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The Route and Timing of Hydrogen Sulfide Therapy Critically Impacts Intestinal Recovery Following Ischemia and Reperfusion Injury

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Abstract

Purpose—Hydrogen sulfide (H_2S) has many beneficial properties and may serve as a novel treatment in patients suffering from intestinal ischemia-reperfusion injury (I/R). The purpose of this study was to examine the method of delivery and timing of administration of H_2S for intestinal therapy during ischemic injury. We hypothesized that 1) route of administration of hydrogen sulfide would impact intestinal recovery following acute mesenteric ischemia and 2) pre-ischemic H_2S conditioning using the optimal mode of administration as determined above would provide superior protection compared to post-ischemic application.

Methods—Male C57BL/6J mice underwent intestinal ischemia by temporary occlusion of the superior mesenteric artery. Following ischemia, animals were treated according to one of the following (N=6 per group): intraperitoneal or intravenous injection of GYY4137 (H_2S-releasing donor, 50mg/kg in PBS), vehicle, inhalation of oxygen only, inhalation of 80ppm hydrogen sulfide gas. Following 24-hours recovery, perfusion was assessed via laser Doppler imaging, and animals were euthanized. Perfusion and histology data were assessed, and terminal ileum samples were analyzed for cytokine production following ischemia. Once the optimal route of administration was determined, pre-ischemic conditioning with H_2S was undertaken using that route of administration. All data were analyzed using Mann-Whitney. P-values <0.05 were significant.

Results—Mesenteric perfusion following intestinal I/R was superior in mice treated with intraperitoneal (IP) GYY4137 (IP vehicle: 25.6±6.0 vs. IP GYY4137: 79.7±15.1; p=0.02) or intravenous (IV) GYY4137 (IV vehicle: 36.3±5.9 vs. IV GYY4137: 100.7±34.0; p=0.03). This

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Level Of Evidence: N/A

benefit was not observed with inhaled H_2S gas (O2 vehicle: 66.6±11.4 vs. H_2S gas: 81.8±6.0; p=0.31). However, histological architecture was only preserved with intraperitoneal administration of GYY4127 (IP vehicle: 3.4±0.4 vs. IP GYY4137: 2±0.3; p=0.02). Additionally, IP GYY4137 allowed for significant attenuation of inflammatory chemokine production of IL-6, IP-10 and MIP-2. We then analyzed whether there was a difference between pre and post-ischemic administration of IP GYY4137. We found that preconditioning of animals with intraperitoneal GYY4137 only added minor improvements in outcomes compared to post-ischemic application.

Conclusion—Therapeutic benefits of H_2S are superior with intraperitoneal application of an H_2S donor compared to other administration routes. Additionally, while intraperitoneal treatment in both the pre and post-ischemic period is beneficial, pre-ischemic application of an H_2S donor was found to be slightly better. Further studies are needed to examine long term outcomes and further mechanisms of action prior to widespread clinical application.

Keywords

Hydrogen sulfide; GYY4137; Intestinal ischemia; Perfusion; Inflammation

Introduction

Acute mesenteric ischemia (AMI) is a devastating disease that occurs when the blood supply to the intestine is cut off abruptly. The lack of blood flow to the small intestine leads to ischemia, cellular damage, intestinal necrosis and death if left untreated. Despite advances in medical care, mortality rates remain as high as 55-80% [1, 2]. In the pediatric population, intestinal ischemia can readily be observed with malrotation and midgut volvulus, incarcerated hernias, or with adhesive bowel obstructions [3]. Intestinal ischemia can also be seen in other disease pathologies such as congenital heart disease, fibromuscular dysplasia, abdominal compartment syndrome, or aortic thrombosis, to name a few [4]. Currently there are no medical therapies that allow for salvage of the ischemic intestine. Patients that require small bowel resection can often require long term total parenteral nutrition or intestinal transplantation secondary to short gut syndrome. AMI causes significant morbidity and mortality; therefore, new treatment modalities are urgently needed. The discovery and development of new medical therapies to improve intestinal perfusion and decrease cellular compromise would drastically change the medical management of this devastating disease.

One drug that could potentially provide protection in the setting of intestinal ischemia is hydrogen sulfide. Hydrogen sulfide (H_2S) is an endogenously produced gasotransmitter that plays an integral role in many physiological and pathological processes. It is known to regulate cell death and apoptosis, reduce inflammatory processes and provide cytoprotection [5, 6]. It also is known to play a key role in vascular relaxation and angiogenesis [7-9]. Studies in the literature have found it to be protective in the setting of cerebral, cardiac, hepatic, renal and intestinal ischemia-reperfusion injuries [10, 11].

Hydrogen sulfide also plays key roles in the production and modulation of several cytokines and chemokines. Two such chemokines include macrophage inflammatory protein 2(MIP-2) and interferon gamma-induced protein 10 (IP-10). These two chemokines play a prominent role in neutrophil recruitment in the inflammatory response following intestinal ischemia

[12-14]. Additionally, interleukin 6 (IL-6), an acute phase reactant, has been found to promote intestinal hyperplasia and villous growth, while interleukin 10 (IL-10), a key anti-inflammatory cytokine, has been found to be dramatically decreased in the setting of intestinal ischemia [12, 14-16]. H_2S has also been found to induce angiogenesis and vasodilation through vascular endothelial growth factor (VEGF) mediated mechanisms [7].

Prior to widespread therapeutic use, the optimal mode of delivery must be identified. Differences in administration between inhaled hydrogen sulfide gas, direct intraperitoneal application, and intravenous therapy could affect the benefits observed with H_2S treatment. Previous studies have observed benefits with all routes of administration but a study to determine the most efficacious route of administration has not previously been undertaken [17-19]. Furthermore, it is unclear if hydrogen sulfide pre-conditioning can further improve outcomes. If there is benefit to prophylactic administration of H_2S prior to any ischemic episode, H_2S could prove to be a novel preventative therapy for those patients at risk for intestinal ischemia. The purpose of this small scale pilot study was to examine mode of delivery and timing of administration during intestinal ischemic injury. We hypothesized that: 1) route of administration of hydrogen sulfide would impact intestinal recovery following acute mesenteric ischemia and 2) pre-ischemic H_2S conditioning using the optimal mode of administration as determined above would provide superior protection compared to post-ischemic application.

1. Methods

1.1 Animals

The Indiana University Institutional Animal Care and Use Committee approved all experimental protocols and animal use. Male adult wild-type C57BL/6J mice used in this study underwent at least 48 hours of acclimation prior to any experimentation. Normal chow and water were provided and all mice were kept in 12-hour light/dark cycled housing. For all animal experiments there were six mice per group.

1.2 Ischemia-Reperfusion Model

Mice were anesthetized using 3% isoflurane followed by maintenance at 1.5% isoflurane in oxygen. Temperature homeostasis was achieved through use of a heating pad and the abdomen was prepped through hair removal and sterile preparation with 70% ethanol followed by betadine. One milliliter of 0.9% normal saline was injected subcutaneously in all mice pre-operatively to account for intra-operative fluid losses. Post-operative pain was managed with pre-operative subcutaneous administration of analgesia (1mg/kg buprenorphine and 5mg/kg carprofen).

Under sterile conditions, a midline laparotomy was performed and the intestines were eviscerated. The base of the superior mesenteric artery was identified and clamped using an atraumatic microvascular clamp. The intestines were then placed back into the abdominal cavity and the abdomen was temporarily closed using silk suture to prevent evaporative losses. Following 60 minutes of intestinal ischemia, the abdomen was reopened and the atraumatic clamp was removed. The abdominal fascia and skin were then closed in a two-

layer fashion with silk suture. Following surgery, animals were placed in warm cage and allowed to recover. Once fully awake and alert, animals were returned to animal housing.

1.3 H_2S Administration

In order to determine optimum mode of administration, H_2S was administered by the following routes: inhaled, intravenous, or intraperitoneal. A total of six mice were used in each treatment group. Treatments were administered in the post-ischemic period as previously established by our lab [20]. Treatment groups (N=6) included: 1) O_2 only (systemic control, 4L/min), 2) H_2S Gas, (80ppm at 2L/min mixed with 2L/min oxygen), 3) intravenous (IV) PBS (40uL; IV vehicle control), 4) IV GYY4137 (a slow-releasing H_2S donor; 50mg/kg in 40uL of PBS), 5) intraperitoneal PBS (IP; 250ul; IP vehicle control), or 6) IP GYY4137 (a slow-releasing H_2S donor; 50mg/kg in 250uL of PBS). The O_2 vehicle and H_2S gas were administered for one hour following removal of the atraumatic clamp on the SMA. In the intravenous and intraperitoneal therapy groups, treatment was administered immediately after clamp removal.

Once the optimum route of therapy was identified (intraperitoneal, see Results below) we performed pre-conditioning experiments of hydrogen sulfide using this route, and compared pre-ischemic application to post-ischemic application (N=6/group): 1) IP PBS (250ul; IP vehicle control), and 2) IP GYY4137 (a slow-releasing H_2S donor; 50mg/kg in 250uL of PBS). Animals in the pre-ischemia treatment groups were given vehicle or H_2S therapy one hour prior to ischemia.

1.4 Perfusion Analysis

Intestinal mesenteric perfusion was analyzed using a Laser Doppler perfusion Imager (LDI; Moor Instruments, Wilmington, DE). Perfusion images were acquired at baseline, at initial clamping of the superior mesenteric artery and at 24 hours following intestinal ischemia. Using images obtained, a region of interest was created around the entirety of exposed intestines. Using three images from each time point, a flux mean perfusion was acquired for the region of interest. Perfusion was expressed as a percentage of baseline (mean±SEM). After the 24-hour recovery analysis, mice were euthanized with isoflurane overdose and cervical dislocation. Intestinal tissues were explanted for further analyses.

1.5 Intestinal Histological Injury Evaluation

At sacrifice, terminal ileum specimens were harvested, fixed in 4% paraformaldehyde, embedded in paraffin and sectioned for 2μm thickness. Slides were subsequently stained with hematoxylin and eosin. A histological scoring method of intestinal damage was used as previously described: 0, no damage; 1, subepithelial space at the villous tip; 2, loss of mucosal lining at the villous tip; 3, loss of less than half of the villous structure; 4, loss of more than half of the villous structure; and 5, transmural necrosis [21]. All histological sections were evaluated by two blinded authors (NAD, JPW) and scores were averaged.

1.6 Intestinal Cytokine Analysis

Mouse intestinal tissues designated for protein analysis were harvested, snap frozen in liquid nitrogen and stored at -80°C. Once ready to use, intestines were thawed and homogenized in

RIPA buffer (Sigma, St. Louis, MO) with phosphatase and protease inhibitors (1:100 dilution, Sigma, St. Louis, MO) using a Bullet Blender tissue homogenizer (Next Advance, Averill Park, NY). Following homogenization, samples were centrifuged at 12,000 rpm to pellet extraneous tissue and supernatants were collected and placed into fresh Eppendorf tubes. Total protein concentration was quantified with the Bradford assay using a spectrophotometer (VersaMax microplate reader; Molecular Devices, Sunnyvale, CA).

Murine intestinal levels of IL-6, IL-10, IP10, MIP-2, and VEGF were quantified using a Bio-Plex 200 multiplex beaded assay system (Bio-Rad, Hercules, Ca) with customizable multiplex plates for murine inflammatory cytokines (Millipore, Billerica, MA). Assays were performed at 1:20 dilution according to the manufacturer's instructions and are reported in nanograms of cytokine per gram of total intestinal protein (mean±SEM).

1.7 Statistical Analysis

Data were compared using Mann-Whitney U test for nonparametric variables. All statistical analyses were performed using GraphPad Prism 7 (GraphPad Software, La Jolla, CA). All data were reported as the mean±SEM and p-values less than 0.05 were considered statistically significant.

2. Results

2.1 Local Application of Hydrogen Sulfide by Intraperitoneal Injection Provides Superior Protection

Intravenous administration of GYY4137 improved intestinal perfusion compared to intravenous administration of vehicle control (IV vehicle: 36.3±5.9 vs. IV GYY4137: 100.7±34.0; p=0.03) (Figure 1). This beneficial effect was also observed when GYY4137 was administered via intraperitoneal injection (IP vehicle: 25.6±6.0 vs. IP GYY4137: 79.7±15.1; p=0.02). No benefit above oxygen control was observed with inhaled hydrogen sulfide gas (O2 vehicle: 66.6±11.4 vs. H_2S gas: 81.8±6.0; p=0.31).

We then sought to examine how route of therapy impacted histological injury. Herein, we only observed protective benefits to mucosal injury with the direct, local, intraperitoneal application of GYY4137. Compared to IP vehicle control, GYY4137 yielded significantly lower mucosal injury scores (IP vehicle: 3.4±0.4 vs. IP GYY4137: 2±0.3; p=0.02) (Figure 2). This preservation of histological architecture was not observed with IV H_2S administration or with inhaled H_2S gas administration.

Finally, we noted significant benefits with the intraperitoneal route when looking at intestinal inflammation. There was a significant decrease in IL-6 production in those mice treated with IP GYY4137 compared to IP vehicle. Additionally, significant decreases in the production of IP-10 and MIP-2 with IP GYY4137 administration were noted when compared to IV administration. There were no statistically significant differences in IL-10 and VEGF production with any of the three modes of administration (Figure 3).

2.2 Pre-ischemic vs. post-ischemic intraperitoneal application of Hydrogen Sulfide

With our above mentioned results demonstrating improvements in mesenteric perfusion, mucosal injury scores, and improvements in intestinal inflammation only with post-ischemic IP administration of hydrogen sulfide, we elected to investigate the benefits of pre-conditioning. We observed no difference in perfusion benefit with GYY4137 administered either pre-ischemia or post-ischemia (pre-ischemia IP GYY4137: 67.3±4.9 vs. post-ischemia IP GYY4137:79.7±15.1; p=0.2; Figure 4A). However, there was significantly less mucosal injury in those mice that were given IP GYY4137 in the pre-ischemic period (pre-ischemia IP GYY4137:0.9±0.1 vs. post-ischemia IP GYY4137: 2±0.3; p=<0.01) (Figure 4B). No significant differences in measured intestinal cytokines were noted between pre-ischemic and post-ischemic groups (Figure 5).

3. Discussion

Hydrogen sulfide therapy has attracted significant attention as a potent biological mediator. Although the benefits of hydrogen sulfide therapy in have been appreciated in intestinal ischemia models, the optimal route and timing of therapy has not been identified. Herein, we observed that intraperitoneal application of an H_2S donor facilitated improved mesenteric perfusion, decreased intestinal mucosal injury, and decreased intestinal inflammation compared to other routes of administration. Furthermore, applying hydrogen sulfide in the pre-ischemic phase of injury may provide some additional minor benefits, such as slightly less intestinal mucosal injury. We therefore suggest that treating intestinal I/R via local, and direct application to the intestines via intraperitoneal injection in the pre-ischemic period of injury may be the best mode of therapeutic administration.

We have previously appreciated that another H_2S donor, NaHS, also provided therapeutic benefit following intestinal ischemia and the potential downstream effects were mediated through eNOS-regulated pathways [20]. In the current study, we further investigated the treatment mode of administration (intraperitoneal, intravenous, inhalation) and demonstrated that intraperitoneal application was superior to intravenous or inhaled hydrogen sulfide. While several other studies in the literature have noted that H_2S donors have a protective effect on intestinal mucosa [18, 22-26], no other study in the literature has compared head-to-head H_2S administration routes.

The superiority of intraperitoneal application inherently makes sense. If the intestines are either ischemic or recovering from acute ischemia, then the blood flow to these areas of the bowel will likely be marginal, at best. Given that the intravenous and inhaled routes require transport to the intestines via the aorta, superior mesenteric artery, and intestinal capillaries, it stands to reason that these vessels will be modestly compromised. Although the peritoneal surface does likely absorb some of the hydrogen sulfide drug, it is also likely that there is a therapeutic benefit of direct application of the drug to the bowel wall. In this regard, we were able to appreciate improved outcomes with intraperitoneal administration of hydrogen sulfide.

In addition to the benefits of improved mesenteric perfusion and mucosal injury, intraperitoneal H_2S therapy also affected several inflammatory chemokines. We observed a

significant decrease in the production of IL-6, which is a known acute phase reactant. This observation would suggest a direct benefit of intraperitoneal hydrogen sulfide on intestinal inflammation. Additionally, the production of IL-6 following IP GYY4137 was not significantly different between pre and post-ischemic administration of H_2S, thereby suggesting that intestinal inflammation is not readily impacted by the timing of hydrogen sulfide administration. Other studies examining inflammation associated with intestinal injury and use of H_2S donors have also observed decreases in IL-6 and myeloperoxidase production, as well as attenuation of TNF-α and IFN-γ induced damage with H_2S administration [27-29].

Given the prominent role of neutrophils in inflammation following intestinal I/R injury, chemotactic cytokines MIP-2 and IP-10 were investigated. While it is known that these chemokines rise significantly in intestinal ischemia [14, 30], there is limited research on the production of these chemokines following H_2S therapy. In the current study, we observed significant decreases in both IP-10 and MIP-2 with IP GYY4137 compared to IV GYY4137 application. These results corroborated findings from our previous study in which the application of NaHS, a rather short acting hydrogen sulfide donor, decreased both IP-10 and MIP-2 following intestinal ischemia [20]. These data would suggest that the pro-inflammatory chemokine signaling that homes leukocytes to areas of injury is decreased with hydrogen sulfide therapy, and therefore, may inhibit influx of these cells following injury.

In this study, we have demonstrated that the direct and local intraperitoneal use of hydrogen sulfide tends to have the most favorable effect and that both pre and post-ischemic administration are beneficial. Preconditioning with H_2S prior to ischemia, however, may provide some additional minor benefits of recovery. These findings are similar to other studies using H_2S donors in either the pre-ischemic or post-ischemic period [25, 31]. Overall, the findings of this study provide a better understanding of the role of administration route in hydrogen sulfide therapy in the setting of intestinal ischemia.

4. Conclusions

In conclusion, intraperitoneal application of hydrogen sulfide appears to be the superior route of therapy following intestinal ischemia and reperfusion injury. Beneficial effects were observed with improvements in mesenteric perfusion, mucosal injury, and intestinal inflammation. In addition, pre-conditioning of subjects with hydrogen sulfide prior to injury onset may provide some additional advantages, such as even less mucosal injury following ischemia. Further studies need to address the effects of hydrogen sulfide on long term intestinal function and mucosal restitution prior to widespread clinical application.

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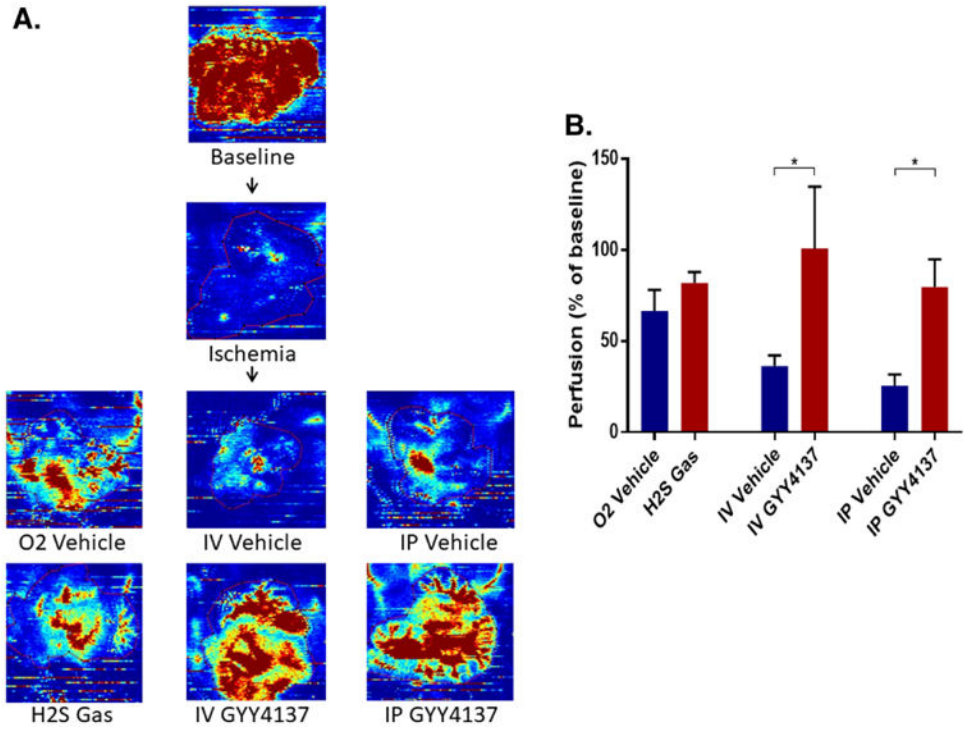

Figure 1. Mesenteric Perfusion

(A) Representative Laser Doppler images assessing mesenteric perfusion with different routes of hydrogen sulfide therapy. (B) Mesenteric perfusion was significantly increased with both intravenous and intraperitoneal application of GYY4137. However, there were no observed improvements in perfusion following treatment with systemic H_2S Gas. (*=$p<0.05$ versus respective vehicle)

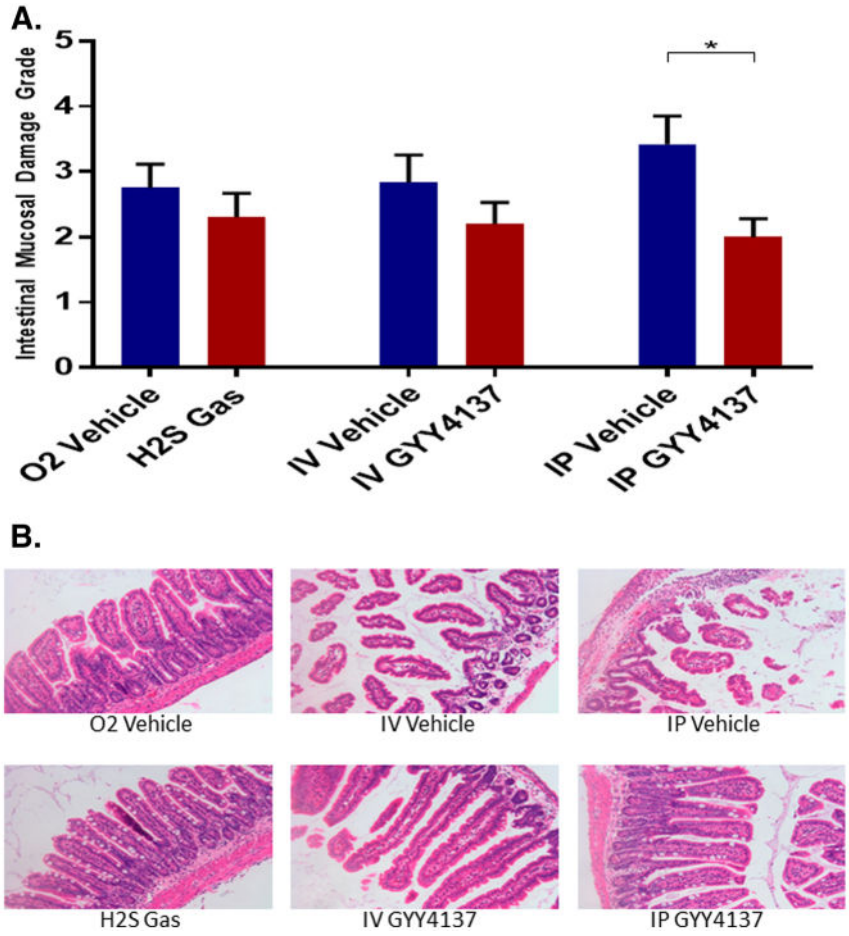

Figure 2. Histological Analysis

(A) Histological scoring of intestinal specimens. A histological scoring method of intestinal damage was used as previously described: 0, no damage; 1, subepithelial space at the villous tip; 2, loss of mucosal lining at the villous tip; 3, loss of less than half of the villous structure; 4, loss of more than half of the villous structure; and 5, transmural necrosis [21]. (B) Representative histology slides of each treatment group (hematoxylin and eosin stain, ×20) demonstrate statistically significant improvements in intestinal histology only with intraperitoneal GYY4137 therapy. (*= $p<0.05$ vs respective vehicle).

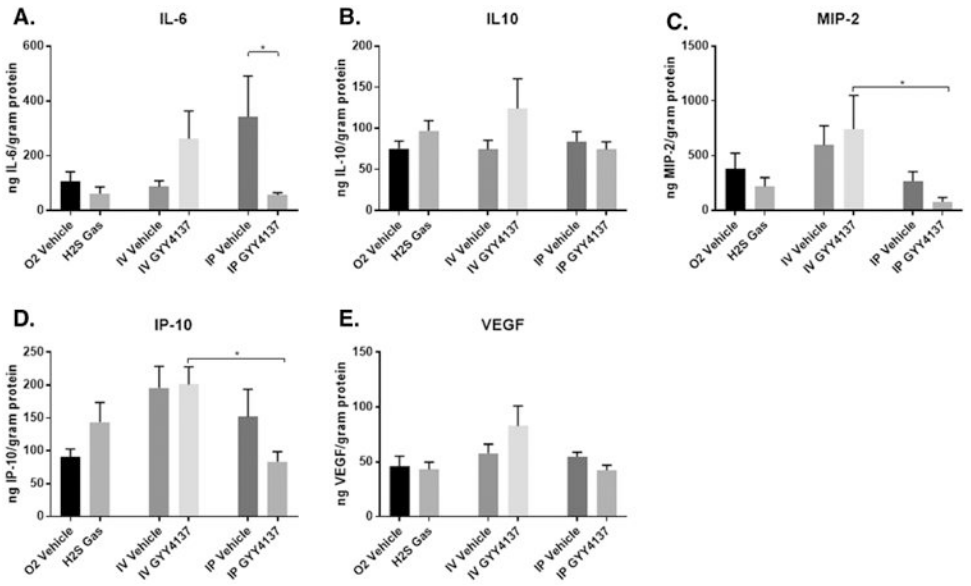

Figure 3. Inflammatory chemokines and growth factors

Intestinal levels of (A) IL-6 were significantly decreased with IP GYY4137 administration compared to IP vehicle. Both (B) IP-10 and (C) MIP-2 production were significantly decreased following IP GYY4137 administration compared to IV GYY4137 administration. (D) IL-10 and (E) VEGF did not appear to be affected by hydrogen sulfide therapy (*=$p<0.05$).

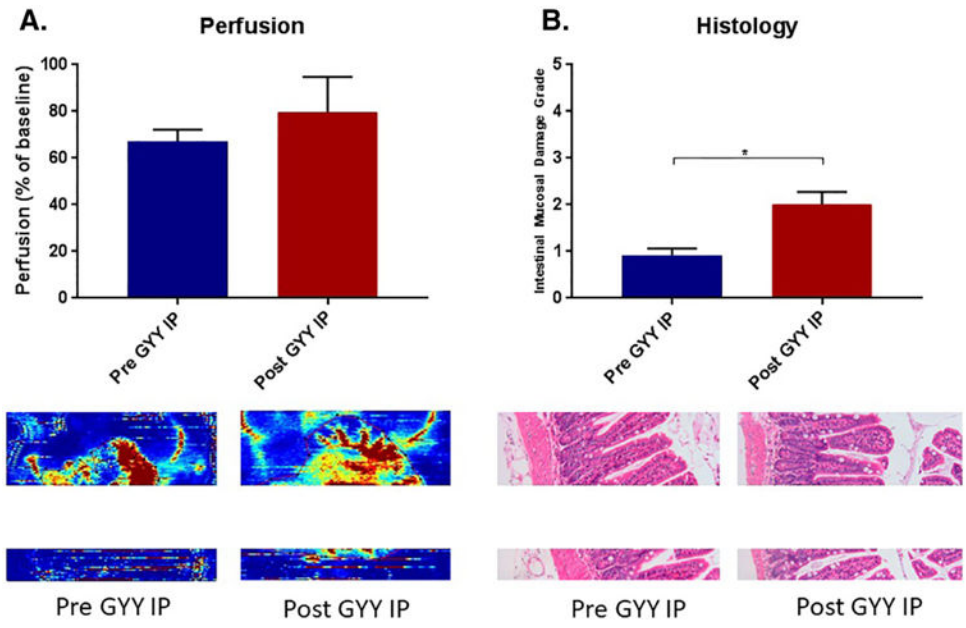

Figure 4. Preconditioning with intraperitoneal GYY4137 Affects Intestinal Histology More than Mesenteric Perfusion

(A) No difference was observed in mesenteric perfusion when pre-ischemic and post-ischemic IP application of hydrogen sulfide was compared. (B) There was a significant decrease in histological injury scores with pre-ischemic application of GYY4137 (*=$p<0.05$ vs. pre-ischemic application). Please see histological scoring system used in *Methods* section.

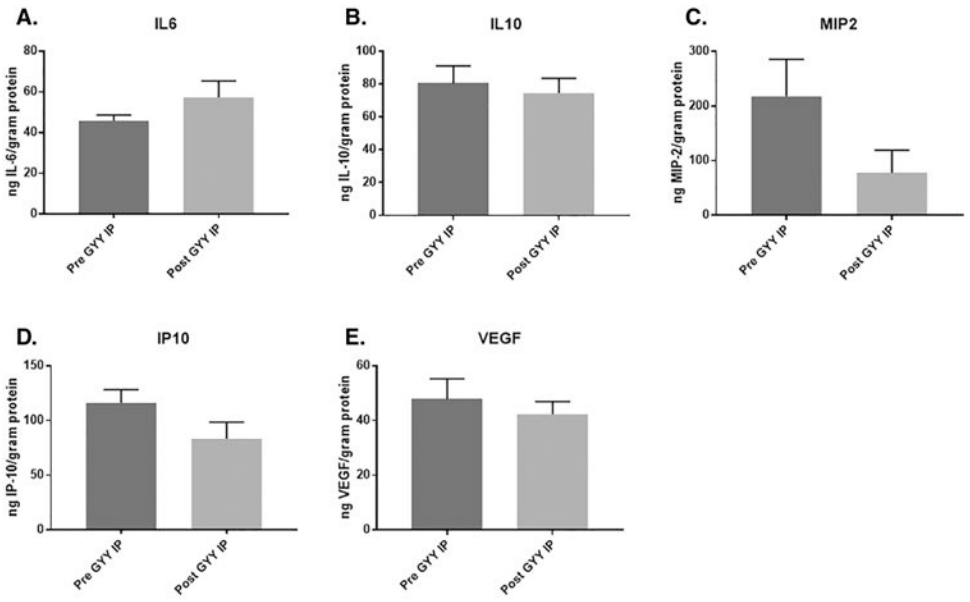

Figure 5. Preconditioning with intraperitoneal GYY4137 Does Not Alter Intestinal Cytokines Compared to Post-ischemic Use

There were no observed differences in intestinal production of several cytokines with regards to pre or post-ischemic intraperitoneal GYY4137 therapy.