HHS Public Access

Author manuscript

J Pediatr Surg. Author manuscript; available in PMC 2020 November 01.

Published in final edited form as:

J Pediatr Surg. 2019 November ; 54(11): 2402–2407. doi:10.1016/j.jpedsurg.2019.02.021.

Dithizone Induced Paneth Cell Disruption Significantly Decreases Intestinal Perfusion in the Murine Small Intestine.

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Abstract

Purpose: Necrotizing enterocolitis is associated with decreased intestinal perfusion and ischemia. Paneth cells, specialized epithelial cells, have been shown to regulate the intestinal vasculature and disruption of these cells has been associated with NEC. We hypothesized that Paneth cell disruption in immature mice intestine would decrease the perfusion of the intestinal microvasculature.

Methods: Paneth cells were disrupted in P14–16 mice using chemical (dithizone) and transgenic (diphtheria toxin) methodology. Six hours after Paneth cell disruption, Dylight 488 was injected directly into the left ventricle and allowed to perfuse for five minutes prior to intestinal harvesting. Tissue samples were evaluated with confocal fluorescence microscopy to quantify intestinal perfusion and samples were quantified by real time RTPCR for gene expression.

Results: Dithizone treatment significantly decreased intestinal perfusion compared to controls ($p<0.01$). However, diphtheria toxin treatment demonstrated no significant difference in perfusion ($p>0.21$). Intestines from all treatment groups had similar PECAM staining, but intestines treated with dithizone had significantly decreased nNOS and iNOS gene expression compared to controls ($p<0.007$).

Conclusions: Paneth cell disruption significantly decreases the perfusion of the small intestinal microvasculature in a dithizone-specific manner. Dithizone has no effect on the amount of microvasculature, but does impact genes critical to nitric oxide signaling likely contributing to mesenteric vasoconstriction.

Keywords

Necrotizing enterocolitis; Animal model; Intestinal microvasculature; Nitric oxide

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Declarations of interest: none

Introduction:

Necrotizing enterocolitis (NEC) remains one of the most common gastrointestinal emergencies acquired by premature infants. It is currently estimated that 30% of patients who develop NEC will go on to require surgical intervention and of those, 50% will die from the disease [1]. While the exact pathophysiology of NEC remains unclear, decreased blood flow in the microvasculature of the small intestine has been postulated to play a critical role in the pathogenesis, as >90% of intestinal tissue affected by NEC have histopathologic evidence of coagulation necrosis [1–4]. Animal models of experimental NEC have demonstrated significantly altered intestinal microvascular blood flow, decreased size of intestinal arterioles, and disrupted villous microvasculature compared to controls [2, 4–7]. In addition, studies that looked directly at the blood flow in the superior mesenteric artery (SMA) in infants found that infants who later developed NEC had an initial high-resistance pattern of blood flow in the SMA on the first day of life [8]. These studies suggest that intestinal microcirculatory dysfunction and altered blood flow may contribute to the subsequent development of NEC. However, the underlying mechanisms by which intestinal hypoperfusion occurs and induces intestinal damage and inflammation that lead to NEC remain unclear.

One possible cause of altered small intestinal blood flow is through Paneth cell disruption. Paneth cells are specialized epithelial cells that were first described in the late 1800's as a granular cell type located within the small intestine at the base of the Crypts of Lieberkühn [9]. This location places Paneth cells just several cell diameters away from the microvasculature as it enters and exits small intestinal villi [10]. The dense granules in Paneth cells contain multiple antimicrobial peptides, angiogenins, cytokines, and vasoactive substances which are released into the crypt lumen constitutively, as well as at higher rates in response to exposure to bacterial antigens [11]. In doing so, Paneth cells are critical for many normal intestinal processes including regulation of the intestinal microbiome, prevention of bacterial translocation, maintenance of intestinal epithelial stem cells, and for regulating the intestinal microvasculature [12, 13]. Disruption of these important cells has significant adverse consequences such as decreased clearance of bacterial pathogens [14, 15], and development of inflammatory bowel disease [16–21]. Several studies have demonstrated a decrease in lysozyme-staining Paneth cells in the intestines affected by NEC compared to controls [20, 22–24]. However, mechanisms linking Paneth cell disruption or dysfunction to altered intestinal microvasculature and the development of NEC have not been previously studied. Taken together, given the close proximity of Paneth cells to the intestinal microvasculature, the vasoactive substances secreted by Paneth cells, and proposed involvement of Paneth cell dysfunction in NEC pathogenesis, we hypothesized that a Paneth cell disruption would significantly decrease the intestinal microvascular perfusion in immature mice.

Material and Methods:

1.1 Mice

All animal experiments were performed according to protocols approved by the Institutional Animal Care and Usage Committees at the University of Iowa (Iowa City, IA) and all mice

were housed in standard conditions until experimentation. All experiments used P14–16 day old C57Bl/6J mice or mice on a C57Bl/6J background. *PC-DTR* mice were generated by inserting a HA-tagged human diphtheria toxin receptor into the cryptdin-2 promotor as previously described [25]. Td Tomato mice were purchased from Jackson Laboratories (stock 007576 mT/mG).

1.2 Paneth cell disruption

Paneth cells were disrupted as previously described [22, 25] via intraperitoneal injection with either 75mg/kg body weight dithizone (Sigma) dissolved in 25mM Lithium carbonate solution (C57Bl/6J mice) or 40ng/g body weight diphtheria toxin in phosphate buffered saline (*PC-DTR* mice). Sham animals were injected with an equivalent volume of buffer alone. All mice were monitored for 6 hours following injection.

1.3 Evaluation of intestinal microvasculature

Intestinal microvasculature perfusion was measured as previously described by multiple laboratories including Besner and Hackam [4, 26, 27]. Briefly, mice were anesthetized with a one-time intraperitoneal injection of ketamine (80mg/kg) and Xylazine (10mg/kg). Mice were then placed on a warming pad and a midline chest wall incision was made to visualize the left cardiac ventricle. Using a 30g needle, 50μL of 20mg/mL Dylight-488 (AbCam) was injected into the left ventricle over 10 seconds. The heart rate was monitored for 3–5 minutes following the injection to ensure adequate perfusion through the intestinal microvasculature. Mice were then euthanized and the terminal third of the ileum was identified and harvested. Harvested sections were evaluated by cross sectional and whole mount preparations [4]. For whole mount intestines, ileal segments were washed with PBS and opened longitudinally before fixation in a 4% paraformaldehyde solution shaken gently at 4°C overnight. Tissue samples were rinsed with cold PBS three times followed by incubation with 10% sucrose/PBS for 4 hours at 4°C and then incubated in 30% sucrose/PBS overnight at 4°C. Whole mount preparations were mounted on glass slides with the villous side up. Cross sectional tissue samples were fixed as above, frozen in OCT medium, and cut in 50μm thick sections for further evaluation.

1.4 Immunohistochemistry and microscopic quantification

Whole mount samples were washed with PBS and blocked with 5% normal goat serum. Anti-CD31 (BD Biosciences) was applied (at 1:50 dilution) and incubated at 4°C overnight in 1% NSG/TBST prior to secondary incubation with goat anti rat A-568 (BD Biosciences) (1:500 dilution). Whole mount and cross-sectional tissues were viewed using a Zeiss LSM 710 confocal microscope. Image J was used to quantify the Dylight 488 fluorescence per high powered field using a 10x objective to obtain a quantitative measurement of the circulating intestinal blood volume. To determine tissue quantity of eNOS, cross-sectional samples were washed with PBS and blocked with 5% normal goat serum. eNOS polyclonal antibody (Thermo Fischer Scientific) was applied (at 1:100 dilution) and incubated at 4°C overnight in 1% NSG/TBST prior to secondary incubation with anti rat IgG (Vector Laboratories).

1.5 Real Time RT-PCR

Ileal samples were homogenized using a TissueLyser LT (Qiagen) as previously described [22, 28, 29]. RNA was isolated using RNeasy Plus Mini Kit (Qiagen) according to the manufacturer's directions. RNA concentration and quality were determined using a NanoDrop 1000 Spectrophotometer (Thermos Fisher Scientific). Quantitative real-time reverse transcription-polymerase chain reaction (qRT-PCR) was performed using Taqman Gene Expression Assays for nNOS(1), iNOS(2), and eNOS(3) (Life Technologies). qRT-PCR reactions were run in C1000 Thermal Cycler (Eppendorf) using the CFX96 Real-Time PCR Detection System (Bio-Rad). Fold change in gene expression was determined by normalizing gene expression to β-actin in each sample. The 2ΔΔ-CT method was used to compare gene expression levels between samples.

1.6 Statistical Analysis

All experiments were performed in at least triplicate and specific sample sizes are noted in the results section. Microvasculature blood volume in the whole mount and villi were compared using non-parametric Student T-test to determine statistical significance using Graph Pad Prism v7.03. Quantitative RT-PCR was analyzed as described above. In all experiments, differences were considered to be statistically significance if $p<0.05$.

Results

Dithizone-induced disruption of Paneth cells resulted in a significant decrease in intestinal microvascular perfusion as demonstrated by decreased Dylight 488 fluorescence of the small intestine compared to controls. This decrease was found in both the whole mount ileal tissue (dithizone 22.64 ± 5.03 average fluorescent units vs. control 45.89 ± 6.55 average fluorescent units, $p<0.01$) as well as in the individual villi when measured in cross section (dithizone 15.28 ± 1.43 average fluorescent units vs. control 20.77 ± 1.36 average fluorescent units, $p<0.01$) (Figure 1). To determine if the decreased perfusion following dithizone treatment was due to Paneth cell disruption, we used a second, complimentary method of Paneth cell disruption. Transgenic mice with a human diphtheria toxin receptor inserted in their Paneth cells (*PC-DTR*) [25] were given a single intraperitoneal injection with diphtheria toxin to induce selective necrosis of their Paneth cells. Interestingly, mice treated with diphtheria toxin had no significant difference in the Dylight 488 fluorescence in either whole mount or cross section compared to controls (diphtheria 23.09 ± 7.52 average fluorescent units and 29.78 ± 1.73 average fluorescent units vs. control 20.05 + 5.57 average fluorescent units and 24.77 ± 1.96 average fluorescent units, $p=0.75$) (Figure 2).

To examine if dithizone-induced decreased intestinal microvasculature perfusion was due to destruction or alteration of the intestinal microvasculature, intestinal samples from all treatment groups were stained for the endothelial marker CD31 (PECAM-1). Histologic examination showed similar CD31 staining in all treatment groups (Figure 3).

As there were no structural differences in the microvasculature due to dithizone treatment, we next examined small intestinal tissue NOS gene expression as nitric oxide is a potent vasodilator previously postulated to play a role in the pathogenesis of NEC [26]. Mice

treated with dithizone had significantly decreased gene expression of nNOS (dithizone 0.49 vs. control 1.10, p=0.007) and iNOS compared to control (dithizone 0.72 vs. control 1.00, p=0.003). There was a trend towards decreased eNOS expression following dithizone compared to control (dithizone 0.92 vs. control 1.22, p=0.10, n=4). There was also no significant difference in any of the NOS gene expression in diphtheria toxin treated mice compared to controls (p>0.08) (Figure 4). To evaluate whether the decreased eNOS gene expression was associated with decreased intestinal tissue levels of eNOS, immunohistochemical staining for eNOS was performed on distal small intestines from control and dithizone treated mice. Histologic examination showed decreased eNOS staining in dithizone treated groups compared to controls (Figure 4).

Discussion

NEC is the single most devastating intestinal disorder seen in premature infants. The bowel injury that occurs with NEC is believed to be due to microbial dysbiosis with bacterial translocation across the immature intestinal epithelial layer, regional hypoxia with free radical formation and tissue necrosis [10, 22, 30, 31]. Hypoperfusion of the intestinal microvasculature has been theorized to play a role in either the initial development or the progression of intestinal injury seen in NEC [1, 3, 7]. However, mechanisms linking hypoperfusion with the development of NEC remain poorly defined. Paneth cell dysfunction has been shown in adults to disrupt adult intestinal microvasculature [13], and studies from our group have shown that Paneth cell disruption in immature animals followed by enteral dysbiosis results in intestinal injury and inflammation that is comparable to other animal models (hypoxia-hypothermia model and LPS + PFA model) and human tissue affected by NEC [22, 25, 32].

To determine if Paneth cell dysfunction alone could induce disturbances in the intestinal microvasculature in the immature mice, we used two established complimentary methods of disrupting Paneth cell biology [22, 25, 32, 33] and measured the resulting effects on microvasculature perfusion. Our data demonstrate that Paneth cell disruption in immature mice can result in a significant decrease in intestinal perfusion compared to controls, but that these effects are method-dependent. This divergent outcome is likely due to the fact that our Paneth cell disruption models work through different mechanisms while still inducing similar phenotypic results [33]. These data support the emerging hypothesis that Paneth cells help to regulate not just the intestinal microbiome, but host physiology as well.

Experimentally, a commonly used methodology for Paneth cell disruption is through administration of the heavy metal chelator dithizone. Dithizone is thought to affect Paneth cells because of their high concentration of zinc [14, 22, 34]. Initial studies in rats showed that 5 minutes following dithizone treatment, zinc-dithizonate complexes are seen within the Paneth cells, and within 60 minutes, these cells degenerate, become necrotic, and are subsequently shed into the crypt lumen [34]. However, recent studies by our group showed that treatment with dithizone significantly altered several autophagy genes including Atg10, *Atg4a, Atg12, and Ambra1* suggesting that dithizone may disrupt murine Paneth cells by induction of autophagy pathways [33]. Our lab has also shown that treatment of neonatal mice with dithizone significantly decreases Paneth cell counts by ~50% with no effect on the

number of mucin-positive Goblet cells [22] and no effect on barrier function, serum zinc levels, serum glucose levels, or serum cytokine levels [25]. However, as dithizone is a non-specific chelator, it remains very possible that it may have unintended off-target effects on either the host or its associated microbial community.

To help mitigate these potential issues, we utilized a transgenic mouse with specific sensitivity to diphtheria toxin. C57Bl/6J mice are naturally resistant to diphtheria toxin as they lack a receptor that can bind to diphtheria toxin (DTR). However, transgenic *PCDTR* mice express human DTR on the cell surface of their Paneth cells. Following exposure, diphtheria toxin selectively binds to DTR inducing receptor-mediated endocytosis [35]. Upon entry into the cytoplasm, diphtheria toxin inactivates elongation factor 2 which inhibits protein synthesis and results in necrotic death of the Paneth cell [35, 36]. Our lab has previously shown that treatment of PC-DTR mice significantly decreases the quantity of granule-positive Paneth cells per crypt and the expression of both the Paneth cell markers cryptdin and lysozyme [25].

While both these methods can selectively disrupt and decrease the number of intestinal Paneth cells, the results of our study show that only dithizone reduces the perfusion of the intestinal microvasculature. This novel effect of dithizone on the mesenteric perfusion is not due to damage or disruption of the intestinal microvasculature as the immunohistochemical staining for platelet endothelial cell adhesion molecule-1 (PECAM-1) of the intestinal vasculature was similar in all treatment groups. However, our data suggests that dithizone may cause vasoconstriction through altered NOS gene expression as dithizone treatment results in a significant decrease in RNA expression of neuronal nitric oxide synthase (nNOS) and inducible nitric oxide synthase (iNOS) as well as a trend towards decreased endothelial nitric oxide synthase (eNOS).

Nitric oxide (NO) is a potent vasodilator and has been implicated in regulating vascular tone, inflammation, and tissue repair [37]. NO is produced by the conversion of arginine to citrulline by nitric oxide synthase (NOS) [37]. There are three isoforms of nitric oxide synthase (NOS), each encoded by a separate gene: endothelial NOS (eNOS), neuronal NOS (nNOS), and inducible NOS (iNOS) [38]. eNOs and nNOS are constitutively expressed at low levels whereas iNOS is not expressed under normal conditions but induced in pro-inflammatory states. Nitric oxide is the primary vasodilator in the newborn intestinal circulation [3]. Studies have shown that the concentration of NO in the mesenteric artery and the response of the mesenteric vasculature to various NO mediated vasodilators are greater in newborns than in adults [1, 3, 39]. This makes the neonatal intestine more sensitive to changes in NO levels where even subtle changes may result in significant alterations in the hemodynamic parameters of the intestine. This may conceivably place premature infants at risk for the development of intestinal ischemia. Our results suggest that dithizone results in a significant downregulation of nNOS and iNOS expression. In addition, it also causes decreased eNOS expression, although significance was night quite reached, likely related to the small sample size. The decreased eNOS gene expression was accompanied by a decreased amount of eNOS within the small intestinal as seen with immunohistochemical staining. Therefore, the decrease in various NOS isoforms within the distal small intestine,

could subsequently lead to an overall decrease in the total nitric oxide levels, which could then result in increased vasoconstriction leading to decreased intestinal perfusion.

Interestingly, prior data from rodent models of NEC have shown a significant increase in inflammatory cytokines, iNOS mRNA expression, NO production, and enterocyte apoptosis following gavaging Cronobacter sakazakii to immature mice to induce significant intestinal injury consistent with NEC [40]. Similar results have also been reported with animal models of NEC that utilize formula feeding and hypoxia or LPS [41–43]. However, it is important to note that all of these animal models result in significant tissue injury and elevation of inflammatory cytokines which act as potent inducers of NO production. Talavera et al has shown that intestinal epithelial cells exposed to inflammatory cytokines resulted in a significant increase in iNOS mRNA expression and NO production greater than direct LPS exposure [42]. In addition, these same studies have shown that increased NO production results in significant enterocyte apoptosis and necrosis while inhibiting repair mechanisms leading to increased intestinal injury, intestinal barrier failure, and bacterial translocation [40–43]. While these studies attempted to understand the role of NO isoforms in NEC-like injury, our study was designed to understand the specific effect of Paneth cell disruption on the blood flow of the intestinal microvasculature and were not designed to induce tissue injury. Therefore, the results of our study likely differ from those previously published because Paneth cell disruption with dithizone or diphtheria toxin administration alone does not produce significant intestinal injury or inflammation that is required for the increased iNOS expression or NO production as previously reported.

In conclusion, our data shows that dithizone-induced Paneth cell disruption in immature mice results in a significant decrease in the intestinal perfusion compared to controls and a concurrent decrease in nNOS and iNOS gene expression and tissue eNOS levels. However, this effect is importantly method-dependent as Paneth cells necrosis using a transgenic diphtheria toxin model results in neither response. This study provides additional insight on Paneth cell biology and their role on regulating the intestinal microvasculature. This data also advances our understanding of the NOS signaling pathway and downstream effects which are critical due to the role they play in the pathogenesis of NEC.

Acknowledgements:

Funding: SJM is supported by the National Institutes of Health DK097335 and the Carver College of Medicine at the University of Iowa. MG is supported by K08DK101608, R03DK111473 and R01DK118568 from the National Institutes of Health, March of Dimes Foundation Grant No. 5-FY17–79, the Children's Discovery Institute of Washington University and St. Louis Children's Hospital, and the Department of Pediatrics at Washington University School of Medicine, St. Louis.

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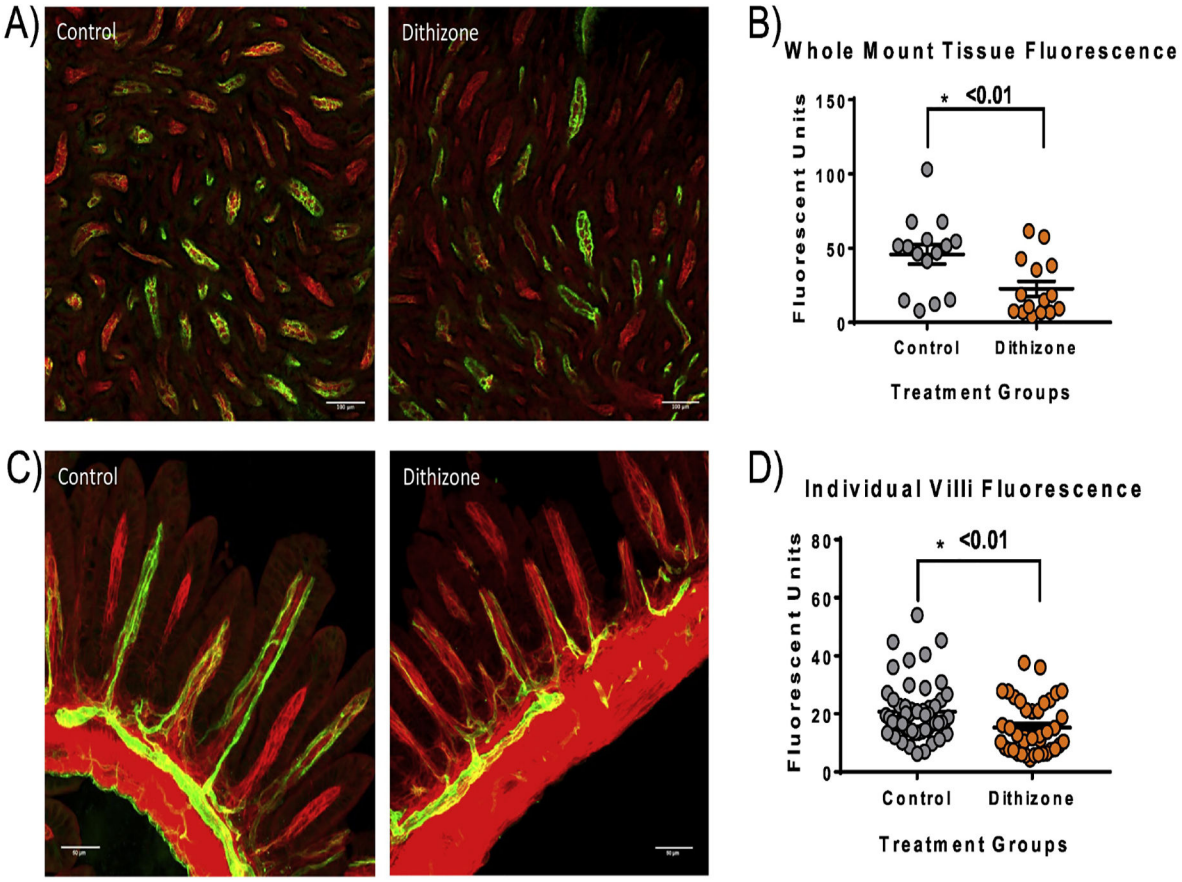

Fig 1. Paneth cell disruption with dithizone significantly decreases the small intestine microvascular perfusion.

(A) Representative confocal fluorescent microscopy whole mount ileal images from mice treated with dithizone compared to controls showing decreased intestinal microvascular perfusion following dithizone treatment. (B) Dithizone treated mice had significantly decreased green Dylight 488 fluorescence in the whole mount intestine compared to controls ($p < 0.01$). (C) Representative confocal fluorescent microscopy images of the ileum in cross section from mice treated with dithizone compared to controls showing decreased perfusion of the individual intestinal villi compared to control. (D) Dithizone treated mice had significantly decreased green Dylight 488 fluorescence in the individual small intestinal villi compared to controls ($p<0.01$). Scale bar equals 100μm.

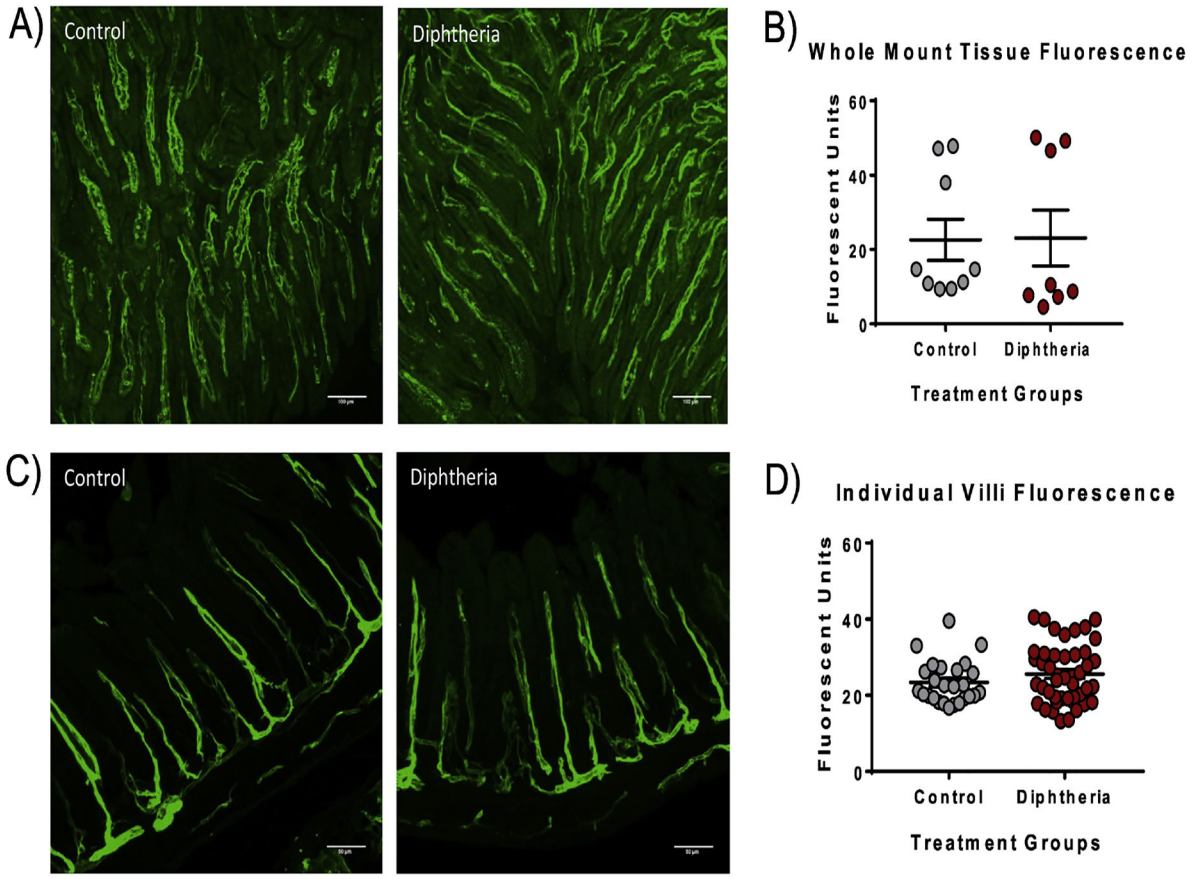

Fig 2. Paneth cell disruption with diphtheria toxin results in no significant change in the small intestine microvascular perfusion.

(A) Representative confocal fluorescent microscopy whole mount ileal images from mice treated with diphtheria toxin compared to controls showing similar intestinal perfusion. (B) The diphtheria toxin treated mice had no significant difference in the green Dylight 488 fluorescence in the whole mount intestine as controls ($p=0.96$). (C) Representative confocal fluorescent microscopy images of the ileum in cross section from mice treated with diphtheria toxin compared to controls again showing similar intestinal perfusion in the individual villi as the control. (D) The diphtheria toxin treated mice had no significant difference in the green Dylight 488 fluorescence in individual small intestinal villi compared to the controls ($p=0.21$). Scale bar equals 100μm.

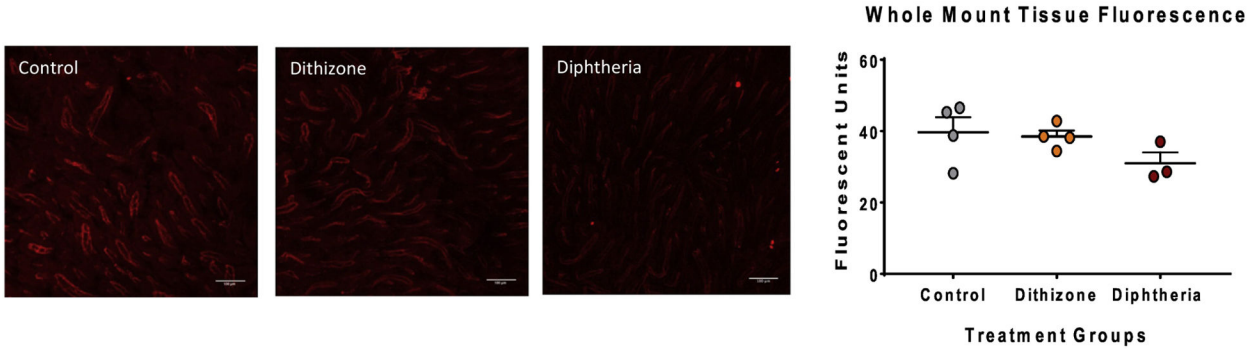

Fig 3. PECAM staining of the intestinal microvascular endothelium was similar among all treatment groups.

(A) Representative confocal fluorescent microscopy whole mount images of the intestine stained for CD31 (red fluorescence) from each treatment group (Control, Dithizone, and Diphtheria toxin) shows similar staining among all three groups. Scale bar equals 100μm. (B) There was no significant difference in the average fluorescence of CD31 among all treatment groups (p=0.27).

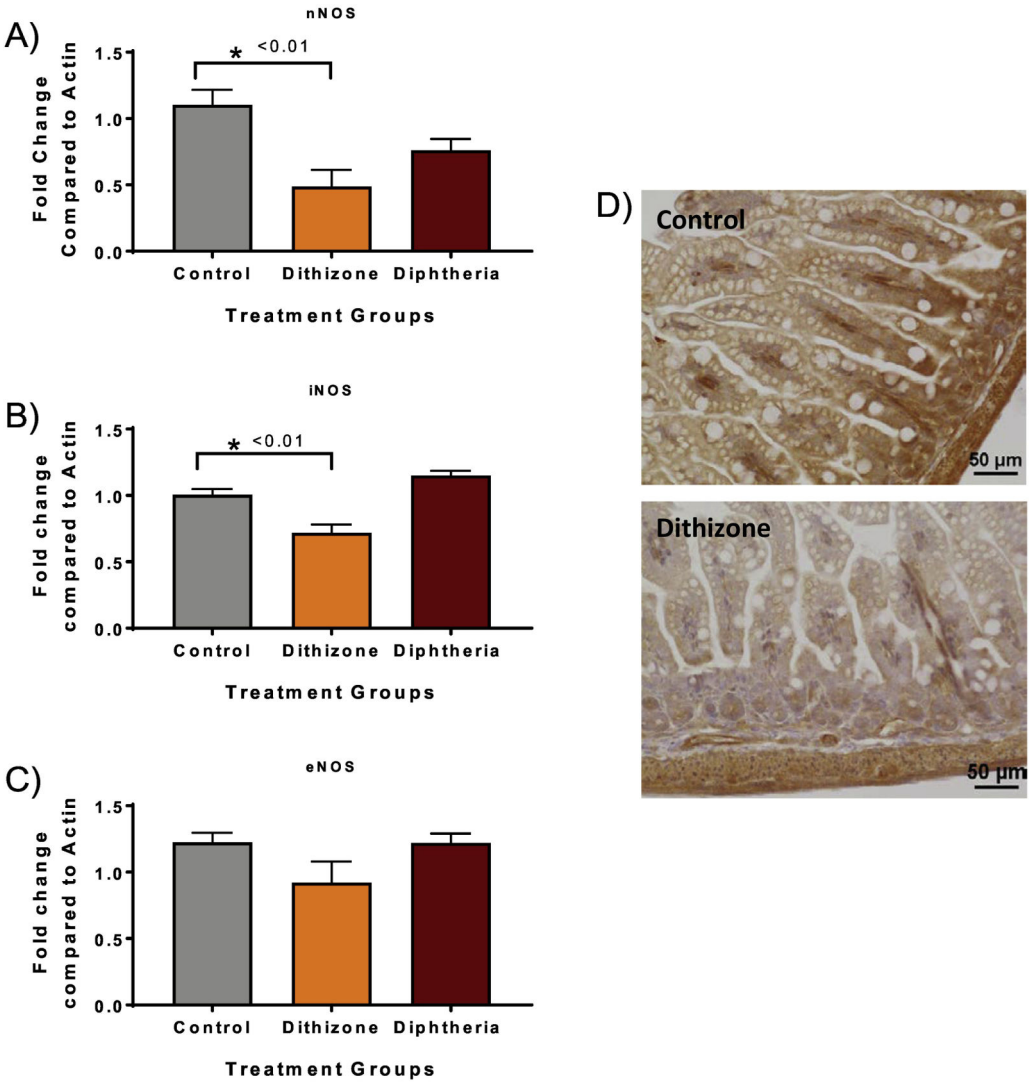

Fig 4. Paneth cell disruption with dithizone results in a significant decrease in small intestine mRNA expression of NOS genes.

(A) Dithizone treated mice had significantly decreased mRNA expression of nNOS ($p<0.01$) and (B) significantly decreased mRNA expression of iNOS compared to controls ($p<0.01$). (C) Dithizone treated mice had a trend towards decreased eNOS expression but significance was not quite reached ($p=0.10$). Diphtheria toxin treated mice had no significant difference in any NOS gene expression compared to controls. (D) Immunohistochemical staining for eNOS (brown) in the small intestine from control and dithizone treated mice showed decreased eNOS staining in the dithizone treated group. Scale bar equals 50µm.