary human vascular smooth muscle cells (VSMC) proliferation and migration. In contrast, it promoted human umbilical endothelial cells (EC) proliferation and migration.
Finally, both NaHS and the H2S-releasing gel limited IH in human great saphenous vein segments obtained from vascular patients undergoing bypass surgery, with the H2S-releasing gel showing efficacy at a 5x lower dose than NaHS.
Our data suggest local perivascular H2S release as a new strategy to limit VSMC proliferation and IH while promoting EC proliferation, hence re-endothelialization.

Research Data Related to this Submission

There are no linked research data sets for this submission. The following reason is given:
Data will be made available on request

Nanomedicine: Nanotechnology, Biology, and Medicine

Lausanne, April 12, 2019

Dear Editor,

Please find enclosed our manuscript entitled "**A new H_2S-releasing peptide hydrogel limits the development of intimal hyperplasia in human vein segments**" by Longchamp et al., which we submit for publication to *Nanomedicine: Nanotechnology, Biology, and Medicine.*

Arterial occlusive disease is the leading cause of death in Western countries. Contemporary therapies, including angioplasties, stenting and bypass surgery, still suffer from high failure rates due to re-occlusive vascular wall adaptations, which result in costly and complex recurrent end-organ ischemia, and often lead to loss of limb, brain function, or life. Hydrogen sulfide (H_2S) is an endogenous gasotransmitter with vasculoprotective properties. However, given its highly reactive and hazardous nature, H_2S is difficult to administer. Here, we developed a novel self-assembling H_2S-releasing hydrogel and tested its benefits on restenosis using human vein segments obtained from patients undergoing bypass surgery. Our findings suggest local perivascular H_2S release as a new strategy to limit VSMC proliferation and restenosis while promoting EC proliferation, hence re-endothelialization.

The submitted manuscript is a revised version of manuscript **jbmt47934**, which was submitted to ***Biomaterials*** in February 2019. In this revised version, we addressed a number of issues raised by the reviewers to clarify the methods and translational potential of our H_2S-releasing peptide hydrogel.

We believe these data to be of interest to the readership of the *Nanomedicine: Nanotechnology, Biology, and Medicine*, describing a new hydrogel with therapeutics potential for the clinical treatment of restenosis in vascular patients.

All authors have read and approved the submission of the manuscript. We certify that this manuscript, or any part of it, has not been published and will not be submitted elsewhere for publication while being considered by the journal *Nanomedicine: Nanotechnology, Biology, and Medicine.*

Thank you very much for your editorial consideration.

Sincerely,

Dr. Florent Allagnat and Prof. John Matson

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***Graphical Abstract: Text**

Graphical abstract

In this work, we describe a novel Hydrogen Sulfide (H_2S)-releasing peptide that self-assembles into nanofibers and forms a gel in presence of calcium. This hydrogel capable of localized slow release of H_2S inhibited the development of intimal hyperplasia (IH) in a model of static culture of human great saphenous vein segments obtained from vascular patients undergoing bypass surgery.

*Graphical Abstract: Image

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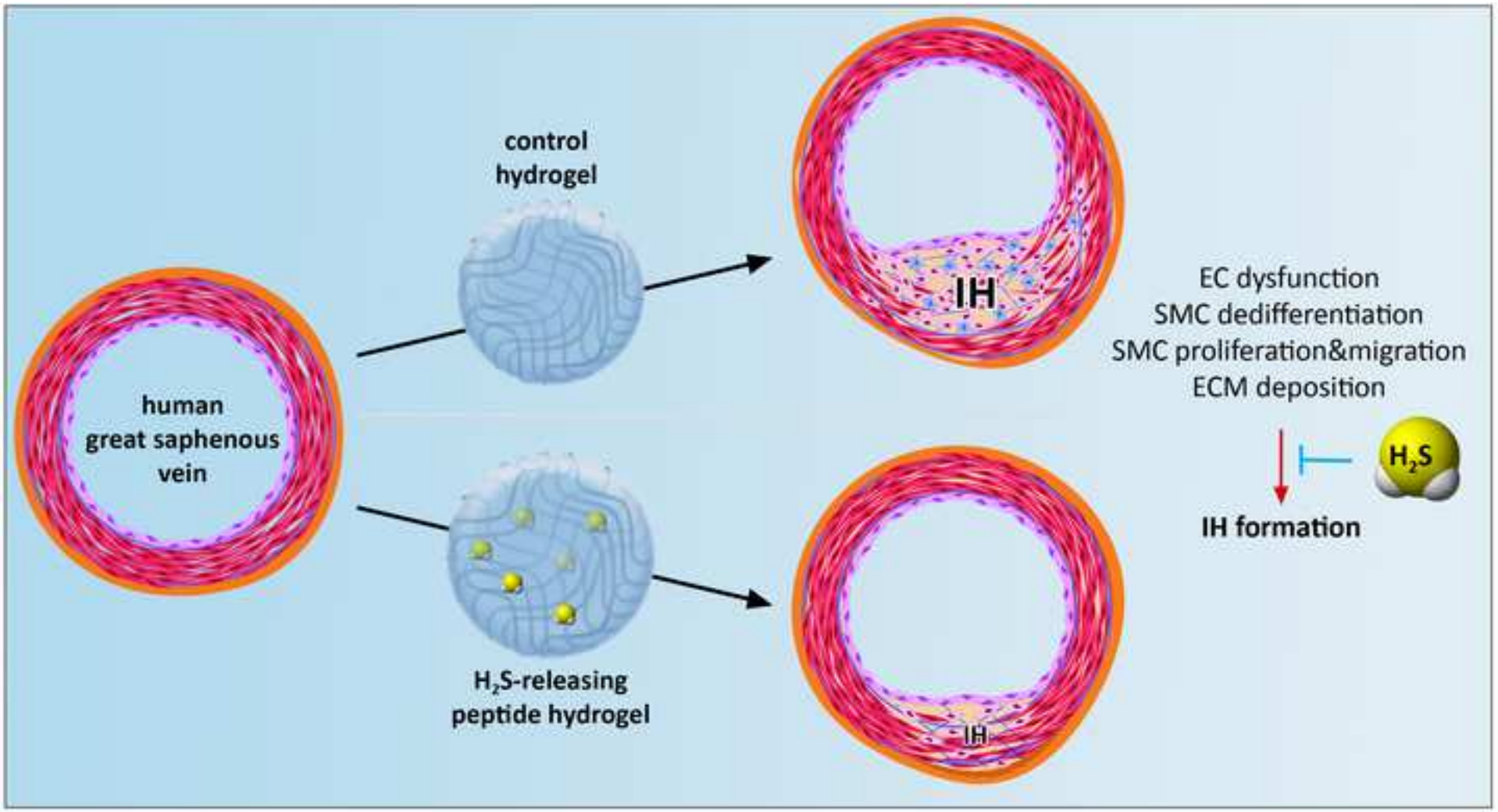

A new H_2S-releasing peptide hydrogel limits the development of intimal hyperplasia in human vein segments

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Competing Interests' Statement: The authors confirm that there are no known conflicts of interest associated with this publication and there has been no significant financial support for this work that could have influenced its outcome.

Original article

Running title: H_2S decreases VSMC proliferation and intimal hyperplasia

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Abbreviations

CAC: critical aggregation concentration

CD: Circular dichroism

EC: endothelial cells

HUVEC: human umbilical vein EC

H_2S: hydrogen sulfide

IH: intimal hyperplasia

L-Cys: L-Cysteine

OBHA: O-benzyl hydroxylamine

PCNA: proliferating cell nuclear antigen

SATO: S-aroylthiooxime

SM22α: smooth muscle 22 alpha

VSMC: vascular smooth muscle cells

Author Contributions

FA, JBM, JMC and SD designed the project

FA, AL, KK, DM, SD and CD performed the experiments

FA, JBM, AL, KK, DM and SD analyzed the data

FA, JBM, AL, KK and DM wrote the manuscript

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Keywords: Intimal Hyperplasia; Smooth Muscle Cells; Proliferation; hydrogen sulfide; hydrogel

ABSTRACT

Currently available interventions for vascular occlusive diseases suffer from high failure rates due to re-occlusive vascular wall adaptations, a process called intimal hyperplasia (IH). Naturally occurring hydrogen sulfide (H_2S) works as a vasculoprotective gasotransmitter *in vivo*. However, given its reactive and hazardous nature, H_2S is difficult to administer systemically. Here, we developed a novel hydrogel capable of localized slow release of precise amounts of H_2S and tested its benefits on IH.

The H_2S-releasing hydrogel was prepared from a short peptide attached to an *S*-aroylthiooxime H_2S donor. Upon dissolution in aqueous buffer, the peptide self-assembled into nanofibers, which formed a gel in presence of calcium. This new hydrogel delivered H_2S over the course of several hours, in contrast with fast-releasing NaHS.

The H_2S-releasing peptide/gel inhibited primary human vascular smooth muscle cells (VSMC) proliferation and migration, while promoting human umbilical endothelial cells (EC) proliferation and migration. Both NaHS and the H_2S-releasing gel limited IH in human great saphenous vein segments obtained from vascular patients undergoing bypass surgery, with the H_2S-releasing gel showing efficacy at a 5x lower dose than NaHS.

Our data suggest local perivascular H_2S release as a new strategy to limit VSMC proliferation and IH while promoting EC proliferation, hence re-endothelialization.

Background

Despite remarkable technological advances, the rate of restenosis due to intimal hyperplasia (IH) one year following endovascular reconstruction or bypass surgery reaches 30% [1]. IH is driven by the dysfunction of endothelial cells (EC) lining the inner part of the vessels, which results in a reprogramming of the vascular smooth muscle cells (VSMC) from a contractile differentiated phenotype to a proliferating and migrating phenotype. These cells proliferate and secrete extracellular matrix, leading to the formation an occlusive neo-intima layer at the site of injury [2]. The available systemic drug therapies used to prevent restenosis are generally poorly tolerated and show narrow therapeutic ranges [3,4]. Localized treatments include drug-eluting angioplasty balloons and stents, which limit VSMC proliferation and reduce IH, but they also delay re-endothelization, limiting their efficacy and prolonging the need for anti-thrombotic medication.

H_2S is an endogenous gasotransmitter, along with nitric oxide (NO) and carbon monoxide (CO) [5]. Enzymatically produced *in vivo* by cystathionine γ-lyase (CSE), cystathionine β-synthase (CBS), and 3-mercaptopyruvate sulfurtransferase (3-MST), H_2S regulates numerous signaling pathways and physiological processes, including blood pressure, inflammation, metabolism, redox balance and, overall, cellular homeostasis [6,7]. In humans, serum H_2S concentration declines with age [8] and the circulating levels of H_2S are reduced in patients suffering from cardiovascular diseases [9,10] and diabetes [11].

To tap the therapeutic potential of this gasotransmitter, numerous synthetic H_2S donors have been developed for exogenous administration. H_2S donors are molecules that release H_2S by either hydrolysis or in response to a specific trigger, such as a thiol or other nucleophiles [12]. However, H_2S delivery via small molecule donors is often limited by short release periods, low water solubility, lack of target specificity, and toxicity in some cases [12,13]. To address these

shortcomings, H_2S donors have been incorporated into water-soluble polymers, micelles, hydrogels, nanofibers, and films, in an attempt to limit toxicity and extend the H_2S release period [14-18]. Despite these improvements, there is still a growing need for H_2S-releasing materials capable of delivering H_2S directly at a site of interest for an extended period of time [19,20]. Peptide-based hydrogels have been widely used for tissue engineering and regenerative medicine [21], but only a handful of reports detail their use in delivering gasotransmitters [22-24].

Here, we developed and tested the safety and therapeutic potential of a type of self-assembling aromatic peptide amphiphile, employing an H_2S-releasing *S*-aroylthiooxime (SATO) group as the aromatic component. We found that our H_2S-releasing hydrogel promotes EC function while inhibiting VSMC expansion and IH formation in human veins, at a significantly lower dose than NaHS.

METHODS

Chemicals

Rink Amide MBHA resin and 9-fluorenylmethoxy carbonyl (Fmoc) protected L-amino acids were purchased from P3biosystems and used as received. HBTU, *N*-methylpiperidine, DBU and other reagents for peptide synthesis were purchased from commercial vendors and used as received unless otherwise noted. The solvents employed for peptide synthesis were reagent grade.

Peptide synthesis and purification

Peptides were synthesized either manually or using a Liberty 1 microwave-assisted peptide synthesizer (CEM) using solid-phase peptide synthesis (SPPS) via standard Fmoc protocol as described previously [24]. 4-Formyl benzoic acid was coupled to the N-terminus of the peptide on resin using HBTU and DIEA in DMF. After cleavage and isolation, peptides were dissolved in water containing 0.1% NH_4OH and filtered through a 0.45 µm PTFE filter before purification. Purification by preparative-scale reverse phase-high performance liquid chromatography (RP-HPLC) was carried out on an Agilent Technologies 1260 Infinity HPLC system, eluting with a gradient of 2% ACN to 90% ACN in milliQ H_2O over 33 min using an Agilent PLRP-S column (100Å particle size, 25 x 150 mm) and monitoring at 220 nm. To both mobile phases was added 0.1% NH_4OH to aid in solubility. Fractions were analyzed by mass spectrometry (Advion ExpressIon Compact Mass Spectrometer), and product-containing fractions were combined, rotovapped to remove ACN, and lyophilized (LabConco).

The lyophilized peptide FBA-IAVEE was dissolved in dry DMSO and reacted with *S*-benzoylthiohydroxylamine (SBTHA) in the presence of catalytic TFA to afford the final SATO-FBA-IAVEE peptide. Peptide FBA-IAVEEEE was similarly reacted with *O*-benzyl

hydroxylamine hydrochloride (OBHA·HCl) in dry DMSO, but without using TFA, to afford the non-H_2S-releasing control peptide OBHA-FBA-IAVEEEE. Peptides were dissolved in a mixture of phosphate buffer (100 mM at pH 7.4) and acetonitrile (5:2 v/v) and filtered through a 0.45 µm PTFE filter before purification. Purification was carried out using RP-HPLC, eluting with a gradient of 2% ACN to 90% ACN in milliQ H_2O without any additives. The protocol for analysing and recovering the peptides was the same as described above. The final peptides were dissolved in milliQ water and distributed into aliquots (100 µg each). Aliquots were frozen, lyophilized, and stored at -20 ºC.

Critical aggregation concentration (CAC) measurements

Nile red stock solution in acetone (1 mg/mL) was diluted in milliQ water to a concentration of 0.01 mg/mL and was used to make all peptide solutions. A peptide stock solution was prepared at 4 mg/mL in the Nile red stock solution and was further diluted to the concentration of 3 mg/mL, 2 mg/mL, 1 mg/mL, 0.5 mg/mL, 0.25 mg/mL, 0.1 mg/mL, 0.01 mg/mL, 0.001 mg/mL, and 0.0001 mg/mL. All peptide dilutions were vortexed/sonicated for a few seconds, then 300 µL of each was transferred to a 96-well plate, and the plate was allowed to sit in dark for 15-20 min. Florescence spectra were recorded using a Varian Cary Eclipse fluorescence spectrophotometer (FL1105M003) with an excitation wavelength of 550 nm. Florescence intensity measured at 628 nm was plotted against log[concentration], and the final CAC values were estimated to be the point of intersection between the linear fits of high and low concentration regimes.

Circular Dichroism (CD) Spectroscopy

CD spectra were measured at room temperature using a Jasco J-815 CD spectrometer (Jasco Inc.) with a preset N_2 flow at 120 mL/min. The range of wavelengths employed was 250 to 190 nm (50

nm/min) with a response time of 8 s. Samples for both Pep-H_2S and Pep-Ctrl were freshly prepared at 10 mM (20 µL) in 1X PBS (pH 7.4) and were analysed using a dismountable quartz cuvette with path length of 0.2 mm with 3 iterations for each sample. Raw spectra were converted to mean residual ellipticity for comparison.

Hydrogelation

Both Pep-H_2S and Pep-Ctrl formed hydrogels in PBS solutions at physiological pH upon addition of 4 µL of $CaCl_2$ solution (200 mM in water) to 90 µL of peptide solution (10 mM in 1X PBS). The final concentration of $CaCl_2$ in the hydrogels was 8 mM.

Rheology

Rheological experiments were done on an AR-2000 (TA instruments) using a 25 mm parallel plate geometry. Buffered peptide solutions (240 µL, 1 wt.% peptide, ~10 mM) were prepared for each peptide in 1X PBS (pH 7.4) and quickly transferred to the rheometer's bottom geometry. Gelation was initiated upon addition of 10 µL $CaCl_2$ solution (200 mM in water) and the resulting solution was mixed thoroughly with a pipet tip to ensure homogeneity. After allowing the solution to gel for 10 min, the upper geometry was lowered to a pre-set gap of 500 µm, and a dynamic time sweep was performed at a frequency of 1 Hz and 0.5% strain to measure storage (G') and loss (G") moduli. Each time sweep was followed by a dynamic frequency sweep (0.010-100 Hz at 0.5% strain) and a strain sweep (1 Hz at 0.5-100% strain).

Morphology analysis via TEM

Peptide solutions (10 mM in 0.05 M phosphate buffer at pH 7.4) were prepared and allowed to age overnight, then diluted with water to 500 µM. Next, 10 µL of the peptide solution was deposited on a carbon-coated copper TEM grid (300 mesh, Electron Microscopy Sciences), allowed to sit for 5-6 min, and then gently blotted with filter paper. The grid was then washed by adding a drop of MilliQ water, allowing it to stand for 1 min, and then blotting with filter paper. Samples were stained with 10 µL of a 2% uranyl acetate aqueous solution for 5-6 min, blotted with filter paper, and allowed to dry in air before TEM observation. Images were taken on a Philips EM420 TEM with a slow scan CCD camera.

H_2S release measurements using an electrode probe

H_2S release from the peptides was measured amperometrically using an electrode probe (ISO-H2S-100-CXX, World Precision Instruments). A solution of either Pep-H_2S or Pep-Ctrl (20 µL of 0.1 mM solution in 1X PBS) was placed in an inner well inside a specially designed glass vial equipped with a stir bar. An additional 76 µL of 10 mM PBS buffer at pH 7.4 was then added to the well, followed by 4 µL of cysteine solution (5 mM in water). The final concentrations in the inner well were 20 µM in peptide and 0.2 mM (10 eq.) in cysteine. The well was immediately covered with the gas-permeable membrane (Breathe easier, Diversified Bioteck), and PBS buffer at pH 7.4 (4.95 mL) mixed with 50 µL of diethylenetriaminepentaacetic acid (DTPA) solution (10 mM in water) was added into the vial, covering the inner well. The H_2S-selective microelectrode was then immersed in the PBS solution, and the output signal was recorded. Similarly, H_2S release from Pep-H_2S at 200 µM was measured by mixing 17 µL of peptide solution (1 mM) with 83 µL of PBS in the well followed by addition of 1 µL (10 eq.) cysteine solution (200 mM in water). H_2S release from Gel-H_2S was measured by placing 96 µL of the peptide solution (10 mM in 1X PBS)

in the inner well followed by 4 µL of $CaCl_2$ to form a hydrogel. 10 µL cysteine solution (200 mM in water) was added, the well was covered with the membrane, and output signal was measured as above. Calibration was carried out as previously reported. [25]

Cell culture

Human veins were obtained from donors who underwent lower limb bypass surgery and static vein cultures were performed as previously described [26]. Briefly, 5 mm segments of vein were kept in culture for 7 days in RPMI-1640 Glutamax supplemented with 10 % FBS and 1% antibiotic solution (10,000 U/mL penicillin G, 10,000 U/mL streptomycin sulphate) in cell culture incubator at 37°C, 5% CO_2 and 5% O2. The cell culture medium was changed every 48 h. 5-mm segments of vein were harvested after culture and fixed in 4% formalin and paraffin-embedded for histological analysis. Five distinct vein segments coming from five different patients were used in this study.

Human smooth muscle cells were also prepared from these human saphenous vein segments as previously described [26]. Briefly, primary smooth muscle cells were cultured from human saphenous veins from a similar cohort used for *ex-vivo* perfusion. Veins explants of 1–2 mm were plated, luminal side down, on the dry surface of a 6-well culture plate, previously coated with 1% Gelatin type B (Sigma-Aldrich). Explants were gently covered with one drop of RPMI, 10% FBS medium, and placed overnight in a 37°C, 5% CO_2, 5% O_2 environment. The next day, the culture medium was carefully added to the wells, taking care not to detach the explants. VSMC, as identified by positive staining for SMA (Abcam, ab5694), and desmin (Dako, M 0760) were maintained in RPMI1640 supplemented with 10 vol. % FBS at 37°C, 5% CO_2 and 5% O2.

HUVECs purchased from Lonza were maintained in endothelial growth medium 2 (EGM-2; Lonza) at 37°C, 5% CO_2 and 5% O2 as previously described[27]. Passages 1 to 8 were used for the experiments.

Histomorphometry and Immunohistochemistry

5-mm segments of vein embedded in paraffin were cut into 5-µm sections. 2-mm out of 5-mm were cut in 4 series of 10 slides with 5 sections per slides with a 250-µm interval between the series. One slide per series was stained using Van Gieson-elastin (VGEL) staining. 3 images per section were taken at a 100x magnification to cover the whole vein area. 8 measurements of the of the intima and media thicknesses were made by images, evenly distributed along the length of the vein wall [28]. Thus, for each vein, the intima and media thicknesses values are a mean of 4 series x 3 images x 8 measures = 96 independent measures. Morphometric measurements were done by two independent researchers, one of them blind to the experimental groups, using the Leica Qwin® software (Leica, Switzerland).

PCNA immunohistochemistry were similarly performed on paraffin sections. After rehydration and antigen retrieval (TRIS-EDTA buffer, pH 9, 15 min in a microwave at 600 watts), human vein sections were incubated overnight with the proliferating cell nuclear antigen antibody (PCNA; M087901, Dako, Baar, Switzerland), washed and revealed using the EnVision +/HRP, DAB+ system according to manufacturer's instructions (Dako, Baar, Switzerland), and counterstained with hematoxylin [29]. One slide per series was assessed and 3 images per section were taken at a 100x magnification. The PCNA and hemotoxlin positive nuclei were manually counted by two independent observers unaware of the conditions.

Immunofluorescent staining

Cell immunostaining was performed on cells grown on glass coverslips (10^6 cells per well in 24 well plates) and fixed for 5 min in −20 °C methanol. BrdU immunostaining was performed as previously described using mouse anti-BrdU (BD Bioscience 55627, 1:200) and fluorescent-labelled anti-mouse secondary antibodies (AlexaFluor 568; 1/500, Thermo Fisher Scientific). BrdU positive nuclei were automatically detected using the ImageJ software and normalized to the total number of DAPI-positive nuclei.

Double immunostaining for calponin and smooth-muscle 22 alpha (SM22α) was performed on VSMC grown on glass coverslips and fixed for 5 min in −20 °C methanol. Cells were then permeabilized in PBS supplemented with 2 wt. % BSA and 0.3 vol. % Triton X-100 for 30 min, blocked in PBS supplemented with 2 wt. % BSA and 0.01 vol. % Tween 20 for another 30 min, and incubated overnight with the primary antibodies (mouse anti human calponin, DAKO; M3556;1/200 and rabbit anti human SM22α, Abcam; 4106; 1/400). Cells were then washed 3 times 5 min in PBS supplemented with 2 wt. % BSA and 0.01 vol. % Tween 20, and incubated for 1 h at room temperature with a mix of fluorescent-labelled secondary antibodies (anti-rabbit AlexaFluor 488 and anti-mouse AlexaFluor 568; 1/500).

Live-cell hydrogen sulfide measurement

Free sulfide was measured in cells using the SF_7-AM fluorescent probe [30] (Sigma-Aldrich cat: 748110). The probe was dissolved in anhydrous DMF at 5mM. 10^5 cells per well were plated in a 96 well plate. 24 hours later, 5 μM SF7-AM was added to VSMC or HUVEC and fluorescence (Ex: 495 nm/Em: 520 nm) was measured continuously in a Synergy Mx fluorescent plate reader at 37°C before and after addition of various donors as indicated. Linear regression of the SF_7-AM

fluorescent signal was calculated during the linear part of the curves generated to deduce the H_2S release rate.

Transmigration assays

The chemotactic-induced transmigration of human cells across a matrix barrier was investigated using a Boyden chamber made of a polycarbonate membrane insert with 8-mm pores (Falcon; BD Biosciences) placed in 24-well culture plates. Confluent VSMCs or HUVECs were trypsinized and resuspended in RPMI 1640 medium without supplements and plated onto the transwell (10^5 cells in 300 µL). Transwells with VSMC were placed in a 24-well plate with 400 µL of EGM-2 culture medium after 48 h in contact with HUVECs. Transwells with HUVECs were placed in a 24-well plate with 400 µL of fresh complete EGM-2 culture medium. In experiments with gels (Gel-H_2S and Gel-Ctrl), 10 µL/mL of gel (1 wt. %) was placed in the 24 well plate. After 8 h, 5 µg/mL calcein-AM (Thermo) was added to the well to stain the cells on the outer surface of the membrane of the transwell. After 30 min and two washes with PBS, fluorescence was measured using a fluorescent plate reader with an excitation wavelength of 485 nm and an emission wavelength of 530 nm. Cells were also imaged using an inverted fluorescent microscope (Leica AG).

Statistical analyses

All experiments were quantitatively analysed using GraphPad Prism® 6, and results are shown as mean ± SEM. One-way ANOVA tests were performed followed by multiple comparisons using *post-hoc* t-tests with the appropriate correction for multiple comparisons.

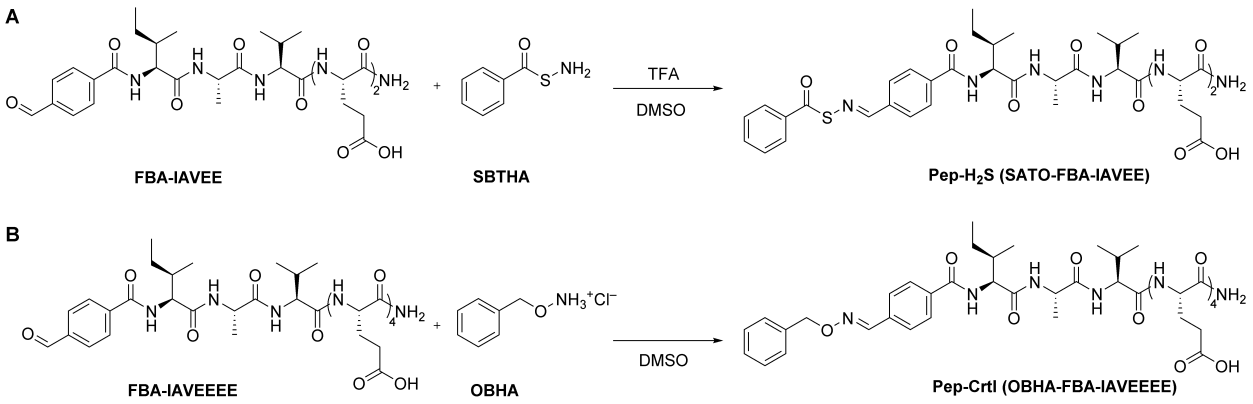
Ethics statement

Written, informed consent was obtained from all vein donors for human vein and VSMC primary cultures. The study protocols for organ collection and use were reviewed and approved by the Centre Hospitalier Universitaire Vaudois (CHUV) and the Cantonal Human Research Ethics Committee (http://www.cer-vd.ch/, no IRB number, Protocol Number 170/02), and are in accordance with the principles outlined in the Declaration of Helsinki of 1975, as revised in 1983 for the use of human tissues.

RESULTS

Design and synthesis of an H_2S-releasing peptide and a control peptide

Self-assembling aromatic peptide amphiphiles rely on a combination of aromatic stacking of N-terminal aromatic groups and hydrogen bonding in a short peptide chain consisting of 2-6 amino acids to drive self-assembly in aqueous solution [31-34]. We based our peptide design for this study on our recent report on the first H_2S-releasing aromatic peptide amphiphile [23]. Using an N-terminal SATO group as the aromatic component and a pentapeptide sequence modified from our original design to increase gel stiffness, the sequence used here for the H_2S-releasing peptide (termed Pep-H_2S) was SATO–FBA–IAVEE, where SATO represents the aromatic H_2S donor, FBA represents 4-formylbenzoic acid, and IAVEE represents the peptide sequence using 1-letter codes. We also prepared a control peptide (termed Pep-Ctrl) incapable of releasing H_2S with the sequence OBHA–FBA-IAVEEEE, where OBHA represents *O*-benzyl hydroxylamine. Pep-Ctrl contains an oxime linkage in place of the acylthiooxime linkage in Pep-H_2S, but the peptides are otherwise identical. In initial rheological studies, we found that two additional C-terminal Glu residues were needed in Pep-Ctrl compared with Pep-H_2S to afford hydrogels with similar storage moduli. We speculate that stronger aromatic stacking and/or hydrogen bonding in the OBHA component vs. the SATO component leads to this requirement for a longer hydrophilic sequence, which tends to decrease storage modulus, in Pep-Ctrl.



Scheme 1: peptide structures

A) Synthesis of Pep-H_2S (sequence SATO-FBA-IAVEE). B) Synthesis of Pep-Ctrl (sequence OBHA-FBA-IAVEEEE).

Peptides FBA-IAVEE and FBA-IAVEEEE were synthesized using Fmoc-based solid-phase peptide synthesis. The FBA units were coupled to the peptides on-resin before cleavage and purification of these two peptide-aldehydes. The two peptide aldehydes were then further derivatized to form the final aromatic peptide amphiphile products (**Scheme 1**). Pep-H_2S was prepared by condensing the N-terminal aldehyde in FBA-IAVEE with *S*-benzoylthiohydroxylamine to form the final molecule. Pep-Ctrl was synthesized similarly, condensing FBA-IAVEEEE with hydroxylamine OBHA. Both peptides were purified by preparative HPLC after the condensation step, lyophilized, and aliquoted for further analysis.

Peptides self-assembled into nanoribbons

Self-assembly is typically observed in amphiphilic molecules, where the hydrophobic component drives assembly as it excludes water. In dilute solution amphiphiles are molecularly dissolved, but self-assembly occurs above a given concentration, termed the critical aggregation concentration (CAC). Here we used the Nile red assay to determine the CAC for both Pep-H_2S and Pep-Ctrl. The hydrophobic Nile red dye, which is non-fluorescent in a hydrophilic environment, incorporates within the hydrophobic core of the self-assembled nanostructures, resulting in a fluorescent enhancement proportional to the peptide concentration. The CAC is then defined as a point of abrupt change in the fluorescence intensity and refers to a minimum concentration above which the molecule exists primarily in a self-assembled state. In our experiments on Pep-H_2S, we measured a CAC of 0.9 mM (near 1 mg/mL) for both peptides.

With CAC values in hand, we next explored the molecular packing of the self-assembled aromatic peptide amphiphiles Pep-H_2S and Pep-Ctrl. Circular dichroism (CD) spectra were measured for both Pep-H_2S and Pep-Ctrl at 10 mM in 1X PBS buffer to evaluate secondary structure formation. Prominent minima at 217 nm and 220 nm for Pep-H_2S and Pep-Ctrl, respectively, indicated the presence of β-sheets (**Figure 1A**). Typically, β-sheet signals in the CD spectra of short, self-assembling peptides indicates the formation of extended, 1-dimensional nanostructures, which are necessary for gelation [35].

To explore further the self-assembled structures, we used conventional TEM with negative staining to image the nanostructures formed by Pep-H_2S and Pep-Ctrl (**Figure 1B, C**). Aromatic peptide amphiphiles can take on many types of morphologies, including spheres, cylinders, flat or twisted ribbons, sheets, and others [32,36]. When long, one-dimensional objects are observed, these may entangle under certain conditions to form gels. In both cases we observed flat nanoribbons, many several microns in length, with widths around 6-7 nm, which was consistent with the CD spectra depicting β-sheets. The nanoribbons were uniform in width, likely due to the fact that both peptide nanostructures are assembled from pure, single molecules with no molecular weight dispersity.

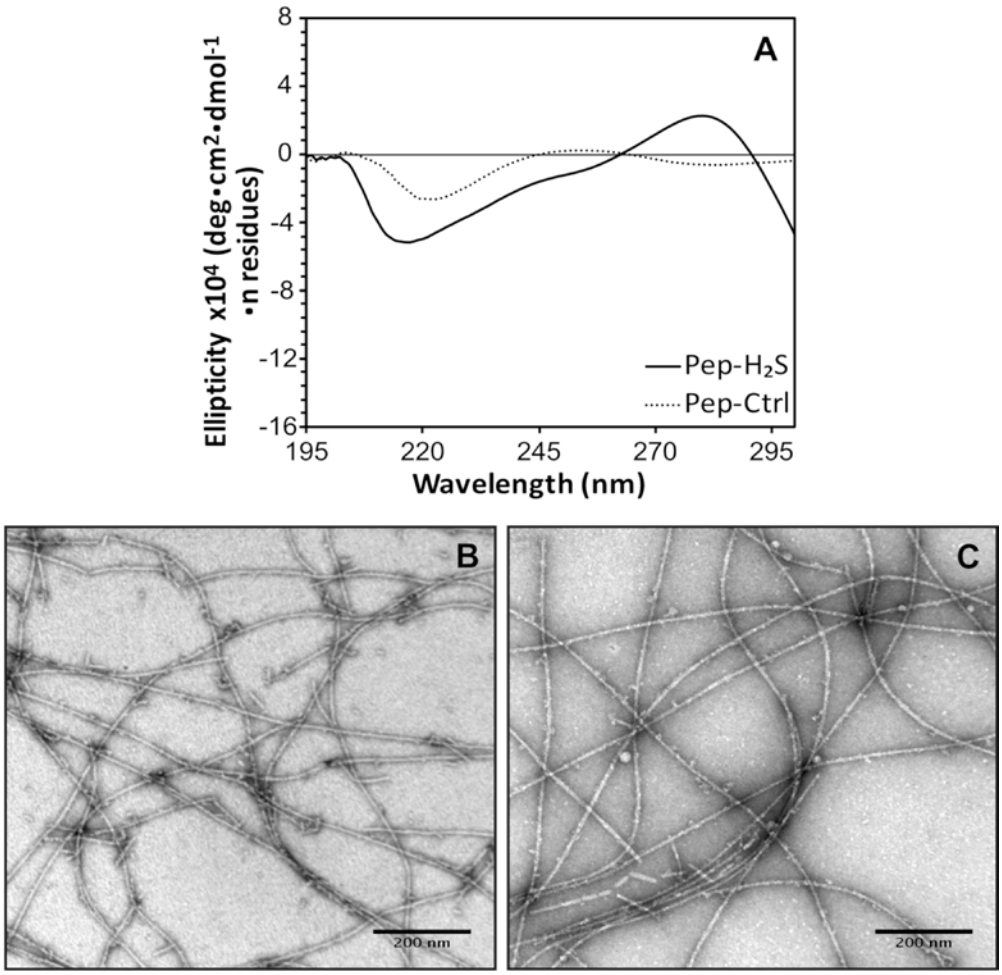


Figure 1: The peptides form long flat nanoribbons

A) CD spectra for 10 mM Pep-H_2S and Pep-Ctrl solution in 1X PBS buffer. Conventional TEM images for **B**) Pep-H_2S and **C**) Pep-Ctrl. Peptides were dissolved at 10 mM in 50 mM phosphate buffer at pH 7.4. Samples were diluted to 500 μM before casting and stained with 2% uranyl acetate solution.

Calcium salts triggered peptide gelation to form soft hydrogels

As noted above, aromatic peptide amphiphiles that form long, one-dimensional aggregates may gel under conditions that promote entanglement of the nanostructures. We found that addition of $CaCl_2$ promoted the rapid gelation of both Pep-H_2S and Pep-Ctrl. This is likely due to two factors: 1) Charge screening of the negatively charged Glu residues, which reside on the nanofiber surface and cause the fibers to repel each other under low salt conditions; and 2) Formation of salt bridges between the nanofibers due to the divalent nature of the calcium ion.

The gelation and viscoelastic properties of the hydrogels, termed Gel-H_2S and Gel-Ctrl when in the gel state, were evaluated by rheological measurements. Peptide solutions were first prepared at 1 wt. % (~10 mM) in 1X PBS, and solutions were added directly to the rheometer. Next, a solution of $CaCl_2$ in water was added to the peptide solutions to afford a final $CaCl_2$ concentration of 8 mM. After a brief waiting period to allow for gelation throughout the sample, the stiffness of each hydrogel was measured. Frequency sweeps of both Gel-H_2S and Gel-Ctrl revealed that the storage modulus (G') was higher than the loss modulus (G") throughout the range tested, indicating that both peptides formed robust, soft hydrogels (**Figure 2**). Gel-H_2S showed a storage modulus of 50 Pa at intermediate frequencies, while Gel-Ctrl showed a storage modulus of 100 Pa. Both hydrogels were soft and easily manipulated by a spatula or syringe. At frequencies above 10 Hz, both hydrogels showed increases in both G' and G". This behavior is characteristic of materials with non-covalent cross-linking such as peptide-based hydrogels [37].

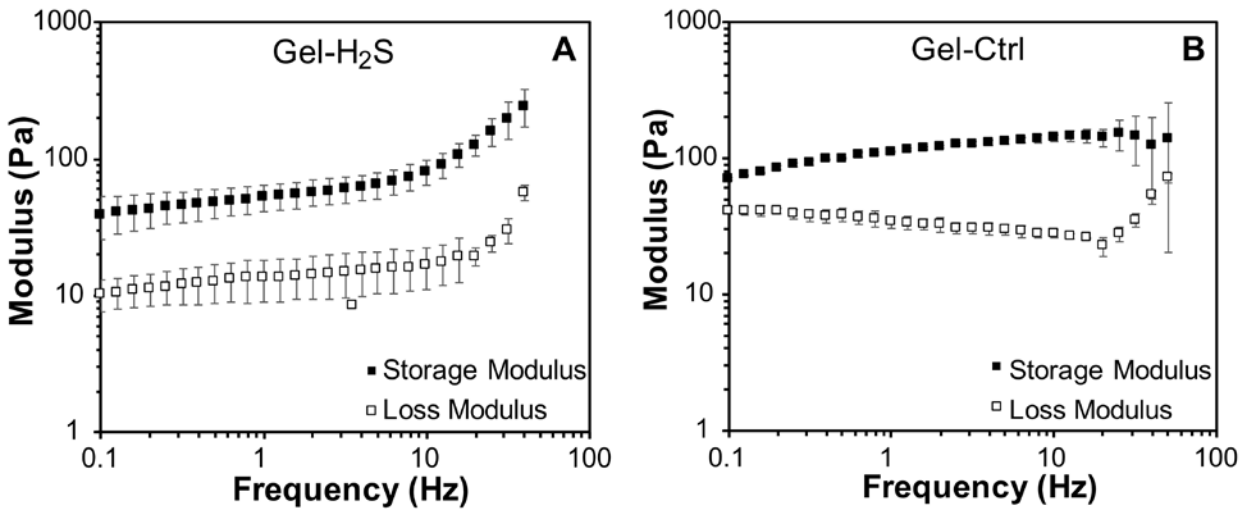


Figure 2: The peptides form soft gels in presence of $CaCl_2$

Frequency sweep oscillatory rheology for hydrogels prepared at 1 wt% in 1 X PBS at pH 7.4 and gelled with $CaCl_2$. A) Gel-H_2S and B) Gel-Ctrl.

Peptide gel exhibited slow and sustained H_2S release

SATOs are thiol-triggered H_2S donors [25], so we aimed to evaluate H_2S release from the peptides in solution and gel form in the presence of thiols. To obtain H_2S release curves using real-time monitoring, we used an H_2S-selective microelectrode probe (**Figure 3**). A solution of Pep-H_2S at 20 µM, triggered with a 10-fold excess of Cys showed a steady rise in H_2S concentration up to 0.2 µM over 120 min, after which it remained steady for another 160 min. Because H_2S is constantly oxidizing and volatilizing, we typically observe peaking concentrations at values much lower than the starting concentration of the H_2S donor. We also measured release at a 10-fold higher concentration to evaluate how this increase would affect the release rate. Similar to the 20 µM case, the H_2S release profile for Pep-H_2S at 200 µM showed a gradual release with a peaking time at 130 min at a concentration of 0.5 µM. As expected, Pep-Ctrl did not show any H_2S release. We also tested release from Gel-H_2S, prepared by addition of $CaCl_2$ to H_2S-Pep, into a large

volume of PBS. Release was slow and steady and continued rising slowly over 220 min up to a peak concentration of 1. µM before tapering off. As measuring H_2S release from Pep-H_2S and Gel-H_2S required different conditions, the peaking times and concentrations cannot be directly compared; however, the peak shapes for the peptide under both solution and gel conditions show slow and steady release, which we expect may be ideal for localized delivery of H_2S.

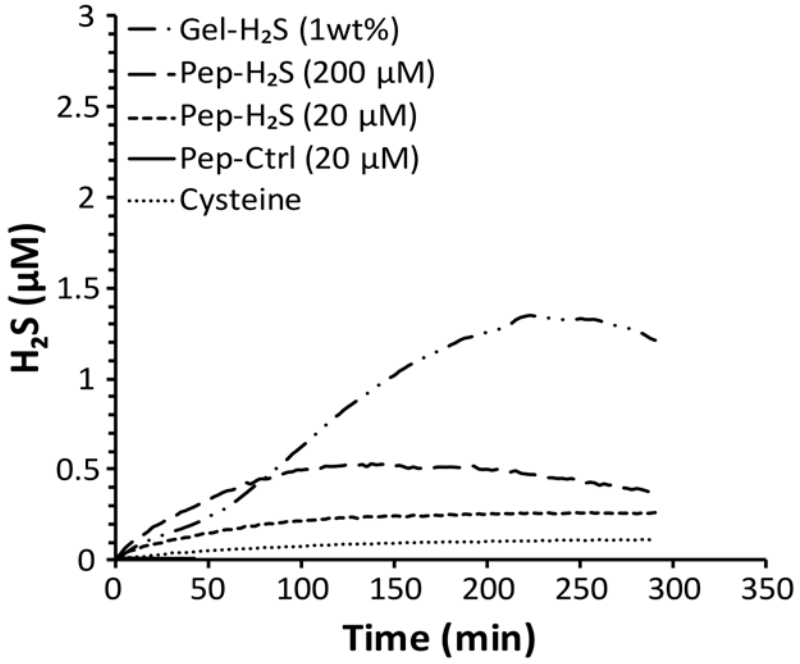


Figure 3: The H_2S-releasing peptide under both solution (Pep-H_2S) and gel (Gel-H_2S) conditions shows slow and steady release in presence of cysteine

H_2S release curves measured on an H_2S-selective electrochemical probe comparing Pep-H_2S (20 µM and 200 µM), Pep-Ctrl, and Gel-H_2S.

SF_7-AM signal in VSMC and HUVEC upon addition of Pep-Ctrl and Pep-H_2S.

Live VSMC or HUVEC were incubated with the fluorescent H_2S probe SF_7-AM, and fluorescence was measured continuously before and after addition of 200 µM Pep-Ctrl, Pep-H_2S or 100 µM NaHS. Experiments were performed in VSMC or HUVEC culture media containing 200 µM L-

Cys. No additional thiol was added to trigger H_2S release. Time-lapse imaging of SF_7-AM in VSMC demonstrated that, in the no treatment condition or in cells exposed to Pep-Ctrl, VSMC produced little endogenous H_2S, as evidenced by the slow and low buildup of the SF_7-AM signal (**Figure 4A**). In contrast, the rate of endogenous H_2S production was two-fold higher in HUVEC in the no treatment condition and in cells treated with Pep-Ctrl, as compared to VSMC (**Figure 4BC**). As expected, addition of NaHS rapidly raised the SF_7-AM signal to similar levels in both cell types, while Pep-H_2S induced a 4-fold slower release in both cell types (**Figure 4C**). Of note, 200 µM Pep-H_2S was not sufficient to surpass significant endogenous SF_7-AM signal in HUVEC, but it significantly raised the signal in VSMC above controls (**Figure 4C**). 20 µM Pep-H_2S was not sufficient to generate detectable amounts of H_2S in either cell type (data not shown).

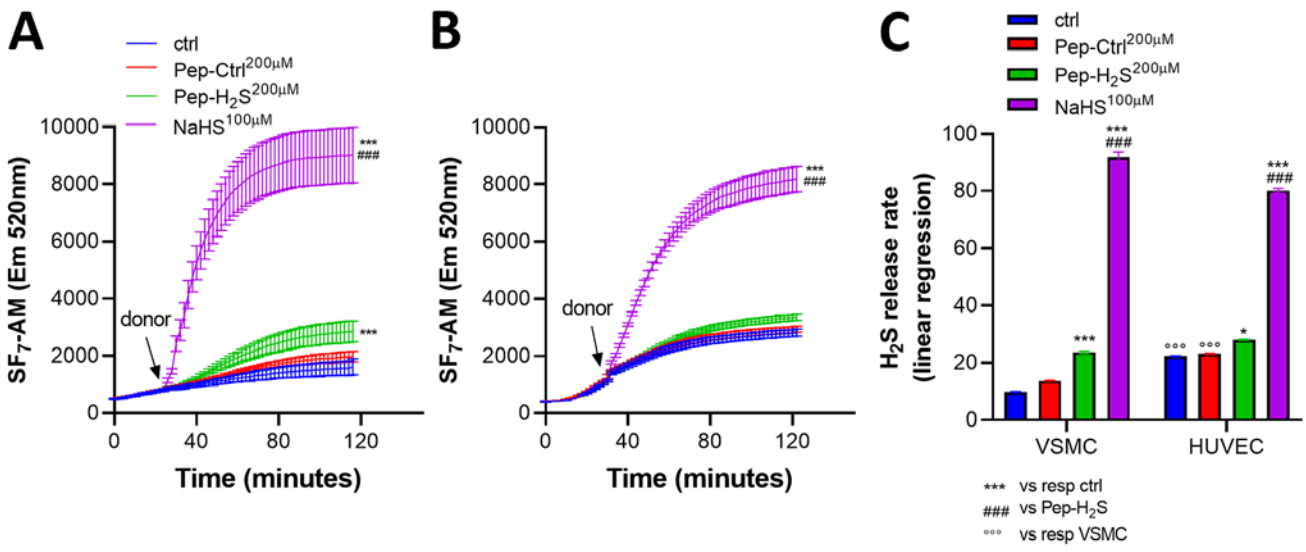


Figure 4: The peptide in solution show slow and steady release in cell culture

SF_7-AM fluorescent signal in live primary human vascular smooth muscle cells (VSMC; **A**) or human umbilical vein endothelial cells (HUVEC; **B**) cultures exposed or not (Ctrl) to 100 µM NaHS, 200 µM Pep-Ctrl or 200 µM Pep-H_2S for the indicated time. **C**) Linear regression of SF_7-AM fluorescence curves to estimate H_2S-releasing rates. Data are mean ± SEM of 4 independent experiments. *P<0.05, ***P<0.001 vs. respective ctrl; $^{###}$P<0.001 vs. Pep-H_2S; °°°p<0.001 vs.

respective condition in VSMC, as determined by two-way ANOVA with post-hoc t-test with Tukey's correction for multiple comparisons.

Ex vivo treatment with the H_2S-releasing gel prevented development of IH in human saphenous vein segments

The soft and robust nature of Gel-H_2S and its sustained release profile led us to explore its application in vascular diseases. To this end, we obtained human vein segments, which were placed in culture for 7 d with or without Gel-H_2S or Gel-Ctrl (10-20 µL gel/mL media). No additional L-Cys was added to trigger H_2S release as the cell culture media (RPMI) contains 200 µM L-Cys. NaHS was also tested as a positive control of exogenous H_2S supply. Gels (10 or 20 µL/mL, equivalent to ~100 or ~200 µM sulfide, respectively) or NaHS (100 µM) were reapplied upon cell culture media change every 48 h.

Histomorphometric analysis of intima and media thickness after 7 d in culture revealed that Gel-H_2S performed similarly to NaHS, with both fully preventing the development of IH, whereas the control gel had no effect (**Figure 5**). For this analysis, we measured the thickness of the intima and media in each vein and calculated the intima over media (I/M) thickness to correct for variations in media thickness. Treatment of human vein segments with Gel-H_2S (10 and 20 µL/mL) and 100 µM NaHS led to a 2-fold reduction in both intima thickness and I/M ratio, while Gel-Ctrl had no effect (**Figure 5**). Importantly, treatment with Gel-H_2S, Gel-Ctrl, or NaHS did not affect the media thickness, suggesting no cytotoxic effect of exogenous H_2S treatment.

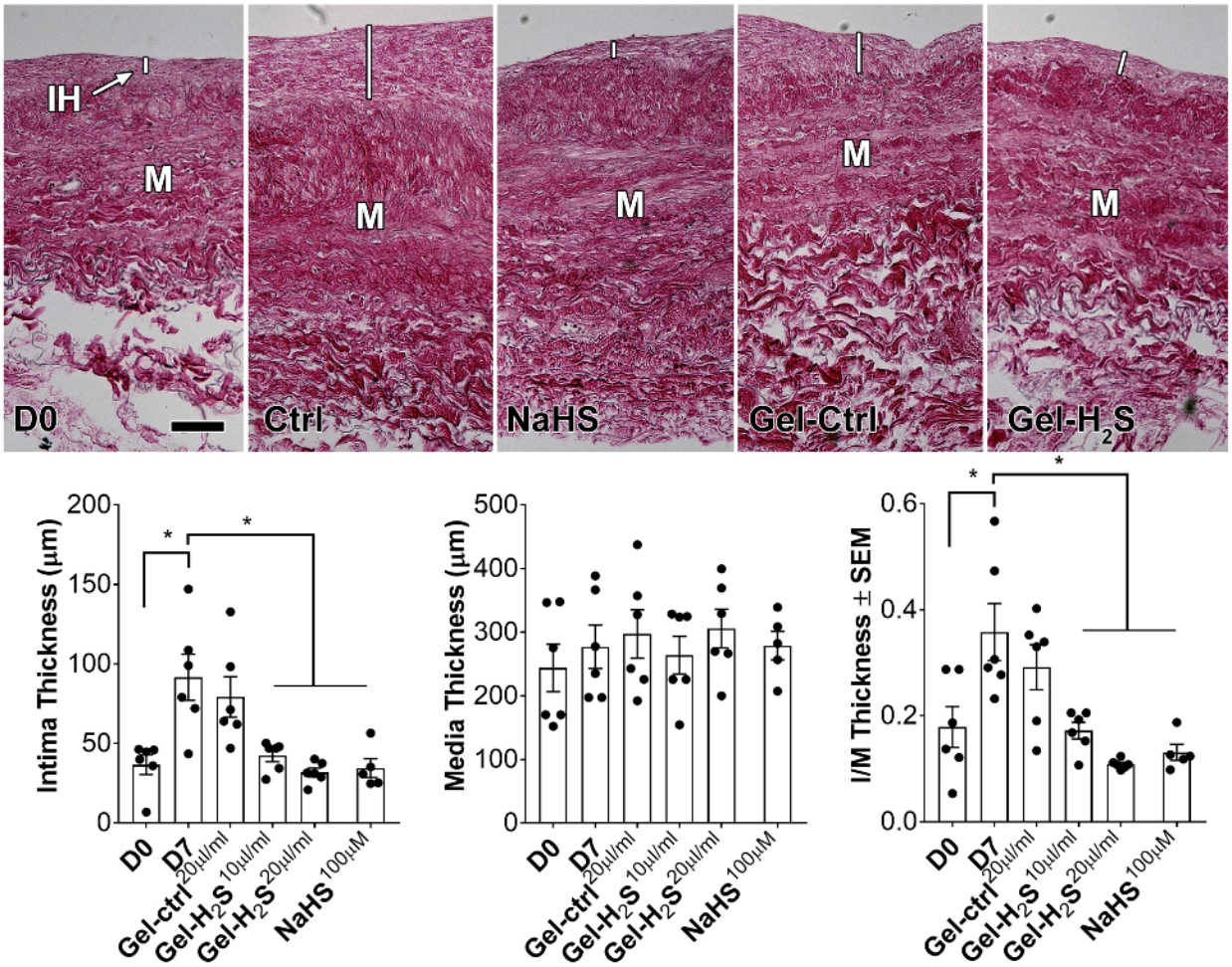


Figure 5: Ex-vivo treatment with the H_2S-releasing gel prevents the development of IH in human saphenous vein segments

Human great saphenous vein segments obtained from donors who underwent lower limb bypass surgery were put or not (D0) in static culture for 7 days in presence or not (D7) of NaHS (100 µM), Gel-Ctrl (20 µL/mL) or Gel-H_2S (10 or 20 µL/mL). Media was changed every two days, with fresh dilutions of H_2S donors. Scale bar represents 200 µm. Data are shown as a scatter plots with mean±SEM of media, intima thicknesses and intima/media ratio (I/M) in 4 vein segments. *P<0.05 vs D7 as determined by one-way ANOVA with post-hoc t-test with Dunnet's correction for multiple comparisons.

Next, we analyzed cell proliferation in the vein samples using PCNA immunostaining. As expected by the increase in neointima generated after 7 days in static culture (**Figure 5**), we

observed a two-fold increase in cell proliferation, mostly localized in the media layer, compared to baseline control (day 0) (**Figure 6**). This increase was prevented by Gel-H_2S, but not by Gel-Ctrl, suggesting that H_2S inhibited VSMC proliferation associated with the formation of IH.

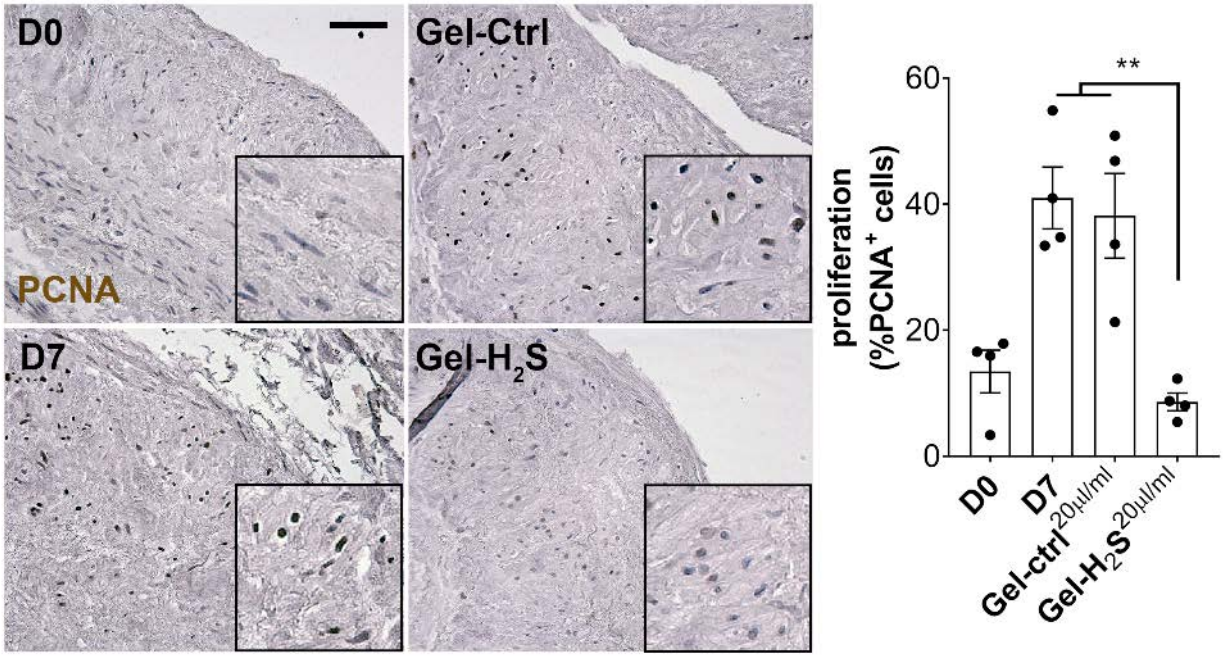


Figure 6: Ex-vivo treatment with the H_2S-releasing gel reduces cell proliferation

Human great saphenous vein segments were put or not (D0) in static culture for 7 days (D7) in presence of Gel-Ctrl (20 µL/mL) or Gel-H_2S (20 µL/mL). Media was changed every two days, with fresh preparation of Gel. Scale bar represents 150 µm. Insets are 3x magnification of main image. Data are shown as scatter plots with mean±SEM of PCNA immunostaining (brown), counterstained with hematoxylin (blue) in 4 distinct veins. **P<0.01 vs D7 as determined by one-way ANOVA with post-hoc t-test with Dunnet's correction for multiple comparisons.

The H_2S-releasing peptide/gel decreased VSMC proliferation and transmigration

To understand further the cell-based effects of Pep-H_2S and Gel-H_2S, we examined human EC and VSMC proliferation and migration *in vitro*. IH is triggered by vessel injury, which leads to EC dysfunction and eventually death. The absence of ECs promotes VSMC dedifferentiation,

proliferation, and migration, leading to IH. Therefore, we first examined how Pep-H_2S and Gel-H_2S affected VSMCs in culture.

In line with our *ex-vivo* data on human vein segments, *in vitro* studies of VSMCs derived from human vein segments revealed that Pep-H_2S inhibited cell proliferation in a dose-dependent manner, while Pep-Ctrl had no effect (**Figure 7A**). Importantly, 10 μM Pep-H_2S had a similar effect as 100 μM NaHS. Media thickness data in human veins (**Figure 5**) suggested that the donors had no cytotoxic effect on VSMCs. To further test this observation, *in vitro* assessment of cell apoptosis using Hoechst-propidium iodide staining was conducted. The results confirmed that Pep-H_2S, Pep-Ctrl, and NaHS had no toxic effect on VSMCs after 48 h of exposure (**Figure 7B**). Thapsigargin (TG) was used as a positive control to induce apoptosis (**Figure 7B**). Finally, using a transmigration assay in Boyden chambers, we observed that, as compared to the pre-conditioned EC culture medium alone ($Ctrl^+$), the addition of NaHS or Gel-H_2S (10 μL/mL), but not Gel-Ctrl, inhibited VSMC transmigration. Interestingly, Gel-H_2S was significantly more potent than NaHS, fully blocking VSMC transmigration (**Figure 7C**).

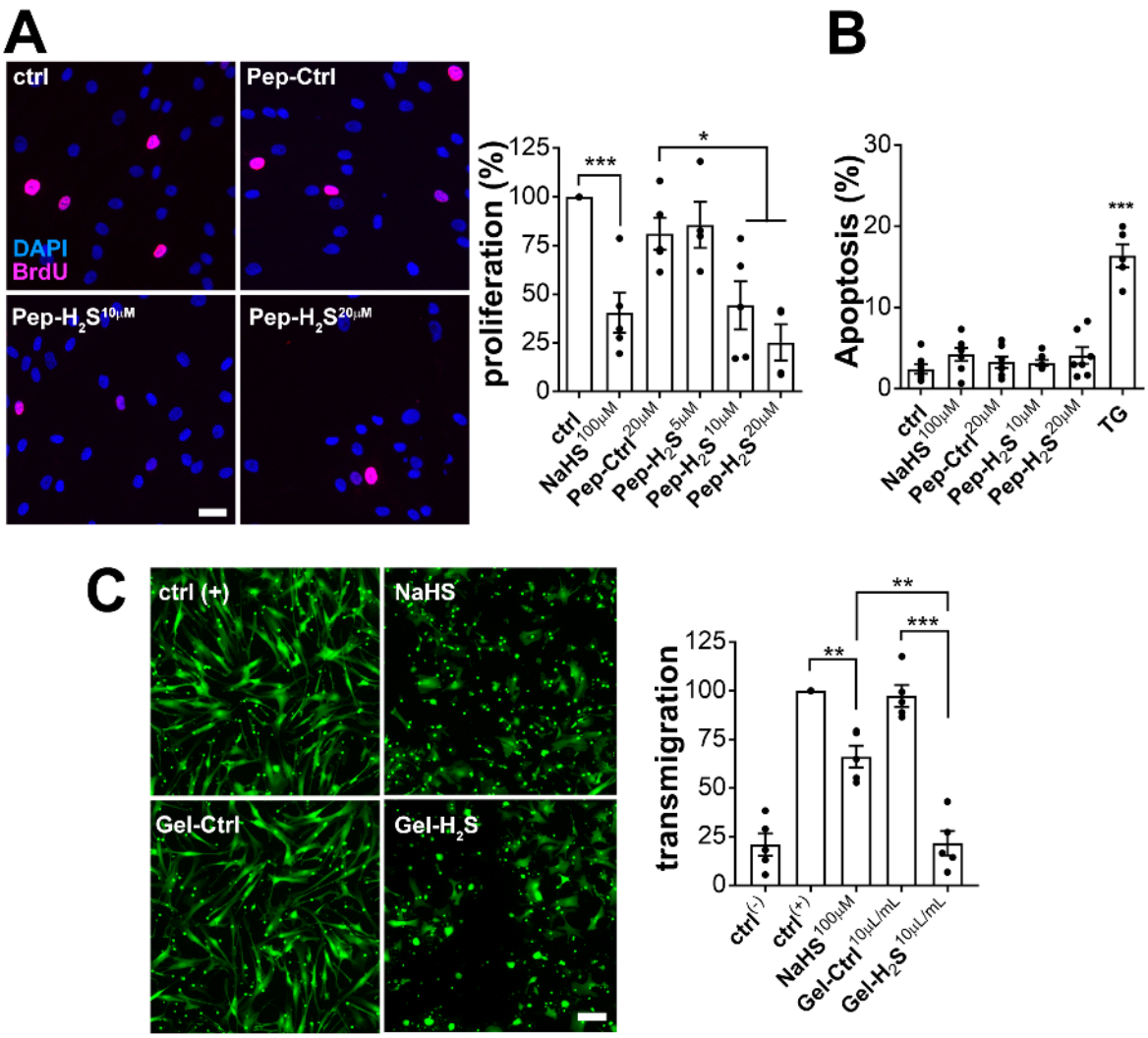


Figure 7: The H_2S-releasing gel inhibits VSMC proliferation and migration

A) Primary human vascular smooth muscle cells (**VSMC**) were exposed or not (Ctrl) to 100 µM NaHS, the control peptide (Pep-Ctrl) or the H_2S-releasing peptide (Pep-H_2S) for 24 h in presence of BrdU. Scale bar represents 20 µm. Proliferation was calculated as the ratio of BrdU-positive nuclei over total DAPI-stained nuclei and expressed as % of proliferation in ctrl condition**. B**) VSMC apoptosis levels after a 48 h exposure to 100 µM NaHS, the control peptide (Pep-Ctrl) or the H_2S-releasing peptide (Pep-H_2S), or a 24 h exposure to 100 nM thapsigargin as positive control. **C)** VSMC transmigration through an artificial membrane toward pre-conditioned medium of HUVECs (EGM-2 medium) supplemented or not ($ctrl^{(+)}$) with 100 µM NaHS, 10 µL/mL of Gel-Ctrl or Gel-H_2S for 16 h. Scale bar represents 50 µm. All data are scatter plots with mean ±

SEM. *P<0.05, **P<0.01, ***P<0.001 vs ctrl$^{(+)}$ as determined by one-way ANOVA with post-hoc t-test with Tukey's correction for multiple comparisons.

We further studied VSMC phenotype. Western blot analyses of calponin and SM22α, two VSMC-specific proteins, did not reveal major changes, although calponin levels tended to decrease in VSMCs exposed to the highest dose of Pep-H_2S (20µM), but not Pep-Ctrl (**Figure 8A, B**). However, immunocytochemistry analysis revealed that NaHS and Pep-H_2S (20µM), disrupted the typical cytoskeleton staining for calponin and SM22α (**Figure 8C**), which may account for the reduced proliferation and mobility of VSMCs exposed to H_2S.

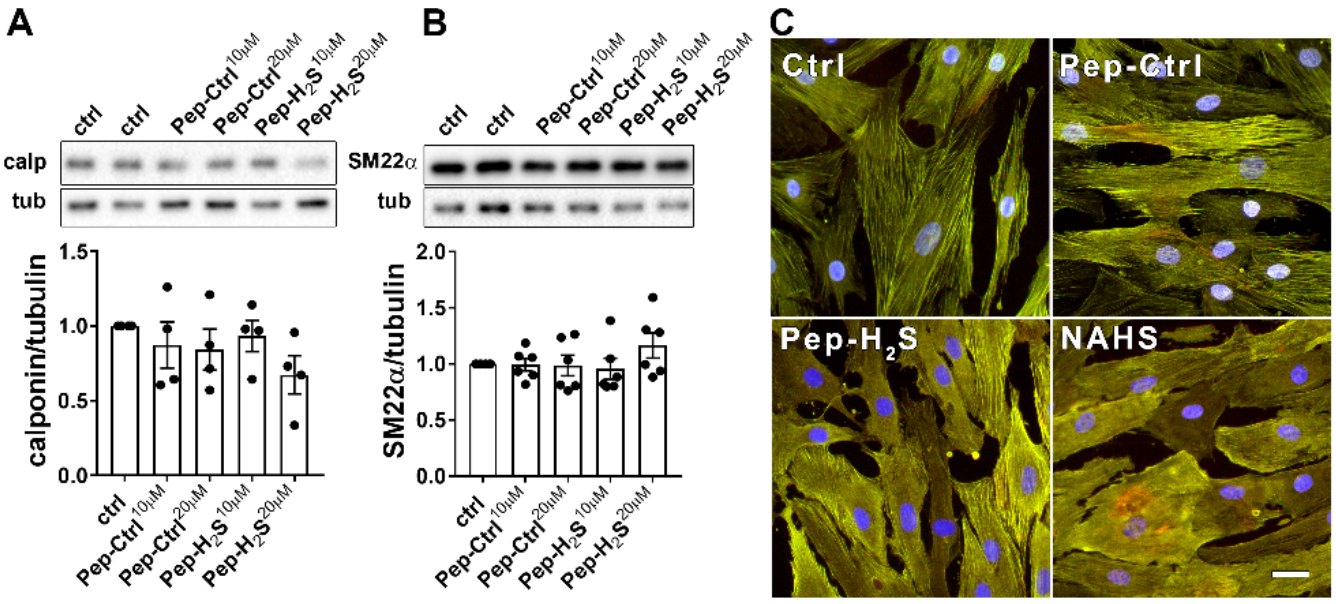


Figure 8: The H_2S-releasing peptide disrupts the VSMC cytoskeleton

Primary human vascular smooth muscle cells (**VSMC**) were exposed or not (ctrl) to NaHS (100 µM), the control peptide (Pep-Ctrl) or the H_2S-releasing peptide (Pep-H_2S) for 24 h. **A, B**) WB analysis of calponin (**A**) and SM22α (**B**), over tubulin levels. **C**) VSMC immunofluorescent staining for calponin (red), SM22α (green), and nuclei (DAPI staining in blue). Images are overlays of the 3 channels, representative of 5 independent experiments. Scale bar represents 20 µm.

The H_2S-releasing peptide/gel increased EC proliferation and transmigration

We then performed similar experiments on human endothelial cells (HUVECs). H_2S promotes EC proliferation, migration, and angiogenesis [38]. As expected, NaHS increased HUVEC proliferation. Interestingly, Pep-H_2S was a more potent stimulator of HUVEC proliferation than NaHS, effective at a 10-fold lower dose than NaHS (**Figure 9A**). We also tested HUVEC transmigration toward full HUVEC culture medium. Consistently, and in contrast with VSMCs, NaHS and the Gel-H_2S (10 µL/mL) promoted HUVEC transmigration (**Figure 9B**).

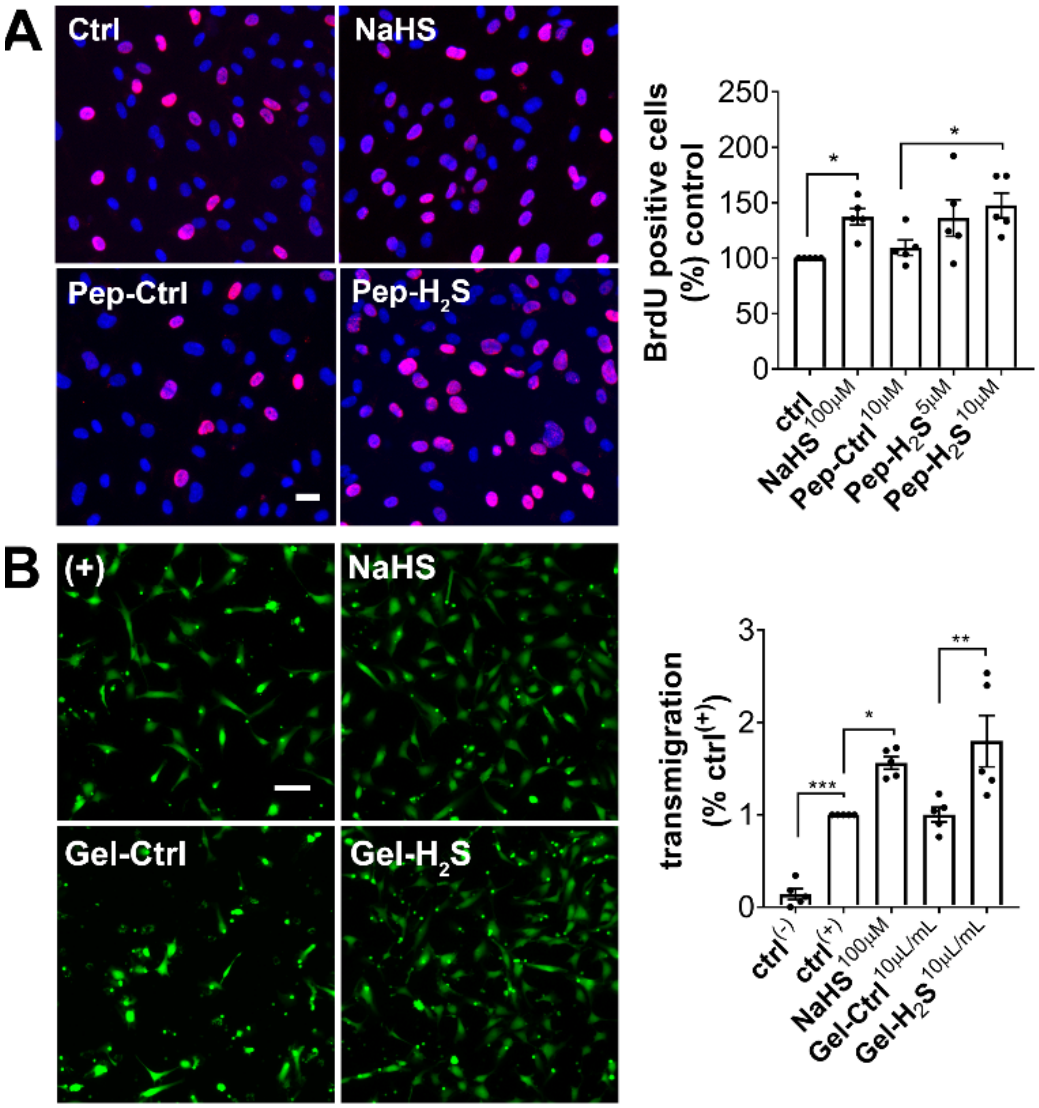


Figure 9: Gel-H_2S promotes HUVECs proliferation and migration

A) Primary human endothelial cells (HUVEC) were exposed or not (ctrl) to 100 µM NaHS, the control peptide (Pep-Ctrl) or the H_2S-releasing peptide (Pep-H_2S) for 8h in presence of BrdU. Proliferation was calculated as the ratio of BrdU-positive nuclei over total DAPI-stained nuclei and expressed as % of proliferation in ctrl condition. Bar scale represents 20 µm. **B)** HUVEC transmigration through an artificial membrane toward EGM-2 medium supplemented or not (+) with 100µM NaHS, 10 µL/mL of Gel-Ctrl or Gel-H_2S for 6h. Data are mean ± SEM of calcein-AM fluorescence signal measured on a fluorescence plate reader (λ_{ex} = 495 nm; λ_{em} = 517 nm). Scale bar represents 50 µm. All data are scatter plots with mean ± SEM. *P<0.05, **P<0.01, ***P<0.001 vs ctrl$^{(+)}$ as determined by one-way ANOVA with post-hoc t-test with Dunnet's correction for multiple comparisons.

DISCUSSION AND CONCLUSIONS

In this work, we developed and evaluated the therapeutic potential of a new H_2S-releasing hydrogel to limit the development of IH in human veins. Previous studies in animal models have reported protection from IH with exogenous NaHS administration in rats [39], rabbits [40] and mice [41,42]. However, human evidence of H_2S donor efficacy against IH was lacking. Here, we report, for the first time in human tissue, that an exogenous H_2S donor inhibits VSMC proliferation and IH in an *ex-vivo* model of human vein culture. We further demonstrate the efficacy against IH of a novel peptide hydrogel with sustained release of low dose of H_2S.

H_2S delivery via small molecule donors is often limited by short release periods, low water solubility, and even toxicity [12]. Controlling the amount of H_2S released is critical as relatively high doses of exogenous H_2S (over 500-1000 ppm) lead to respiratory distress and death, hindering the use of H_2S medications in humans. H_2S donors release H_2S by either hydrolysis or in response to a specific trigger, such as a thiol or other nucleophiles [12]. Here, we employed a thiol-triggered donor motif, which adds a level of control to the release and ensures more sustained release compared to passive hydrolysis. In addition, the peptide we developed, based on an aromatic peptide amphiphile, is highly soluble and self-assembles to form long nanoribbons stabilized by β-sheets, which results in slow H_2S release compared to the salt NaHS, as evidenced by H_2S release profiles showing steady release in the range of hours, in stark contrast to the instantaneous release of H_2S from NaHS. Importantly, Pep-H_2S reduces IH at a 10-fold lower total sulfide dose than NaHS, which is likely due to the slower release kinetic, leading to better H_2S bioavailability. In addition to reduced toxicity, a sustained release strategy is crucial for the prevention of IH in patients because the acute stage of IH development typically occurs during the first 30 days following the intervention [43].

Local biodegradable sheaths, wraps, meshes, membranes, and cuffs have all been tested for perivascular applications. However, these solid solutions adapt poorly to the elasticity of vascular tissue. While solid forms are more stable over time, injectable semi-solid formulations, in particular hydrogels, present exciting alternatives as they are easily positioned and provide adequate coverage of the vessel [44]. Due to their ability to self-assemble into nanoribbons, both Pep-H_2S and Pep-Ctrl spontaneously form hydrogels at 1 wt. % in aqueous solution in the presence of $CaCl_2$. Both gels are soft as measured by rheology, as is typical of self-assembled peptide gels at low concentrations. Gel-H_2S provides extended release compared to Pep-H_2S, with potentially increased efficacy in reducing IH *in-vivo* [43]. While hydrogels offer an interesting alternative to solid perivascular solutions, most current formulations involve the combination of a gel and a drug. The H_2S-releasing gel developed here has advantages over polymer-based gels because it is a fully biodegradable single small molecule with no polydispersity, and it requires no additional covalent crosslinking.

The protective effect of the H_2S-releasing peptide against IH is probably largely imparted by a direct inhibition of VSMC proliferation. Indeed, in line with previous reports [41], we show that Pep-H_2S/Gel-H_2S and NaHS specifically inhibit human VSMC proliferation and transmigration, which are important features of pathogenic synthetic VSMCs involved in IH [2]. We further observed that the H_2S-releasing peptide and NaHS disrupt the normal cytoskeleton architecture of VSMCs, as evidenced by a modified pattern of calponin and SM22α immunostaining. Given the prominent role of cytoskeleton dynamics and remodeling during mitosis and cell migration, this disrupted pattern likely contributes to reduced VSMC proliferation and migration. Whether or not the protection imparted by the donors is directly mediated by H_2S remains unknown. *S*-Aroylthiooxime compounds release H_2S in the presence of thiols with intermediate formation of

thiocysteine (cysteine persulfide) [24]. Thus, the observed biological effects of Pep-H_2S/Gel-H_2S may be attributable, at least in part, to cysteine persulfide or other related reactive sulfide species. Further studies will be conducted to identify the mechanism underlying the effects of H_2S/persulfide on the VSMC cytoskeleton.

In contrast to VSMC, EC proliferation and transmigration is stimulated by Pep-H_2S/Gel-H_2S, consistent with previous reports showing that exogenous H_2S stimulates HUVEC proliferation and migration *in vitro* [27,45] and has pro-angiogenic properties *in vivo* [46,47]. This feature is of particular interest in the context of IH. Indeed, EC dysfunction and death during vascular surgery plays a major role in the development of IH, and the pro-angiogenic effect of H_2S might accelerate endothelium recovery following vascular trauma [2]. Numerous reports have shown that H_2S increases NO synthesis and bioavailability in EC [48]. Interestingly, NO produced and released in EC plays an important role in maintaining the quiescent contractile features of VSMC [2], and many therapies have been developed based on the use of NO donors. However, low tolerance and uncontrolled hypotensive effects have plagued the therapeutic use of NO-releasing drugs [48]. Further studies will focus on measuring the effects of Pep-H_2S/Gel-H_2S on NO production and bioavailability. We believe that H_2S donor-based therapies may hold stronger therapeutic potential than NO and, given that some effects of H_2S are NO-mediated, replace failed NO-based strategies.

Currently available local therapies, such as drug-eluting stents and balloons, are coated with non-specific cytotoxic (paclitaxel) and cytostatic (sirolimus) drugs. These devices may improve long-term vessel patency when compared with standard “bare” stents and balloons [49,50]. However, their long-term effect on patients outcome remains unclear and recent evidence suggest a negative outcome for paclitaxel-coated devices and increased rate of complications such as late stent thrombosis, and delayed re-vascularization [51]. Thus, local perivascular application of an H_2S-

releasing gel might provide a unique therapeutic opportunity, with benefits on both VSMCs and ECs, without systemic toxicity. In future efforts, we will evaluate the therapeutic potential of the perivascular application of our Gel-H_2S at the site of vascular trauma following surgery in animal models of IH. Overall, we propose that application of such H_2S-releasing self-assembling peptide hydrogels may constitute a viable solution to limit IH in human vein grafts.

Data Availability: The raw/processed data required to reproduce these findings cannot be shared at this time due to technical or time limitations

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