Research

Sildenafil As a Rescue Agent Following Intestinal Ischemia and Reperfusion Injury

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Abstract

Background: Acute mesenteric ischemia carries a significant morbidity. Measures to improve blood flow parameters to the intestine may ameliorate the disease. Sildenafil, a PDE5 inhibitor, has been shown to increase cyclic GMP and has been shown to prevent the effects of ischemia when given before injury. However, its effects as a rescue agent have not been established. We therefore hypothesized that sildenafil, when given as a rescue agent for intestinal ischemia, would improve mesenteric perfusion, limit intestinal epithelial injury, and decrease intestinal leukocyte chemoattractants.
Methods: Eight to twelve-week-old male C57Bl6J mice underwent laparotomy and temporary occlusion of the superior mesenteric artery for 60 minutes. Following ischemia, reperfusion was permitted and prior to closing the abdomen, sildenafil was injected intraperitoneally in a variety of concentrations. After 24 hours, reperfusion was reassessed. Animals were euthanized and intestines evaluated for histologic injury and leukocyte chemoattractants.
Results: Post-ischemic administration of sildenafil did not improve mesenteric perfusion following intestinal ischemia and reperfusion injury. However, sildenafil did improve histologic injury scores in low dose treated groups. No difference was noted in histological injury with 100 mg/kg dose, and all members of the 1000 mg/kg group died and had significantly elevated intestinal injury scores compared to vehicle. Epithelial protection was not facilitated by the leukocyte chemoattractants RANTES, Mip1a, MCP, KC, or GCSF.
Conclusion: Administration of sildenafil following intestinal ischemia appears to protect the intestines via an epithelial mechanism rather than by promoting vascular dilation and improved blood flow to the mesenteric bed.

Hannah Moore is a third-year medical student interested in pediatrics or pediatric neurology. "I have always wanted to work with kids," she said, "and I have always been interested in neurology beginning with my neuroscience undergraduate degree at Indiana University." Her summer experience through IMPRS taught her the importance of following ideas and experimental hypotheses to the very end and keeping an open mind, because there may be findings or significant results that were not thought of or anticipated.

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 and/or necrotic 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 AMI.

In this regard, sildenafil has been observed to decrease the detrimental effects of intestinal ischemia and end organ injury when given prophylactically before an ischemic insult [5-7]. Sildenafil is a phosphodiesterase five (PDE5) inhibitor that works to decrease the conversion of cyclic guanosine monophosphate (cGMP) to GMP. This effect works to promote smooth muscle relaxation and improved blood flow to organs via cGMP-dependent protein kinase-dependent activation of K channels [8] (Figure 1).

It is often unclear which patients will develop intestinal ischemia until the insult happens. Although it is good to have preventative measures, there also needs to be appropriate rescue agents that can ameliorate injury or even rescue the intestine following injury. Therefore, it is likely that the use of sildenafil could improve and/or protect intestinal function and mesenteric artery integrity following injury. This protection would facilitate improved intestinal blood flow and decreased proinflammatory leukocyte influx into the injured tissue as measured by Regulated on Activation, Normal T Cell Expressed and Secreted (RANTES), Macrophage Inflammatory Protein 1 alpha (Mip1a), Monocyte Chemoattractant Protein (MCP), Neutrophil Activating Protein (KC), and Granulocyte Colony Stimulating Factor (GCSF).

We surmised that sildenafil, when given as a rescue agent after ischemia, would function much like it does when given prophylactically before injury. We hypothesized that sildenafil would stabilize the mesenteric vasculature and improve intestinal mesenteric perfusion, decrease intestinal epithelial injury, and limit leukocyte chemoattractants in the small intestinal tissues when given as a rescue agent following intestinal ischemia.

Method

Animals

The Indiana University Institutional Animal Care and Use Committee approved all experimental protocols and animal use. Male adult wild-type C57BL/6J mice weighing 25-30 grams 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. Animals were bred in house and treated in humane fashion according to the "Guide for the Care and Use of Laboratory Animals" [9].

Ischemia-Reperfusion Model

The murine intestinal ischemia and reperfusion (IR) protocol was performed as we previously described [10, 11]. Briefly, 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 subcutaneous administration of analgesia (1 mg/kg buprenorphine and 5 mg/kg carprofen) given immediately following surgery.

Under sterile conditions, a midline laparotomy was performed and the intestines were eviscerated. 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 suture. Following surgery, animals were placed in warm cage and allowed to recover. Once fully awake and alert, animals were returned to animal housing.

Drug Administration

Sildenafil was obtained through a generous donation from Pfizer (New York, NY). It was reconstituted from its lyophilized powder in PBS daily for experimentation. Following removal of the vascular clamp from the superior mesenteric artery, sildenafil in PBS vehicle (250ul) was administered intraperitoneally. Experimental groups were: 1) PBS vehicle, 2) 0.01 mg/kg sildenafil, 3) 1.0 mg/kg sildenafil, 4) 10 mg/kg sildenafil, 5) 100 mg/kg sildenafil, and 6) 1000 mg/kg sildenafil.

Perfusion Analysis

Intestinal mesenteric perfusion was analyzed using a Laser Doppler perfusion Imager (LDI; Moor Instruments, Wilmington, DE) as we have previously described [11]. Perfusion images were acquired at baseline, at initial clamping of the superior mesenteric artery and at 24 hours following intestinal ischemia (N=8/group). 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 data was normally distributed and expressed as a percentage of baseline (mean±SEM). After the 24-hour recovery analysis, mice were

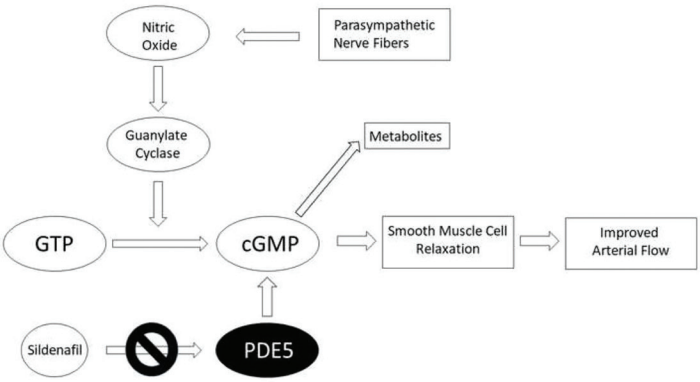


Figure 1: Mechanism of action of Sildenafil. Sildenafil inhibits PDE5, which then works to increase the concentration of cGMP. Elevated cGMP promotes vascular dilation by activation of potassium channels within the vascular bed.

euthanized with isoflurane overdose and cervical dislocation. Intestinal tissues were explanted for further analyses.

Histology Injury Score

Following euthanasia of experimental groups, terminal ileums were harvested and fixed in 4% paraformaldehyde with subsequent dehydration in 70% ethanol (N=8/group). Paraffin-embedded sections were prepared and stained with hematoxylin and eosin. Histological scoring of the depth of tissue injury was performed as previously described: 0, no damage; 1, subepithelial space at the villous tip; 2, loss of mucosal lining of 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 [12, 13]. Sections were evaluated blindly by three observers. Data were not normally distributed and are presented as median and interquartile range.

Intestinal Chemokine 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 Regulated on Activation, Normal T Cell Expressed and Secreted (RANTES), Macrophage Inflammatory Protein 1 alpha (Mip1a), Monocyte Chemoattractant Protein (MCP), Neutrophil Activating Protein (KC), and Granulocyte Colony Stimulating Factor (GCSF) 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 1:25 dilution according to the manufacturer's instructions and are reported in nanograms of chemokine per gram of total intestinal protein. Data were not normally distributed, and so are expressed as median and interquartile range. Experiments were repeated to insure accuracy (N=12-13/group).

Statistical Analysis

Data were assessed for normalcy by the Shapiro-Wilk and KS normality tests. Normally distributed data were compared with Student's t test while nonparametric data were compared with Mann Whitney U test. All statistical analyses were performed using GraphPad Prism 7 (GraphPad Software, La Jolla, CA). Parametric data were expressed as mean ± SEM while nonparametric data were reported as median and interquartile range. p-values less than 0.05 were considered statistically significant.

Results

Perfusion

Mesenteric perfusion was noted to be depressed following clamping of the superior mesenteric artery. Once the clamp was removed, reperfusion was allowed (Figure 2). Sildenafil did not offer any significant improvements over vehicle when measuring mesenteric perfusion following intestinal IR. The highest dose of sildenafil tested was 1000 mg/kg. All animals died at this dose, and therefore, their perfusion was counted as 0 since they died before the 24 hour assessment. As a result, the 1000 mg/kg dose had significantly lower perfusion (0% +/- 0%) compared to vehicle (53.03% +/- 11.35) or any of the other doses of sildenafil tested (Figure 3, $p<0.05$).

Histologic Injury

Intestinal epithelial injury scores were significantly improved for the lower doses of sildenafil compared to vehicle (vehicle: 3 (IQR 1.75), 0.01mg/kg: 2 (IQR 2), 1.0mg/kg: 2 (IQR 1.75), 10.0mg/kg: 3 (IQR 3), $p<0.05$). There was no difference between vehicle and the 100 mg/kg dose (3 (IQR 2.75), $p=.309$). The 1000 mg/kg (5 (IQR 0)) dose was significantly worse than vehicle ($p<0.05$), and significantly

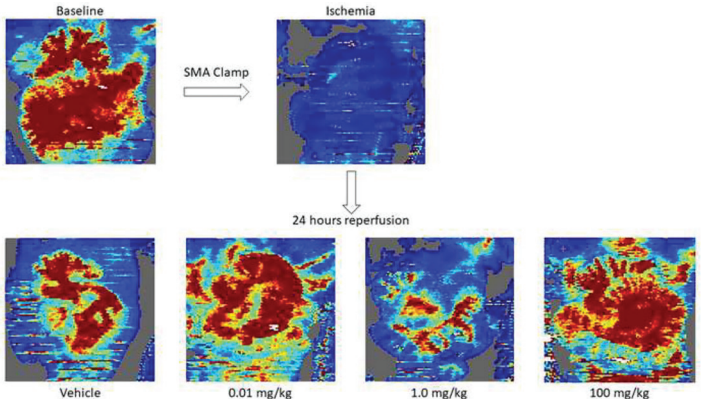
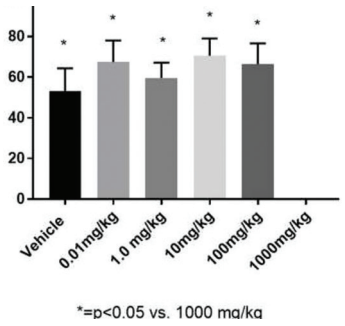


Figure 2: Mesenteric Perfusion. Representative Laser Doppler images assessing mesenteric perfusion with varying doses of sildenafil.

Figure 3: Sildenafil Did Not Improve Mesenteric Perfusion. Mesenteric perfusion was not altered with the majority of doses tested. However, the highest dose tested, 1000mg/kg resulted in significantly lower perfusion compared to any of the other tested groups (*=p<0.05 versus respective vehicle).



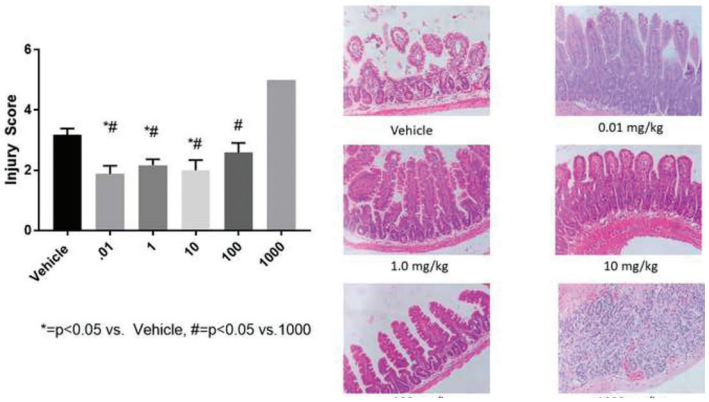


Figure 4: Intestinal Epithelial Injury. Intestinal epithelium had significantly less injury for the majority of sildenafil doses tested compared to vehicle. However, the highest dose tested, 1000mg/kg resulted in significantly worse mucosal damage compared to any of the other tested groups (*=p<0.05 versus vehicle, #=p<0.05 vs. 1000mg/kg).

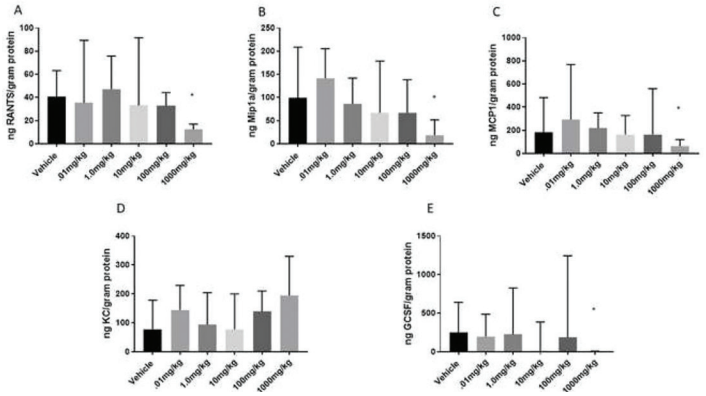


Figure 5: Leukocyte Chemokines. Intestinal levels of (A) RANTES, (B) Mip1a, (C) MCP, (D) KC, and (E) GCSF were not significantly different between vehicle and low/moderate sildenafil treated groups following intestinal IR injury. High dose sildenafil maintained lower levels of 4 of the 5 chemokines tested. (*=p<0.05 vs. Vehicle)

worse than all other tested doses of sildenafil (p<0.05, Figure 4).

Intestinal Chemokines

Intestinal lysates were normalized to total protein concentration. Sildenafil did not offer any significant improvements over vehicle with regard to the leukocyte chemoattractants RANTES, Mip1a, MCP, KC, or GCSF (Figure 5). The 1000 mg/kg dose was noted to have significantly lower RANTES, Mip1a, MCP, and GCSF compared to vehicle (p<0.05).

Discussion

Sildenafil has previously been shown to confer intestinal protection when given prior to ischemic injury [5-7]. Therefore, it seemed natural to expect that it would also provide protection when given following injury. Herein, we observed that sildenafil offered no improvements in mesenteric vascular perfusion, but did decrease the level of intestinal epithelial injury following ischemia. The highest dose of sildenafil was actually detrimental to animals as the 1000mg/kg dose yielded 100% mortality at 24 hours following injury. This translated into no mesenteric perfusion at 24 hours and the highest degree of mucosal injury.

Protective effects have been appreciated in intestinal injury with pretreatment of sildenafil. In a study by Soydan et al., sildenafil pretreatment prevented ischemia induced impairment of acetylcholine responses which could lead to dysmotility and impairments in vascular tone [7]. A 2005 study suggested that sildenafil releases endogenous mediators that work to increase nitric oxide production, which then activates guanylate cyclase to increase cyclic GMP levels. Elevations in these levels can then work to further vasodilate the end organ vascular bed [14]. It is difficult to understand why sildenafil offered no improvements in mesenteric perfusion when used as a rescue agent following intestinal IR. Perhaps a longer period between ischemia and measurement of reperfusion (greater than 24 hours) would have yielded notable differences. However, other studies that we have performed in the past have shown measurable differences in both perfusion and histologic injury after only 24 hours of reperfusion [10, 11].

Previous studies have also suggested that the generation of oxygen free radicals interferes with cellular function through the disruption of ionic homeostasis [15]. These radicals may exacerbate the injury seen following intestinal IR. Additionally, leukocytes have been observed to infiltrate the injured intestine following injury [16], and therefore it would be expected that leukocyte chemoattractants would be elevated as well [17]. However, herein, we did not see any alterations in common leukocyte chemoattractants following low to moderate dose sildenafil administration. This would suggest that the protective mechanism observed is more complex than simply limiting inflammation or leukocyte infiltration within the intestinal epithelium. The 1000 mg/kg dose did demonstrate significantly lower levels of four of the five chemokines measured. This is likely due to the severe ischemia that developed secondary to cardiovascular collapse, and the inability of the circulation to deliver chemokines and leukocytes to the intestinal tissue bed.

The discrepancy between sildenafil's ability to provide protection when given prior to injury versus its limited ability to facilitate rescue following injury is indeed perplexing. In our study we saw only epithelial (intestinal mucosa) protective qualities of sildenafil, but not endothelial (mesenteric vasculature) protection. Given that the superior mesenteric artery was clamped for 60 minutes it is likely that endothelial damage occurred in the mesenteric vessels. It is possible that this damage progressed beyond a state that would allow sildenafil to promote vasodilation and improved perfusion, although several other cellular and drug compounds have been shown to promote vasodilation in the post-ischemic period [10, 18]. Therefore, it is likely something specific to the compound's bioavailability or to the length of time that it takes from drug delivery to onset of action.

Previous studies have also suggested that sildenafil has specific epithelial protective properties as well. A study of cardiomyocytes suggested that the preconditioning of these cells prior to hypoxia was able to decrease necrosis and apoptosis. When eNOS was pharmacologically inhibited or genetically ablated these effects were attenuated [19]. Separate studies on acute lung injury have suggested that sildenafil can also decrease lung epithelial injury independent of vascular dilatation. In this model, sildenafil decreased epithelial leakiness, oxidative damage, and apoptosis [20]. These data would confirm our findings of epithelial protection and suggest a role for sildenafil beyond its vasodilatory properties.

Multiple dose ranges from 0.01 mg/kg all the way up to 1000mg/kg were tested. 0.01, 1.0, and 10.0 mg/kg did not alter mesenteric perfusion or intestinal leukocyte infiltration, but did appear to limit intestinal epithelial damage. The typical dose for humans with erectile dysfunction is between 50-100 mg, which is approximately 0.7-1.5 mg/kg for a typical 70 kg male [21]. This contrasts with the treatment of pulmonary hypertension in infants from the Sildenafil in Treatment-Naïve Children, Aged 1-17 Years, With Pulmonary Arterial Hypertension trials (STARTS-1 and STARTS-2) which used ranges from less than 1.5 mg/kg/day to greater than 7.5 mg/kg/day [22]. We therefore felt that we had an appropriate range of therapy based on previous well-established human trials.

Given the lack of effectiveness of the lower doses to improve mesenteric perfusion, we also elected to try 100 and 1000 mg/kg, which would be considered supra-therapeutic, at nearly 10 to 100 times the doses used in the trials previously noted. 100 mg/kg had no effect compared to vehicle. It offered no improvement in perfusion, mucosal injury, or chemoattractant concentrations within the intestine. However, 1000 mg/kg resulted in cardiovascular collapse and prompt demise within the first 12-24 hours. Clearly this dose had a profound effect on mesenteric perfusion and mucosal injury, albeit an adverse one. It is unclear why the lower doses had no effect on the animal's perfusion, but a measurable difference in mucosal injury, while the largest dose had a completely detrimental effect within the first 12-24 hours. It is likely that the distribution of specific isoforms of PDE5 are

most pronounced in the lungs and central cardiovascular system, and less available in other end organ vascular beds [23]. This would explain why sildenafil has an effect on lungs and erectile tissue, but minimal to no effect on the mesenteric vascular bed. However, this does not explain why sildenafil given prior to injury promotes improved intestinal blood flow while given following injury only appears to protect the epithelial tissue of the intestine.

This study has several limitations. The first is the animal model used to assess intestinal ischemia. In reality, most patients likely develop segmental areas of intestinal ischemia rather than pan-intestinal ischemia. Therefore, this model may be more severe than what is seen clinically. That being said, we feel that because it is more severe than what may be seen clinically, any benefit seen in the model should be translatable to the clinical setting.

Conclusion

Intestinal ischemia is a devastating clinical problem. Post-ischemic application of sildenafil does not appear to improve mesenteric endothelial protection through improvements in mesenteric blood flow but does appear to limit intestinal epithelial injury. This protection is not mediated by ameliorating leukocyte infiltration, as leukocyte chemoattractants were similar between vehicle and treated groups. Further studies are needed to explore the endothelial and epithelial disparities associated with the post-ischemic application of sildenafil as a rescue agent following intestinal IR.

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