





ORIGINAL RESEARCH

Periprocedural Hydrogen Sulfide Therapy Improves Vascular Remodeling and Attenuates Vein Graft Disease

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BACKGROUND: Failure rates after revascularization surgery remain high, both in vein grafts (VG) and arterial interventions. One promising approach to improve outcomes is endogenous upregulation of the gaseous transmitter-molecule hydrogen sulfide, via short-term dietary restriction. However, strict patient compliance stands as a potential translational barrier in the vascular surgery patient population. Here we present a new therapeutic approach, via a locally applicable gel containing the hydrogen sulfide releasing prodrug (GYY), to both mitigate graft failure and improve arterial remodeling.

METHODS AND RESULTS: All experiments were performed on C57BL/6 (male, 12 weeks old) mice. VG surgery was performed by grafting a donor-mouse cava vein into the right common carotid artery of a recipient via an end-to-end anastomosis. In separate experiments arterial intimal hyperplasia was assayed via a right common carotid artery focal stenosis model. All mice were harvested at postoperative day 28 and artery/graft was processed for histology. Efficacy of hydrogen sulfide was first tested via GYY supplementation of drinking water either 1 week before VG surgery (pre-GYY) or starting immediately postoperatively (post-GYY). Pre-GYY mice had a 36.5% decrease in intimal/media+adventitia area ratio compared with controls. GYY in a 40% Pluronic gel (or vehicle) locally applied to the graft/artery had decreased intimal/media area ratios (right common carotid artery) and improved vessel diameters. GYY-gel-treated VG had larger diameters at both postoperative days 14 and 28, and a 56.7% reduction in intimal/media+adventitia area ratios. Intimal vascular smooth muscle cell migration was decreased 30.6% after GYY gel treatment, which was reproduced in vitro.

CONCLUSIONS: Local gel-based treatment with the hydrogen sulfide-donor GYY stands as a translatable therapy to improve VG durability and arterial remodeling after injury.

Key Words: hydrogen sulfide ■ vascular remodeling ■ vein graft disease

In lower extremity bypass surgery, autologous vein grafts remain the superior choice to achieve successful revascularization.¹ Although success rates are surpassing other conduits,² vein graft primary patency nevertheless falls toward 60% 1 year postintervention³ with vein graft disease (VGD) as the main effector in prompting graft occlusion mid- to long-term

post implantation.⁴ The intimal hyperplasia seen in VGD, that is, thickening of the intimal wall by vascular smooth muscle cell (VSMC) growth,⁵ also drives open and endovascular arterial intervention failure. Taken together both these components render the vascular fibroproliferative response to injury a major unaddressed clinical challenge in cardiovascular surgery practice.

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CLINICAL PERSPECTIVE

What Is New?

- This work evaluates a gel containing a slow-releasing hydrogen sulfide prodrug that can be applied locally and directly onto the artery or graft during the surgical procedure.
- One-time periprocedural application of this hydrogen sulfide gel limits arterial intimal hyperplasia and attenuates vein graft disease.

What Are the Clinical Implications?

- High failure rates after (cardio)vascular surgery interventions remain a significant clinical problem, with adverse vascular remodeling as a major contributor to high failure rates.
- This one-time, local, and periprocedural approach holds significant translational potential in improving vascular remodeling after (cardio)vascular surgery operations.

Nonstandard Abbreviations and Acronyms

DR	dietary restriction
GYG	hydrogen sulfide releasing prodrug
H₂S	hydrogen sulfide
I/M+A	intimal/media+adventitia
NaHS	sodium hydrosulfide
RCCA	right common carotid artery
SF₇-AM	fluorescent probe binding free hydrogen sulfide
VGD	vein graft disease

An emerging scientific field holding translational promise employs the concept of preconditioning of the patient, before surgery, against surgical stress/injury via short-term dietary restriction (DR). The efficacy of DR, including the restriction of calories, proteins, or specific amino acids, is established in a wide range of preclinical surgical models, including surgical trauma,^{6,7} renal^{8,9} and hepatic^{8,10,11} ischemia-reperfusion injury, focal stroke in the brain,¹² poststenosis arterial hyperplasia,¹³ and hindlimb ischemia,¹⁴ without decelerating postinterventional wound healing processes.¹⁵

One mechanism by which DR derives its benefit is via increased production of endogenous hydrogen sulfide (H₂S),¹⁶ a gaseous vasodilator and transmitter molecule¹⁷ with anti-inflammatory and cytoprotective potential.¹⁸ In the vasculature, H₂S is mainly enzymatically derived, with cystathionine γ -lyase being the most abundant H₂S producing enzyme.¹⁹ In the

setting of vascular injury in rodent models, DR-induced augmentation of endogenous H₂S protects from ischemia-reperfusion injury²⁰ and accelerates neovascularization after hindlimb ischemia.¹⁴ Genetic (cardiac-specific) overexpression of cystathionine γ -lyase protects from heart failure after transverse aortic constriction,²¹ while knockdown of the same gene leads to increased neointima formation after carotid ligation, via increased VSMC migration.²²

In venous bypass surgery, DR-induced increased endogenous H₂S production (via cystathionine γ -lyase up-regulation) attenuates inward remodeling and improves vein graft adaptation to its arterial environment, partly via limiting both VSMC migration and neutrophil transmigration toward the intimal layer.²³ Upregulation of endogenous H₂S therefore possesses the capability to improve outcomes in various settings of (vascular) surgical injury. Nonetheless, translational barriers remain, not least of which includes the requirement for planning of the dietary intervention, as we found in a recent pilot study of DR in patients scheduled for vascular surgery.²⁴

Thus DR-mimetic drugs, including direct H₂S administration, are an attractive alternative approach. Although systemic treatment with various H₂S donors shows therapeutic potential in a wide range of preclinical disease models,²⁵ including a single dose immediately before hepatic ischemia reperfusion injury,¹⁶ its potential in vascular reconstructions has yet to be fully explored. Furthermore, the ability to use local delivery may address potential safety issues regarding systemic treatments, thereby improving clinical translatability. Here, we tested the potential of H₂S to mitigate intimal hyperplasia and VGD, using a single, local periprocedural application.

METHODS

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Experimental Animals

All animal experiments were approved by the appropriate Harvard Medical Area or Brigham and Women's Hospital Institutional Animal Care and Use Committee (04475 and A4752-01 respectively) and in accordance with the NIH guidelines. All surgical experiments were performed on C57BL/6 mice (male, 10–12 weeks old, Stock No: 000664, Jackson Laboratory), fed a high-fat diet (Research Diets D12492, 60% fat) during the experiment. Mice were housed 4 to 5 per cage and maintained on a 12-hour light-dark cycle at 22°C with 30% to 50% humidity. All in-vivo experiments were conducted in male mice to limit intergroup variability while assessing

intervention effectiveness, thereby lowering the number of experimental animals needed.

Vein Graft Surgery

Vein graft surgery was performed as described previously.²⁶ Briefly, mice were anesthetized with 5% isoflurane and maintained under 2% to 3% isoflurane via a nose cone for the duration of the procedure. The mouse was placed on a heating pad to maintain perioperative body temperature. After removal of fur in the neck region a neckline incision was performed. After dissection of the right common carotid artery (RCCA) from its surrounding tissues, the artery was ligated with an 8-0 nylon suture. Vascular clamps were placed at the proximal and distal arterial ends and the carotid wall was everted over a polyetheretherketone cuff. The thoracic inferior caval vein from a donor mouse was harvested just before the start of the vein graft procedure in the recipient and stored in ice cold sterile 0.9% NaCl supplemented with heparin (100 UI/mL). After everting the carotid artery of the recipient over the cuff, the donor caval vein was sleeved and then sutured over both ends of the cuff with an 8-0 nylon suture, creating an end-to-end anastomosis. After grafting of the caval vein, vascular clamps were removed, and blood flow was restored. The incision was closed with 6-0 Vicryl sutures. Postoperatively animals received warm lactate ringer solution (0.5 mL, subcutaneous) and buprenorphine (0.1 mg/kg, subcutaneous).

Focal Carotid Stenosis Procedure

A focal stenosis was created as described previously to generate a hemodynamically induced arterial intimal hyperplastic response in the setting of flowing blood.²⁷ Briefly, mice were anesthetized with 5% isoflurane and maintained under 2% to 3% isoflurane via nose cone for the duration of the procedure. After dissection of the RCCA from its surrounding tissues, a 35-gauge blunt needle mandrel was placed longitudinally along the RCCA and tied with a 9-0 nylon suture approximately 2 to 2.5 mm proximal to the bifurcation. The needle mandrel was then removed and skin closed with a 6-0 Vicryl suture. Postoperatively mice received warm lactate ringer solution (0.5 mL, subcutaneous) and buprenorphine (0.1 mg/kg, subcutaneous).

Systemic Treatment with GYY4137

For systemic treatment, mice were randomized in pre-GYY, post-GYY, and control groups. Based on our previous work,¹⁶ in the pre-GYY treatment group, 250 $\mu\text{mol/L}$ GYY4137 (Sigma, cat# SML0100) (GYY) was supplied in the drinking water for 1 week preceding vein graft surgery and replaced fresh after

3 days. Immediately post surgery, drinking water was replaced with regular (without GYY). In the post-GYY treatment group, 250 $\mu\text{mol/L}$ GYY was supplemented in the drinking water immediately postoperative until harvest at postoperative day 28 and replaced weekly.²⁸

Periprocedural Local Treatment With GYY

For periprocedural local treatment with GYY, a 40% Pluronic gel (Sigma, cat# P2443) was created the day before surgery (40% w/v, 4-g Pluronic gel, 10 mL sterile 0.9% NaCl) and kept at 4°C overnight under continuous stirring. Mice were randomized into GYY treatment or vehicle groups. Ten minutes before application of the gel onto the vessel/graft, GYY was dissolved into a stock solution (0.9% NaCl) and resuspended in 100 μL of 40% Pluronic gel to a final concentration of 250 $\mu\text{mol/L}$. During the focal stenosis procedure, 100 μL gel containing GYY or vehicle only was applied immediately after stenosis creation. During bypass surgery, the gel (GYY or vehicle) was applied onto the caval vein after it was grafted into the RCCA but before opening the proximal and distal vascular clamps. Immediately after enveloping the graft with the gel, vascular clamps were opened, and blood flow was restored.

Vein Graft/Focal Carotid Stenosis Harvest

Animals were anesthetized (induction under 5% isoflurane, 1–2% continued isoflurane to maintain anesthesia), anesthetic depth was confirmed by toe pinch. A percutaneous cardiac puncture was performed to obtain whole blood, followed by a thoracotomy. Mice were then euthanized via exsanguination by cutting and removing the caval vein. Perfusion was performed with lactate Ringers solution via the left ventricle for 3 minutes, then switched to 3 minutes of perfusion-fixation with 10% formalin. To harvest the graft/RCCA, a midline neck incision was made, the graft/RCCA was excised en-block and fixed in 10% formalin for 24 hours. After 24 hours the tissue was transferred to a 70% ethanol solution and then processed for paraffin embedding.

Graft and Artery Processing for Histology

After paraffin embedding, both vein grafts and RCCA were cut with a microtome in 5 μm sections and mounted on slides. Grafts were sectioned at regular intervals of 200 μm , starting from the proximal cuff until 1000 μm post proximal cuff. Arteries were cut at regular intervals of 200 μm , starting at 200 μm proximal from the focal stenosis, until 2800 μm proximal from the stenosis. After section cutting, a Masson-trichrome histology staining was performed. In short, slides were deparaffinized to 95% ethanol, 3 minutes in 5% picric acid (in 95% ethanol), tap water

wash, 3 minute stain in working Harris Hematoxylin Solution (Fisher Scientific, cat# 245–678), tap water wash, stained with 1% Biebrich Scarlet in 1% acetic acid (Fisher Scientific, cat# A38S-500) for 3 minutes, quick rinse in distilled water, 1 minute stain in 5% Phosphomolybdic/Phosphotungstic acid solution then stained with 2.5% light green SF yellowish in 2.5% acetic acid (Fisher Scientific, cat# A38S-500) for 4 minutes, rinsed in distilled water and rinsed in 1% acetic acid solution (Fisher Scientific, cat# A38S-500) for 2 minutes. After dehydration with Xylene slides were covered with a cover glass employing Permount (Electron Microscopy Science, cat# 17986-05).

Immunohistochemistry

For fluorescent immunohistochemistry, slides were first preheated to 60°C in a vacuum oven for 30 minutes. Afterwards, slides were immediately deparaffinized (Xylene 3×5 minutes, 100% ethanol 3×5 minutes, 70% ethanol 5 minutes, 50% ethanol 5 minutes, deionized water 5 minutes). After deparaffinization, antigen retrieval was performed for 30 minutes at 97°C in citrate buffer (pH 6.0 in PBS, Abcam, ab93678) and slides were cooled to room temperature (RT) afterwards. Next, slides were incubated with 10% goat serum (Life Technologies, cat# 50062Z), in PBS with 0.3 mol/L glycine (Aijnomoto, cat# R015N0080039), for 1 hour at RT. Consecutively, slides were incubated with primary antibodies for smooth muscle cell- α (SMC- α ; mouse anti-mouse, Abcam, ab7817, 1:800 dilution) and Ki-67 (Ki-67 (rabbit anti-mouse, Abcam, ab16667, 1:100 dilution) o/n at 4°C for SMC- α – Ki-67 double staining. The next day, slides were washed 3 times in PBS+0.05% tween and incubated in secondary antibody for 2 hours at RT. For SMC- α +Ki-67 double staining, slides were incubated with Alexa Fluor 647 (goat anti-mouse, A-32728, 1:600 dilution) and Alexa Fluor 568 (goat anti-rabbit, A-11011, 1:600 dilution). After secondary antibody incubation, slides were washed in PBS+0.05% tween 3×5 minutes and mounted with DAPI (Vector, CB-1000) and imaged by confocal microscopy.

Histology and Immunohistochemistry Analyses

All histology and immunohistochemistry analysis were done by a blinded observer. Vein grafts were excluded from analysis in case of complete occlusion of the graft/RCCA at postoperative day (POD) 28. Based on our previous vein graft experiments with endogenous H₂S treatment,²³ our initial systemic GYY experiments were conducted with n=10/group. After systemic GYY experiments (n=10/group), 2 control, 2 pre-GYY, and 2 post-GYY vein grafts were occluded at POD 28. Observed differences between experimental groups after systemic GYY treatment led us to increase group size from

n=10 to n=18 for local (one-time) treatment with GYY. For local GYY experiments (n=18/group), 0 vehicle and 2 local GYY vein grafts were occluded at POD 28. For focal stenosis experiments (n=10/group), 1 vehicle and 0 local-GYY RCCA were occluded at POD 28. Failure rates after both vein graft and focal stenosis experiments were comparable to previously conducted experiments by microsurgeon (~10%). Brightfield images of vein graft and carotid artery cross-sections were taken with a Zeiss Axio A1 microscope (Carl Zeiss). For vein grafts, histomorphometric analysis was performed on 5 cross-sections per vein graft (200–400–600–800–1000 μ m) using Image J 1.51p (Java 1.8.0_66). Luminal, intimal (I), medial, and adventitial (M+A) areas and circumferences were measured for each cross-section with 3 cross-sections per slide, then averaged per slide. Next, I/M+A area, intimal thickness, M+A thickness, I/M+A thickness ratios, and corrected luminal area were calculated as described previously,²⁹ to account for morphometric changes induced by vein graft processing. For collagen measurements, Masson-trichrome stained slides were processed via the color deconvolution tool in Image J and percentage of total vein graft layer occupied by the deconvoluted green channel was then calculated. For VSMC+Ki-67 analysis, images were processed in Image J 1.51p by measuring intimal and M+A total area based on DAPI positive cells, followed by color thresholding of the SMC- α positive cells. Area occupied by SMC- α was then calculated as a percentage of total area in that respective vein graft layer. Proliferating VSMCs were defined as SMC- α /Ki-67 double-positive cells, counted per vein graft layer and normalized to mm². For histomorphometric analysis of carotid artery cross-sections, luminal, I, and M circumferences and area were measured, followed by calculation of I/M area, M thickness, I/M thickness ratio, and corrected luminal diameter.

Duplex Ultrasound Biomicroscopy

A Vevo 2100 imaging system with 18 to 70 MHz linear array transducers (VisualSonics Inc., Toronto, ON, Canada) was employed for high resolution in vivo ultrasonography of vein grafts and carotid arteries poststenosis creation. At POD 14 and 28 mice were anesthetized via 5% isoflurane inhalation and maintained under 2% to 3% via nose cone inhalation, body temperature was controlled via heating pad. To measure vessel cross-dimensional sections, M-mode was employed. Three luminal axial images were taken (at proximal, mid, and distal graft/poststenosis) and mean vessel diameters were calculated.

Primary VSMC Isolation

Primary VSMCs were harvested from murine aortas as described before.³⁰ Briefly, C57BL/6 (4–6 weeks old)

mice were anesthetized (induction under 5% isoflurane, 1–2% continues isoflurane to maintain anesthesia) and a thoracoabdominal incision was performed after which the thoracic aorta was exposed. Primary euthanasia occurred via removal of the cava vein followed by exsanguination. The left cardiac ventricle was perfused with sterile saline and the perivascular adipose tissue surrounding the aorta was carefully removed with forceps and scissors. The thoracic aorta was removed and placed in Hanks Balanced Salt Solution (Thermo Fisher Scientific, cat# 14025076) with 1% penicillin/streptomycin (Corning, cat# 30-002-CL) on ice. After a brief wash in PBS, aortas were then placed in 6-well plates (n=2 per well) in enzyme digestion solution consisting of 1 mg/mL Collagenase type 2 (Gibco, cat# 17101015), 0.24 mg/mL Elastase (Worthington Biochemical, LS002279), 1 mg/mL Soybean Trypsin Inhibitor (Worthington Biochemical, LS0033570), 1% penicillin/streptomycin in Hanks Balanced Salt Solution at 37°C/5% CO₂ for 10 minutes. After the first digestion the aortas were transferred to another 6-well well containing Dulbecco's Modified Eagle Medium (DMEM) (Thermo Fisher Scientific, cat#10566016), 20% CCS, 1% pen/strep and washed briefly. Next, employing forceps, the adventitia layer of the aorta was carefully everted over the intimal and medial layer of the aorta and removed. After removal of the adventitial layer, the aorta was cut longitudinally, and the endothelium carefully scraped off with angled forceps. The artery was then placed in enzyme digestion solution and incubated for 60 minutes at 37°C/5% CO₂. After the second digestion, the resulting single cell suspension was washed with DMEM (20% CCS, 1% pen/strep), centrifuged for 5 minutes at 1200 rpm/4°C, resuspended in complete DMEM and plated in 24-well plates (Falcon, cat#353047). Cells were passaged at 90% confluency and used for subsequent assays at passage 3-8.

Human Umbilical Endothelial Cell Isolation and Culture

Cords were collected from full-term pregnancies and stored in sterile PBS at 4°C and subsequently used within 7 days. A cannula was inserted in the vein and flushed with sterile PBS. The vein was infused with 0.075% collagenase type II (Worthington, Lakewood, NJ, USA) and incubated for 20 minutes at 37°C. The collagenase solution was collected, and the vein was flushed with PBS. The cell suspension was centrifuged at 1200 rpm for 5 minutes and the pellet was resuspended in complete culture medium, EGTM-2 Endothelial Cell Growth Medium-2 (BulletKit, Lonza). The cells were cultured in plates coated with fibronectin from bovine plasma (Sigma).

Cells were maintained at 37°C in a humidified 5% CO₂ environment. Culture medium was refreshed every 2 to 3 days. Cells were grown until cobblestone morphology was reached. Cells were passed 1:3 using trypsin-EDTA (Sigma) and human umbilical endothelial cell (HUVECs) were used up to passage three.

MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide) Assay

HUVEC and murine VSMCs, both p3-4, were seeded in gelatin coated 96-well plates (0.1% gelatin, Sigma, G9391) in complete medium and left to adhere overnight. Then, 125 to 250 to 500 to 1000 µmol/L solutions of GYY4137 were prepared fresh in complete medium and supplemented in 6 replicate wells per cell type. After 2, 6, and 12 hours of incubation with GYY, 10 µL of MTT (5 mg/mL) was added per well and left to incubate for another 4 hours. After 4 hours of MTT incubation, supernatant was removed and replaced with 75 µL of isopropanol/hydrochloride and incubated for 1.5 hours. Using a plate reader, 96-well plates were then read at an optical density of 590 nm.

Live-Cell Imaging and Analysis

HUVEC and murine VSMCs, both p3-4, were seeded in gelatin-coated, tissue-cell treated 96-well plates in complete medium and left to adhere overnight. Then, 125 to 250 to 500 to 1000 µmol/L solutions of GYY4137 were prepared fresh in complete medium. For both VSMCs and HUVECs, propidium iodide (PI) (1:200) was added to the GYY containing medium and supplemented in duplicate wells. Live imaging was performed using a fluorescence microscope with an automated stage and temperature and CO₂ control (Leica AF6000) taking pictures every 15 minutes at 2 locations/well. The number of cells positive for PI was then counted per image and totaled per well.

In-Vitro Transmigration Assay

VSMCs were serum starved overnight (0.5% CCS) and seeded in the apical chamber of 6.5 mm Transwell inserts with 8 µm pore polyester membranes (Corning, cat#3464). Before seeding 5×10⁴ cells in DMEM per apical chamber, apical membranes were coated with collagen type 1 (Thermo Fisher Scientific, A1048301). VSMCs were seeded with or in absence of 250 µmol/L GYY. In a separate experiment, VSMCs were preincubated with 250 µmol/L GYY for 6 hours before seeding. The bottom chamber contained serum-free DMEM with/without 10 ng/mL platelet-derived growth factor-BB (PDGF-BB, R&D Systems, cat# 520-BB-050). After

6 hours incubation, apical and bottom chambers were washed with PBS twice and fixed in 4% paraformaldehyde (PFA, Chemcruc, cat# sc-281692) for 10 minutes at RT. Next, inserts were washed twice in PBS and incubated with DAPI for 10 minutes at RT in the dark. After washing with PBS, membranes were cut out from inserts and mounted on slides. Per insert, 8 images were taken at 20× magnification, number of migrated VSMCs were then counted with ImageJ and normalized per mm².

Immunocytochemistry

VSMCs were first grown on gelatin-coated slides (0.1% gelatin, Sigma, G9391) till near-confluency then fixed with 4% PFA, washed twice in PBS and permeabilized with 0.01% Triton and incubated with 0.1% bovine serum albumin in PBS for 45 minutes. After removal of the blocking reagent, slides were incubated with SMC- α primary antibody (mouse anti-mouse, Abcam, ab7817, 1:400 dilution in 0.1% BSA/PBS) o/n at 4°C. After 3 washes with PBS, slides were then stained with Alexa Fluor 647 (goat anti-mouse, A-32728, 1:600 dilution in BSA/PBS) for 2 hours at RT. After 2 washes with PBS, slides were incubated with DAPI mounting medium and covered with coverslips. Slides were imaged by confocal microscope.

Ex-Vivo Measurement of H₂S

To establish the release profile of GYY/NaHS ex vivo, GYY (250 μ mol/L, 1 mmol/L) and NaHS (1 mmol/L) were dissolved in 3 mL PBS and incubated at 37°C. At various time points, solutions were sampled and incubated with 0.25 μ mol/L SF₇-AM (Tocris, cat# 4943) for 30 minutes at 37°C in the dark in a glass bottom 96-well plate (Cellvis, cat# P96-0-N). After incubation, plate was imaged with a GE Typhoon FLA 9500 Laser Scanner (GE Healthcare, cat# 15342) and mean fluorescent intensity of each well was measured with ImageJ.

H₂S Release by GYY in a 40% Pluronic Gel

To assess the release profile of GYY while dissolved in a 40% Pluronic gel. PBS, GYY (1 mmol/L, 250 μ mol/L) and NaHS (1 mmol/L, Sigma, cat# 161527) were dissolved in 3 mL 40% Pluronic gel in separate wells of a 6-well plate, and incubated at 37°C. After gel was solidified, each gel was overlaid with 3 mL of sterile PBS. At various time points, supernatant was sampled and incubated with 0.25 μ mol/L SF₇-AM for 30 minutes at 37°C in the dark in a glass 96-well plate and imaged with a GE Typhoon laser scanner. Starting at 12 hours after first overlaying the Pluronic gel with PBS, supernatant was removed every 24 hours and renewed with fresh 3 mL PBS.

H₂S Release After Local GYY Treatment

C57BL/6 mice (n=4/group) were anesthetized via 5% isoflurane induction then switched to 2% to 3% isoflurane. Midline neck incision was performed and the right and left CCA were exposed via surgical manipulation. Both arteries were then enveloped with the same Pluronic gel with or without 250 μ mol/L GYY. After 30 seconds incubation, midline incision was closed with 6/0 Vicryl suture and postoperatively mice received warm lactate ringer solution (0.5 mL, subcutaneous) and buprenorphine (0.1 mg/kg, subcutaneous). Twenty-four hours after surgery, mice were anesthetized, and the wound bed was reopened. Then, 100 μ L of gel remnant visible in the wound bed was collected and incubated with 0.25 μ mol/L SF₇-AM for 30 minutes in a 96-well plate. Mice were then euthanized via cervical dislocation. After incubation, plates were imaged with the GE Typhoon laser scanner, wells were then analyzed with ImageJ and corrected for background signal (PBS+0.25 μ mol/L SF₇-AM).

Statistical Analysis

Data are expressed as Mean \pm SD. Normality testing was performed with a Shapiro-Wilk normality test. Student's *t* test, 1-way or 2-way analysis of variance was used to analyze normally distributed data. Nonnormally distributed data were analyzed by Mann-Whitney test or Kruskal-Wallis test. All statistical analyses were performed with Graphpad (8.12).

RESULTS

Systemic Therapy With the H₂S Donor GYY Limits Vein Graft Disease

To test the effectiveness of exogenous H₂S in vein graft surgery, we first supplemented 250 μ mol/L GYY to the drinking water of C57BL/6 mice, either throughout the 7 days preceding vein graft surgery (pre-GYY) or starting immediately after surgery and continuing until harvest at POD 28 (post-GYY). Figure 1A exemplifies the experimental design and Figure 1B outlines the vein graft procedure, which encompasses the transplantation of a donor caval vein in a recipient RCCA via an end-to-end anastomosis. At POD 28, all mice were anesthetized and then euthanized via exsanguination, vein grafts were harvested and processed for histology. Figure 1C shows representative histology images of vein graft cross-sections from control, pre-GYY, and post-GYY groups after Masson-trichrome staining at POD 28.

Pretreatment with GYY decreased the I/M+A area and thickness ratios with 36.5% and 35.8% respectively (Figure 1D through E) compared with control mice, whereas there was no difference in control versus

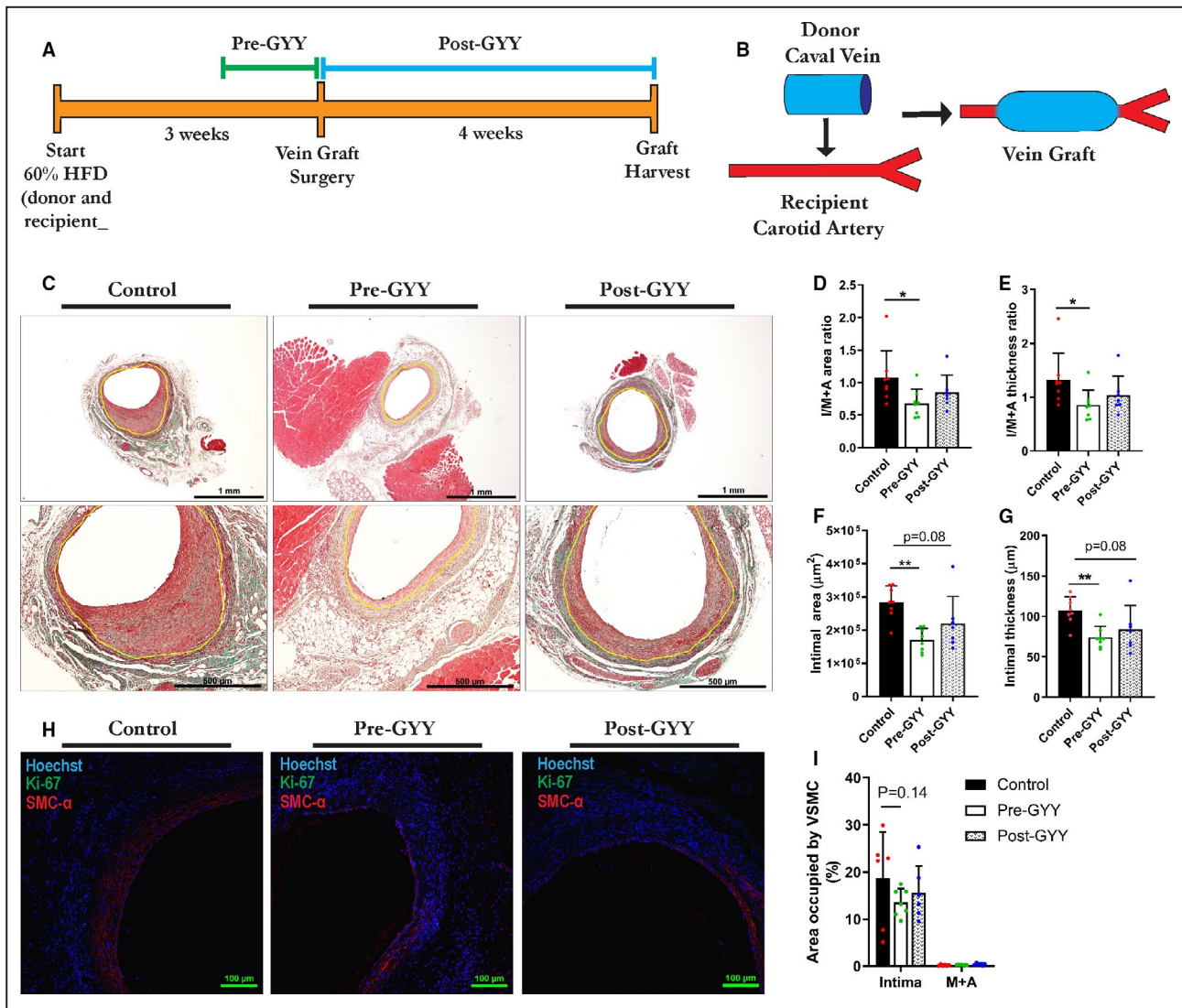


Figure 1. Systemic therapy with the H₂S donor GYY limits vein graft disease.

A, Experimental design: 60% high-fat diet (HFD), 1 week of preconditioning (pre-GYY) or 4 weeks of postconditioning (post-GYY) vs control. **B**, Schematic depiction of the vein graft procedure: end-to-end anastomosis of a donor mouse caval vein into a recipient mouse right common carotid artery. **C**, Representative images of control, pre-GYY, and post-GYY vein grafts harvested at POD 28 after Masson-trichrome staining, with yellow lining highlighting the intimal-M+A border. Scale bars=1 mm or 500 μ m as indicated. **D** through **G**, Morphometric analysis of vein grafts at POD 28 in control, pre-GYY, and post-GYY treated mice as indicated; n=7 to 8/group. Multiple comparisons are via Kruskal-Wallis test with Dunn's multiple comparisons test unless indicated otherwise. **D**, I/M+A area ratios. **E**, I/M+A thickness. **F**, Intimal area. **G**, Intimal thickness. **F** through **G**, One-way analysis of variance with Dunnett's multiple comparisons test. **H**, Vein grafts after VSMC+Ki-67 IHC. Scale bars=100 μ m as indicated. **I**, Percentage of intimal and M+A layers occupied by VSMCs; n=6–7/group; via 2-way analysis of variance with Tukey's multiple comparisons test. Data represented as Mean \pm SD. * P <0.05, ** P <0.01. GYY indicates hydrogen sulfide produg; I/M+A, intimal/media+adventitia; IHC, immunohistochemistry; POD, postoperative day; and VSMC, vascular smooth muscle cell.

post-GYY area and thickness ratios (Figure 1D through E). In concurrence with a decrease in area and thickness ratios, intimal area (Figure 1F), and thickness (Figure 1G) were diminished in the pre-GYY group. Although there was a trend toward decreased intimal area and thickness in the post-GYY group (Figure 1F through G). In both treatment groups, there was no difference in lumen area (Figure S1A), M+A area (Figure S1B) and thickness (Figure S1C). Further analysis of the VSMC content of

pre- and postconditioned vein grafts (Figure 1H) hinted toward decreased intimal VSMC infiltration in the pre-GYY group (Figure 1H through I). Interestingly, in the post-GYY group there was increased M+A collagen content (Figure S1D) and a trend toward increased overall collagen content, signifying an outward remodeling response.

Taken together these data suggest a significant beneficial effect for systemic GYY treatment in attenuating

VGD, with preconditioning yielding an additional beneficial response compared with postconditioning.

Development of a Locally Applicable Pluronic Gel that Ensures Extended H₂S Release Both Ex Vivo and In Vivo

Because of potential translational barriers associated with preconditioning for extended time periods before surgery, we next explored the potential local use of GYY at the time of surgery to prevent potentially dangerous side effects associated with excess H₂S. To understand the kinetics of local delivery of different sulfide donors we first tested the slow and extended release qualities of different sulfide donors ex vivo by dissolving the well-known sulfide donor sodium hydrosulfide (NaHS) at 1 mmol/L in PBS as a positive control, and GYY at 1 mmol/L and 250 μmol/L respectively.²⁸

To first compare the presence of free H₂S at various time points after dissolving slow- and fast-releasing H₂S-donors, we dissolved GYY and NaHS in PBS and took a 100 μL sample at regular intervals. This sample was placed in a 96-well plate and free H₂S was then measured by adding the H₂S-binding molecular probe SF₇-AM.³¹ As expected, PBS-dissolved GYY proved

capable of extended release of H₂S molecules up until 1 day after dissolvment, whereas NaHS-derived H₂S release diminished after 6 hours (Figure 2A). After the 24-hour time point there was no GYY/PBS present due to evaporation. In the same experiment, we compared the sulfide release rate of both donors, by a repeat measurement of the T=0 sample, after SF₇-AM was added. We found that GYY maintained a steady and continuous release of H₂S molecules, as shown by a slow increase in intensity of the SF₇-AM signal, whereas the H₂S molecules released by NaHS rapidly saturated the SF₇-AM signal (Figure 2B).

Next, to enable periprocedural local sustained and targeted release, we tested whether dissolving GYY in a 40% Pluronic gel would extend the release profile of GYY. We dissolved PBS, GYY, or NaHS in 40% Pluronic gel at 37°C, then overlaid the gel with PBS. At regular intervals, the supernatant was tested for free H₂S by incubation with the SF₇-AM probe. Measurements were continued until the Pluronic gel was dissolved in supernatant PBS and consecutively evaporated. Interestingly, we were able to extend the release profile of GYY from 24 to 72 hours by utilizing Pluronic gel as a vehicle, while NaHS was fully released from the gel at 6 hours postdissolvment (Figure 2C).

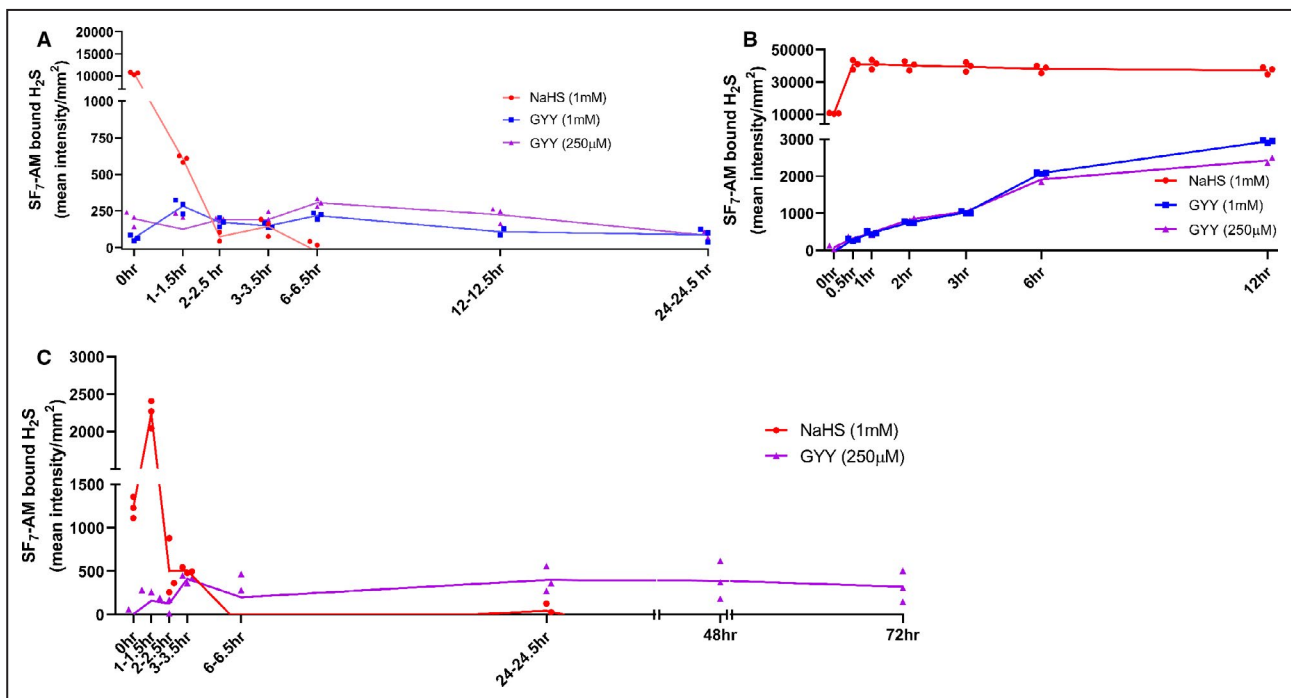


Figure 2. Development of a locally applicable Pluronic gel that ensures extended H₂S release both ex-vivo and in-vivo.

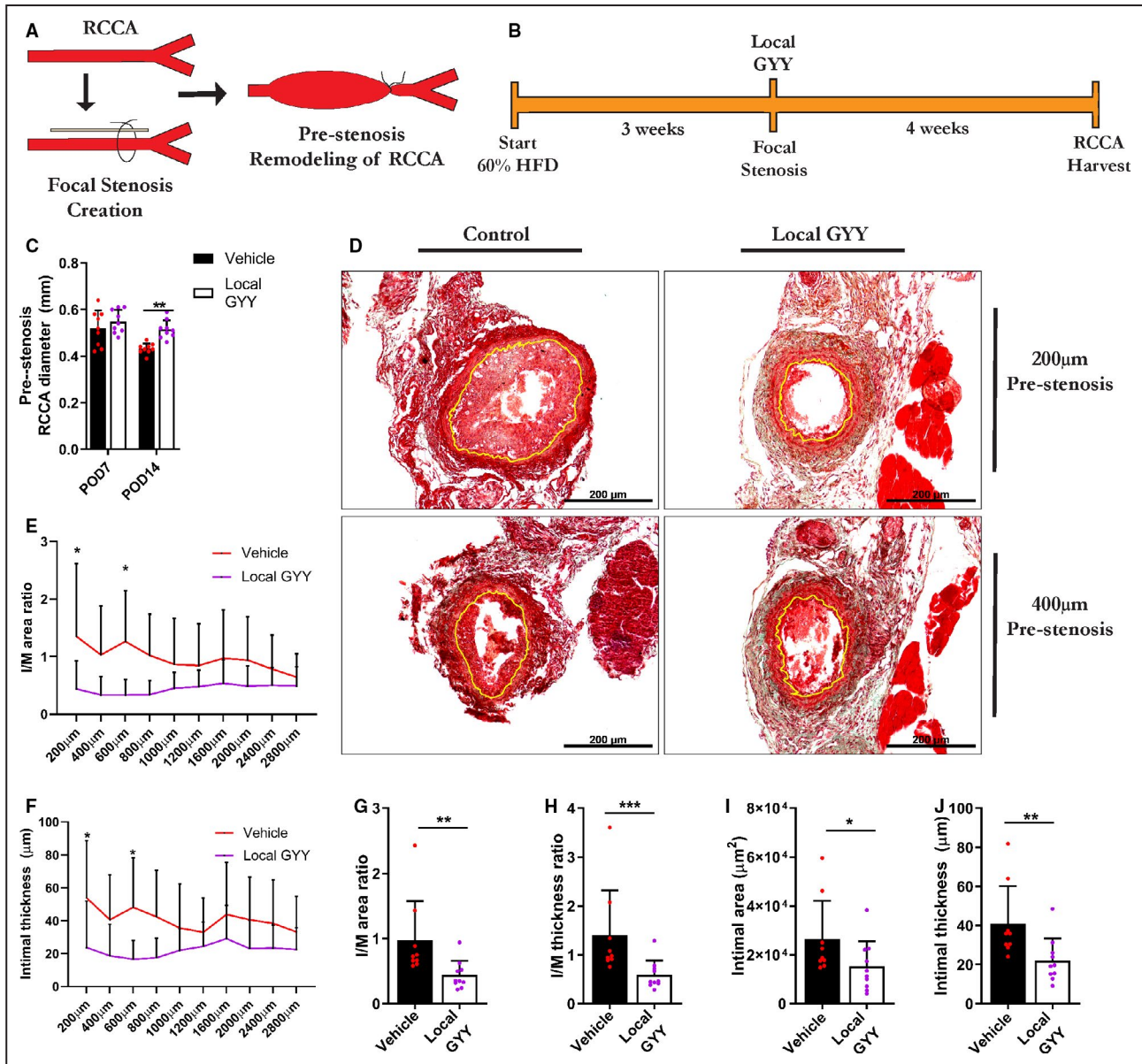
A through **C**, All samples were incubated with 0.25 μmol/L SF₇-AM in a 96-well plate in triplicates for 30 minutes at 37°C and then imaged. Resulting fluorescent intensity was corrected for background signal with a PBS+SF₇-AM control. **A**, NaHS (1 mmol/L), GYY (1 mmol/L, 250 μmol/L), and PBS dissolved in equal volume PBS, H₂S release was measured at regular intervals. **B**, Repeated measurement of same wells in 96-wells plate to assess release rate of respective H₂S releasing compounds. **C**, H₂S-drugs dissolved in equal volume of 40% Pluronic gel and overlaid with equal volume PBS. At regular intervals H₂S release in PBS supernatant was measured. GYY indicates hydrogen sulfide prodrug; H₂S, hydrogen sulfide; NaHS, sodium hydrosulfide; and SF₇-AM, fluorescent probe binding free hydrogen sulfide.

Finally, we evaluated the capability of this GYY gel to release H_2S in vivo by enveloping the left and right common carotid arteries (LCCA/RCCA) in the 40% Pluronic gel with/without 250 $\mu\text{mol/L}$ GYY. At 24 hours postsurgery, we tested for H_2S release from the gel remnants surrounding the LCCA/RCCA. Although sampling was hampered by high background signal because of the presence of blood, there was a trend for an increase in fluorescent intensity after incubation with $SF_7\text{-AM}$ in

the local-GYY group (1 ± 0.37 versus 1.791 ± 0.72 , $n=4$ /group, $P=0.1$) supporting extended H_2S release in vivo.

Local Application of the H_2S Donor GYY Attenuates the Arterial Fibroproliferative Response to Injury

To determine the effectiveness of local therapy with GYY during a vascular procedure, we first tested our



GYG gel in a model of arterial hyperplasia and vascular remodeling. Figure 3A illustrates the employed surgical model of focal stenosis of the RCCA in order to create proximal arterial hyperplasia and remodeling, and Figure 3B outlines the experimental design.

Interestingly, at POD 14 there was an increase in RCCA diameter proximal from the stenosis in the GYG group, as measured by ultrasound (Figure 3C). After harvesting the RCCA at POD 28, a Masson-trichrome staining was performed with representative images in Figure 3D at 200 μm and 400 μm proximal from the stenosis. Figure 3E and 3F visualize the remodeling response at regular distance intervals prestenosis, per I/M area ratio and intimal area respectively. When averaged, the group treated with local GYG showed decreased I/M area and thickness (Figure 3G through H) ratios and decreased intimal thickness (Figure 3J), and there was a trend for decreased intimal area (Figure 3I). Local treatment with GYG had no significant effect on lumen area (Figure S2A), medial area (Figure S2B), or medial thickness (Figure S2C).

Thus, local therapy was effective in mitigating the remodeling response to vascular surgical injury.

Periprocedural H₂S Therapy Protects from Vein Graft Disease and Attenuates Intimal VSMC Migration

Consequently, we set out to test whether this intervention could be advantageous in attenuating VGD and intimal hyperplasia. As outlined in Figure 4A, we next applied our GYG gel locally during the bypass procedure. During the procedure, the vein graft was enveloped with Pluronic gel (vehicle or GYG), just before the vascular clamps were opened. Interestingly, at POD 14 and POD 28, vein grafts treated with GYG showed a sustained increase in vein graft diameters *in vivo* compared with vehicle-treated grafts, as measured by ultrasound (Figure 4B).

After graft harvest at POD 28 and consecutive histology, the local GYG group had increased luminal graft diameters (Figure 4C, representative images), confirmed by an increase in corrected luminal area (Figure S3A), which correlated with the *in-vivo* ultrasound measurements on an individual mouse basis (Figure S3B). After quantification of the vein graft wall, we found that local GYG treatment decreased I/M+A area ratios with 56.7% (Figure 4D) and resulted in a 44.8% reduction in I/M+A thickness ratios (Figure 4E), signifying a strong attenuation of VGD after periprocedural treatment with GYG. Intimal area (Figure 4F) and thickness (Figure 4G) were also decreased combined with an increase in M+A area (Figure S3C) and thickness (Figure S3D). Overall, this pointed toward improved and beneficial adaptation of the vein graft to its new arterial environment, as was shown by a limited

inward remodeling response (Figure 4B through G) and increased outward remodeling (Figure S3C through Figure S3D) after local GYG treatment.

A major hallmark of VGD and consecutive graft failure, is the proliferation and migration of VSMCs from medial and adventitial layers of the vein conduit into the intimal layer.⁵ Interestingly, at POD 28 there was a 30.6% decrease in intimal VSMCs (Figure 4H) in the local GYG group (Figure 4I) whereas there was no difference in proliferating VSMCs at this post-operative time point (Figure 4J), nor was there a difference in collagen deposition (Figure S3E). This pointed toward attenuation of VSMC migration by local GYG treatment as an underlying mechanism for its therapeutic effect; therefore, we examined the effects of GYG on VSMCs *in vitro*.

Periprocedural H₂S Therapy Limits VSMC Migration and Proliferation *In Vitro*

To further investigate this phenotype, we isolated VSMCs from murine aortas and confirmed their origin by immunocytochemistry with α -SMC (Figure 5A). Intracellular H₂S levels were measured with SF₇-AM upon 6-hour GYG stimulation (Figure 5B). Next, we tested whether GYG treatment would impair VSMC migration *in vitro* by either preconditioning VSMCs with GYG (6 hours, 250 $\mu\text{mol/L}$ GYG) or stimulate them with GYG for the duration of the Boyden chamber assay (6 hours). Interestingly, VSMC transmigration was limited both by preconditioning (Figure 5E, 6 hours preconditioning) and costimulating (Figure 5C and 5D peri-GYG) PDGF-BB stimulated VSMCs.

To rule out cell death as an explanation for limited VSMC migration during/after GYG stimulation and to test the toxicity of our compound, we next performed live imaging on VSMC stained with PI during GYG supplementation in various concentrations. Most important, 250 $\mu\text{mol/L}$ did not increase cell death, but of note was that even 1000 $\mu\text{mol/L}$ of GYG did not prove toxic for VSMCs (Figure 5F). Because GYG did not have any detectable toxic effects on VSMC we next assessed whether it influenced metabolic activity/proliferation via an MTT assay. Proliferation was not different after short incubations with GYG (Figure 5G), and medium length incubation slightly amplified VSMC proliferation (Figure 5H). At the 18-hour time point, however, GYG limited VSMC proliferation, especially at higher concentrations (Figure 5I).

Lastly, because endothelial cells, next to VSMC, play an important role in both early and late vascular remodeling,³² we repeated our analysis of the toxicity and metabolic effects of GYG but then on HUVECs. Interestingly, live imaging with PI revealed a slight increase in cell survival compared with control conditions in HUVECs supplemented with 250 $\mu\text{mol/L}$

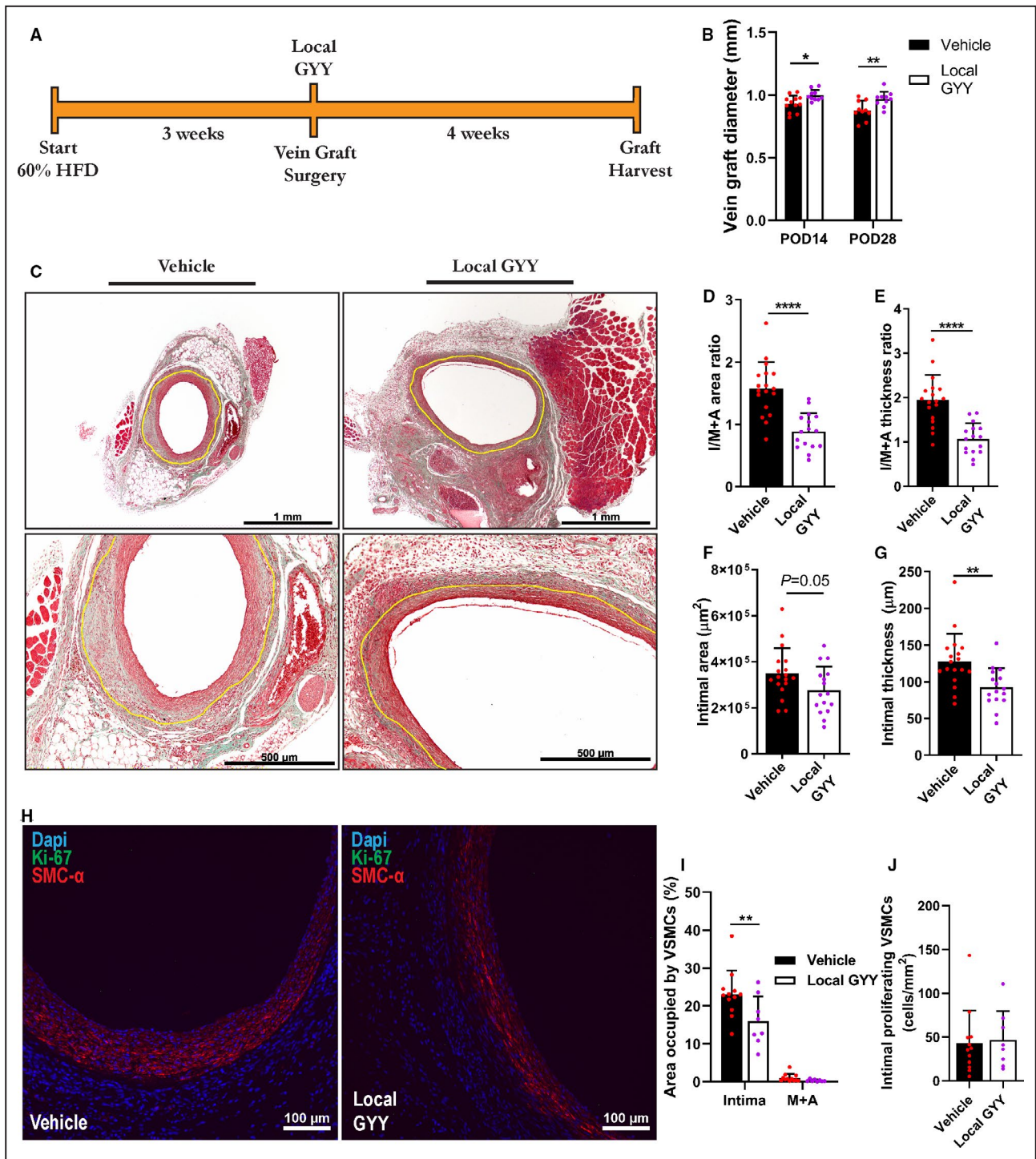


Figure 4. Periprocedural local H₂S therapy protects from vein graft disease by attenuating intimal VSMC migration.

A, Experimental outline with local H₂S application during vein graft surgery. **B**, Ultrasound measurements of vein graft diameters at POD 14 and POD 28 by 2-way analysis of variance with Sidak’s multiple comparisons test; n=10 to 12/group. **C**, Representative images of locally treated vein grafts at POD 28 after Masson-trichrome staining. Yellow lining indicating the intima-media+adventitia border. Scale bars 1 mm/500 μm as indicated. **D** through **G**, Morphometric analysis of vein grafts at POD 28, n=16 to 18/group, Student’s *t* test was performed unless indicated otherwise. **D**, I/M+A area ratios. **E**, I/M+A thickness ratios **F**, Intimal area. **G**, Intimal thickness. **H**, IHC for SMC- α +Ki-67. Scale bars are 100 μm as indicated. **I**, Percentage of area occupied by SMC- α positive cells in intimal and M+A layers, via 2-way analysis of variance with Sidak’s multiple comparisons test; n=8 to 12/group. **J**, Intimal colocalization of VSMC and Ki-67. Data represented as Mean \pm SD. **P*<0.05, ***P*<0.01, *****P*<0.0001. GYY indicates hydrogen sulfide prodrug; HFD, high-fat diet; H₂S, hydrogen sulfide; I/M+A, intimal/media+adventitia; IHC, immunohistochemistry; POD, postoperative day; SMC- α , smooth muscle cell- α ; and VSMC, vascular smooth muscle cell.

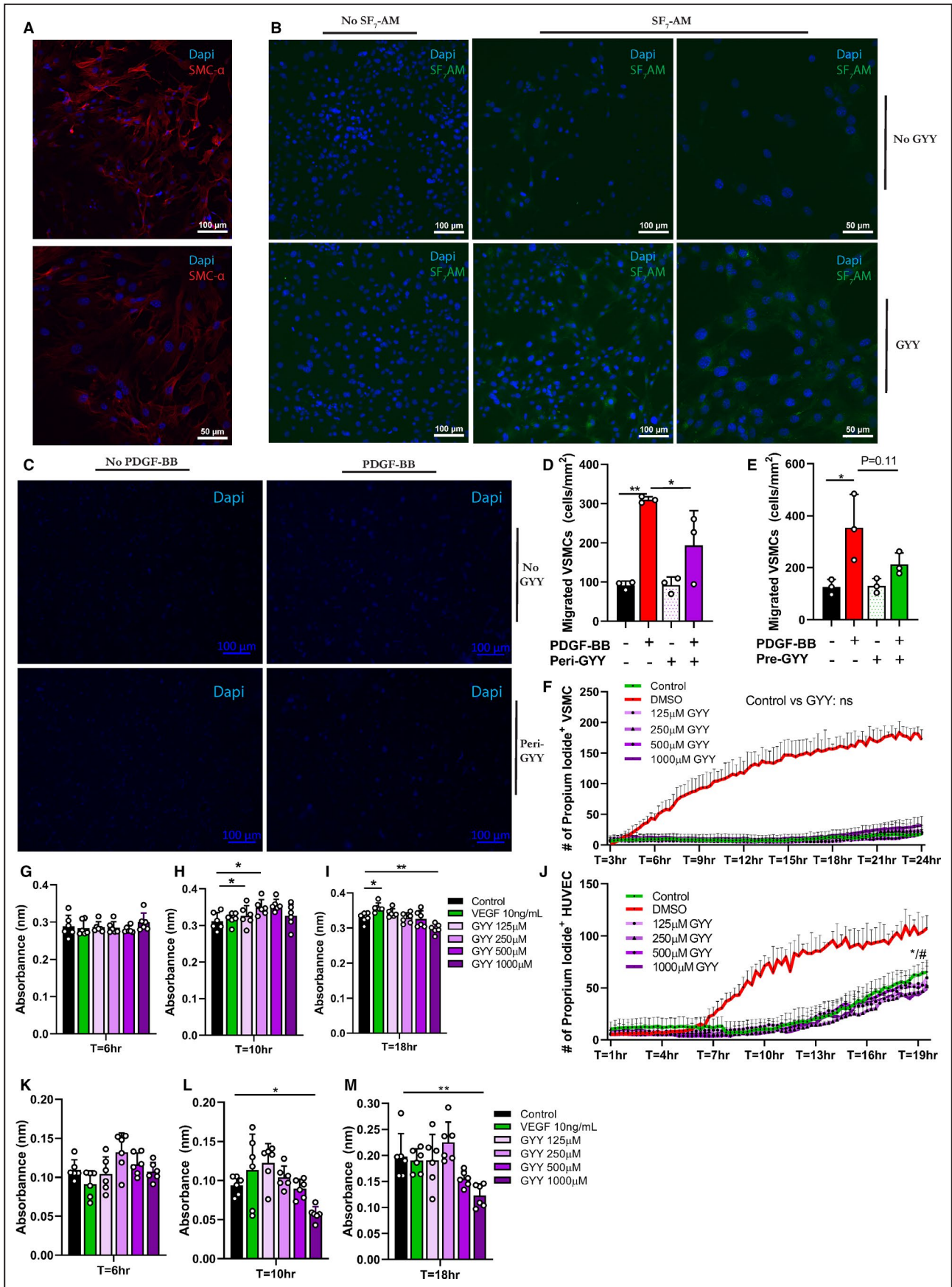


Figure 5. Periprocedural H₂S therapy limits VSMC migration and proliferation in vitro.

A, Representative immunocytochemistry image (20× & 40× magnification) of primary VSMC SMC- α staining. Scale bars 100 and 50 μ m as indicated. **B**, VSMCs after 6 hours treatment with 250 μ mol/L GYY, with/without SF₇-AM incubation (20× & 40× magnification). Scale bars 100 and 50 μ m as indicated. **C**, VSMCs after Transwell migration assay (6 hours in presence or absence of PDGF-BB and/or 250 μ mol/L GYY). **D**, Quantification of Transwell migration assay. Number of migrated VSMCs normalized to per mm² after 6 hours PDGF-BB and/or 250 μ mol/L GYY incubation in triplicates via 1-way analysis of variance with Sidak's multiple comparisons test. **E**, Quantification of Transwell migration assay after 6 hours of PDGF-BB incubation and/or 6 hours of 250 μ mol/L GYY pretreatment. **F**, Live imaging of VSMC positive for PI during incubation with 0 to 1000 μ mol/L GYY or DMSO. Number of PI⁺ VSMC in GYY versus control via 2-way analysis of variance with Dunnett's multiple comparison test. **G** through **I**, MTT assay of VSMC incubated with 0 to 1000 μ mol/L GYY or VEGF (10 ng/mL), 6 replicates per condition. One-way analysis of variance with Dunnett's multiple comparison test. **J**, Live imaging of HUVECs positive for PI during incubation with 0 to 1000 μ mol/L GYY or DMSO. Number of PI-positive HUVEC in GYY versus control via 2-way analysis of variance with Dunnett's multiple comparison test. *#: significant decrease in PI⁺ HUVECs supplemented with 250 μ mol/L and 1000 μ mol/L GYY respectively compared with control at T=18 to 19 hour. **K** through **M**, MTT assay of HUVECs incubated with 0 to 1000 μ mol/L GYY or VEGF (10 ng/mL), 6 replicates per condition. One-way analysis of variance with Dunnett's multiple comparison test. Data represented as Mean \pm SD. *P<0.05, **P<0.01. DMSO indicates dimethyl sulfoxide; GYY, hydrogen sulfide prodrug; H₂S, hydrogen sulfide; HUVEC, human umbilical endothelial cells; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; PDGF-BB, platelet-derived growth factor; PI, propidium iodide; SMC- α , smooth muscle cell- α ; VEGF, vascular endothelial growth factor; and VSMC, vascular smooth muscle cell.

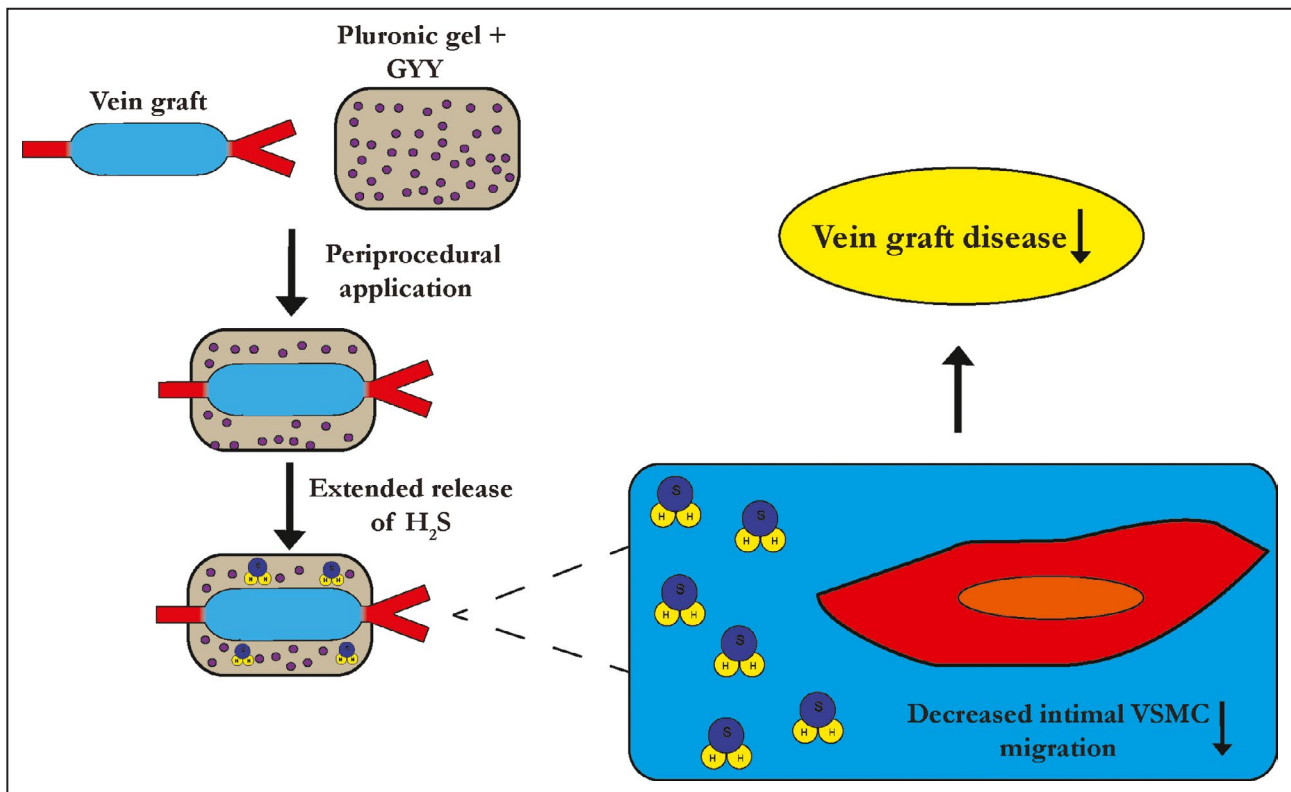
or 1000 μ mol/L GYY at T=18 to 19 hours (Figure 5J), indicative of a potential cytoprotective effect of GYY on endothelial cells. Further analysis of HUVEC proliferation via MTT assays (Figure 5K) and 5M) showed reduced metabolic activity/proliferation after longer periods of incubation (T=18 hour) and high (1000 μ mol/L) GYY concentration (Figure 5M).

Taken together, these data suggest that a single local periprocedural administration of the H₂S donor GYY is able to attenuate VGD and that this is at least

partly through limiting VSMC migration and proliferation. Benefits from local GYY therapy in vascular remodeling could, parallel to limited VSMC migration/proliferation, possibly extend to increased cell survival and cytoprotection in endothelial cells.

DISCUSSION

The prospect of translating short-term dietary preconditioning strategies into everyday surgical practice

**Figure 6. Periprocedural local hydrogen sulfide therapy limits vein graft disease.**

GYY indicates hydrogen sulfide prodrug; H₂S, hydrogen sulfide; VSMC, vascular smooth muscle cell.

appears appealing as such approaches have been tested in certain patient populations. For example, in healthy kidney donors scheduled for transplant surgery, a 2-week protein-calorie restriction diet was proven feasible in terms of compliance.³³ Furthermore, just 1 week of preoperative caloric restriction diet was able to reduce intraoperative blood loss in patients undergoing liver resection.^{34,35} In the vascular surgery patient population, however, with a predisposition toward unhealthy lifestyle choices and increased metabolic disease even a short-term change in dietary patterns may prove challenging from a DR-compliance perspective. In our recent pilot study only about 10% of appropriate patients were able to undergo a defined 3-day dietary intervention before elective vascular surgery.²⁴

Here we established a promising new approach to mitigate graft failure after bypass surgery by local delivery of the H₂S donor GYY during the vein graft procedure. This local periprocedural therapy would circumvent the need for preconditioning via DR or pharmacological therapy (with H₂S donors) and increase the potential for clinical translation. A 1-time periprocedural application of this H₂S gel onto the graft proved sufficient to limit inward graft remodeling, while simultaneously improving vein graft outward adaptations. Furthermore, even when compared with systemic pre- or postconditioning with GYY, local application had increased efficacy in attenuating VGD. Protection from VGD and vein intimal hyperplasia notwithstanding, our H₂S gel was additionally able to reduce arterial hyperplasia and improve arterial remodeling in a mouse model of arterial focal stenosis, thereby bolstering the potential of this intervention beyond vein graft surgery, to extend toward both open and endovascular arterial procedures.

Previous research found that systemic treatment with H₂S donors, via IP injection, attenuated arterial remodeling after balloon angioplasty.³⁶ In a follow-up study the same group showed decreased VSMC proliferation and migration *in vitro* as a result of exogenous H₂S therapy, due to downregulation of matrix-metalloproteinase-2.²² And recently, a slow-releasing H₂S-peptide gel was developed that limited VSMC migration *in vitro* and *ex vivo* in transplanted human vein graft segments.³⁷ Here we show that local (exogenous) H₂S therapy limits intimal VSMC migration *in vivo*, which in turn contributes to protection from graft failure. (Figure 6) This supports our previous work on the role of H₂S in vein grafts, where we found that endogenous upregulation of H₂S (via DR) protected from VGD, also partly via inhibition of the fibroproliferative response.²³ Whether exogenous H₂S therapy (and endogenous) also mitigates dedifferentiated VSMC migration or is involved in VSMC phenotype switching, both important hallmarks in the

intimal hyperplastic response,³⁸ remains to be determined in future studies. However, H₂S is also known for its anti-inflammatory properties.¹⁸ For example, systemic H₂S therapy decreased circulating tissue necrosis factor- α after transient aortic occlusion, implying a decrease in the inflammatory response after vascular injury.³⁹ It is likely that increased locally available H₂S not only directly inhibits VSMC migration but also indirectly, by blocking the influx and activation of immune cells.

A possible concern in exogenous H₂S supplementation is its narrow therapeutic window and the toxicity of H₂S in high concentrations.⁴⁰ We therefore opted for a slow-releasing H₂S donor (GYY4137) as opposed to the more conventional exogenous H₂S treatment with the fast-releasing H₂S donor NaHS. Because GYY continuously releases free H₂S molecules in low concentrations, we circumvented any potential issues of toxicity via oral administration. Furthermore, GYY being a prodrug, its parent molecule ZYJ112 has not shown any biological (or adverse) effects *in vitro* or *in vivo*,⁴¹ which further reinforces the potential of GYY as a safe candidate for (oral) exogenous H₂S treatment.

Despite the translational promise this work holds, there are several limitations to be acknowledged. First, this intervention was tested only on vein grafts and arteries at 28 days postsurgery, because in this model long-term protection cannot be established. Second, although we established protection from VGD at POD 28, the effects of GYY during early vein graft remodeling are unknown. We hope that this work will incite future research on the effects of local H₂S therapy on the different cell types (VSMC, endothelial cells, leukocytes) that play a major role in facilitating or accelerating this remodeling response. Third, only a single concentration of GYY was tested, therefore the most efficacious and optimal dose of GYY in VGD/arterial remodeling is unknown. Lastly, although a high-fat diet was employed to mimic the vascular surgery patient population, other factors were not accounted for (age, sex, underlying comorbidities).

In short, we developed and tested a H₂S releasing gel that can be applied locally during the procedure and that is capable of attenuating both vein graft failure and arterial remodeling. Future directions should focus on exploring its potential in vascular access surgery and endovascular interventions.

ARTICLE INFORMATION

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Disclosures

None.

Supplementary Material

Figures S1–S3

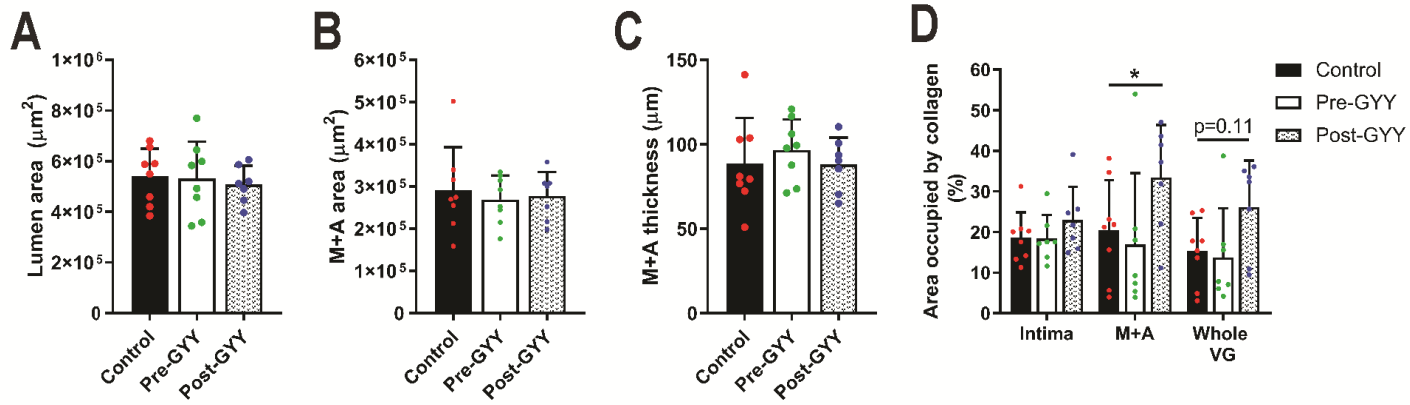
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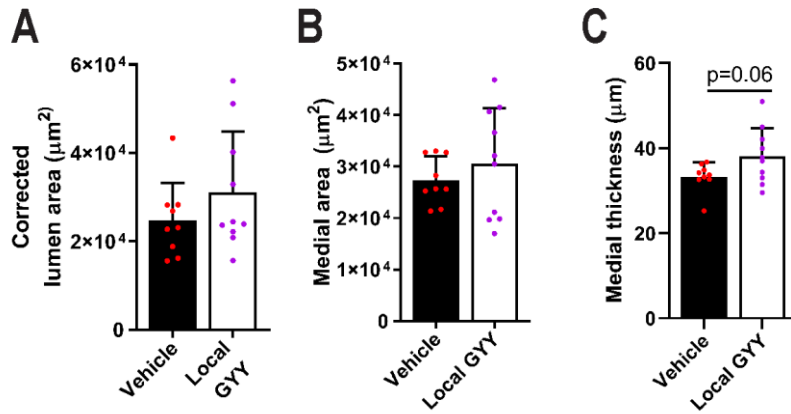
SUPPLEMENTAL MATERIAL

Figure S1. Histomorphometric and collagen analysis after systemic H₂S treatment.



A-D: Corrected lumen area in control, pre- and post-GYY treated vein grafts at POD28. **B:** M+A area. **C:** M+A thickness. **D:** Percent of intimal and M+A layers and whole VG occupied by collagen; $n=7-8/\text{group}$; via two-way ANOVA with Tukey's multiple comparisons test. *Data represented as: Mean \pm SD.* H₂S: hydrogen sulfide, GYY: hydrogen sulfide pro-drug, POD: post-operative day, M+A: media + adventitia, VG: vein graft.

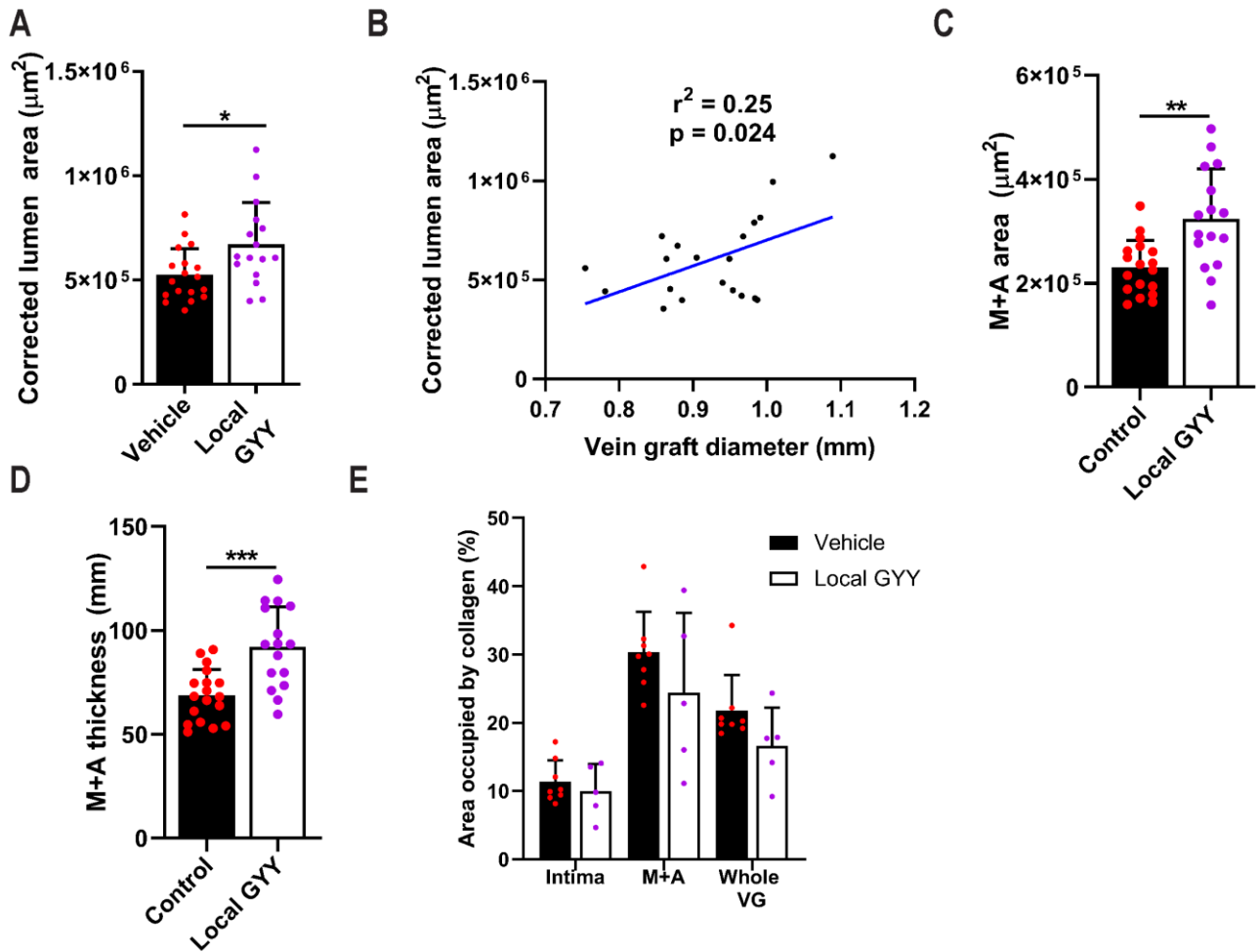
Figure S2. Hystomorphometric analysis of carotid arteries after local H₂S treatment.



A: Averaged corrected lumen area in locally treated carotid arteries at POD28 after focal stenosis procedure.

B: M+A area. **C:** M+A thickness. *Data is represented as Mean \pm SD* H₂S: hydrogen sulfide, POD: post-operative day, M+A: media + adventitia.

Figure S3. Histomorphometric and collagen analysis of vein graft after local H₂S therapy.



A: Corrected lumen area in vehicle and local GYY treated vein grafts at POD28. **B:** Correlation between corrected luminal area after histology and vein graft diameter ultrasound measurements *in vivo*, both at POD28. **C:** M+A area. **D:** M+A thickness. **E:** Percentage of vein graft layers occupied by collagen. Data represented as Mean \pm SD. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. H₂S: hydrogen sulfide, GYY: hydrogen sulfide pro-drug, POD: post-operative day, M+A: media + adventitia.