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Mesenteric neovascularization during spring-mediated intestinal lengthening☆

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ABSTRACT

Background: Short gut syndrome, a condition characterized by inadequate absorption of nutrients owing to decreased bowel length, has minimal avenues for treatment. We have proposed spring-mediated distraction enterogenesis to lengthen bowel in porcine jejunum as a treatment for short gut. We aim to evaluate the extent of mesenteric neovascularization in segments of lengthened bowel via spring-mediated enterogenesis.
Methods: Female juvenile Yucatan pigs underwent laparotomy and insertion of gelatin-encapsulated compressed nitinol springs, held in place with plication sutures, into the jejunum. At surgery and sacrifice, macroscopic mesenteric blood vessels were counted between the plication sites. Histologic samples of the mesentery were obtained to evaluate microscopic vasculature.
Results: A statistically significant increase in macroscopic mesenteric blood vessels was seen after intestinal lengthening (before: 1.9 ± 0.7 vessels, after: 4.7 ± 1.2 vessels, $p = 0.001$). A statistical significance is also seen in the density of arterioles (control: 3.0 ± 3.0 vessels/mm, spring: 7.0 ± 9.0 vessels/mm, $p = 0.01$) and venules (control: 4.0 ± 3.0 vessels/mm, spring: 8.0 ± 8.0 vessels/mm, $p = 0.003$).
Conclusion: Intestinal segments lengthened by intraluminal springs demonstrated total greater number of macroscopic vessels and microscopic blood vessels per length of mesentery as compared to control. This suggests local changes within the mesentery to recruit blood supply to growing intestine.
Level of evidence: N/A
Type of study: Treatment study.

Short gut syndrome (SGS) is a debilitating medical condition that is characterized by malabsorption and malnutrition owing to extensive intestinal loss [1–5]. It is most commonly seen in the pediatric population and has numerous causes such as necrotizing enterocolitis, aganglionosis, intestinal atresia, midgut volvulus, and massive intestinal resection owing to infarctions from abdominal wall defects [1,6,7]. Mortality rates for SGS have been reported to be 20%–40%, and the disease process and its treatments are associated with significant morbidity [2,5,8,9].

While a high-calorie high-protein diet including mineral and micronutrient supplementation is recommended, patients often rely on parenteral nutrition (PN) to provide essential nutrients and vitamins independent of the gastrointestinal system. However, over time, PN is associated with infections, venous thrombosis as well as liver and kidney dysfunction [10,11]. These complications result in significant morbidity and increased risk of mortality [10,11]. Surgical intervention aimed at slowing intestinal transit, increasing absorptive surface, and lengthening the bowel or intestinal transplant has also had limited success highlighting that true enteral autonomy is relatively rare with current treatment methods [7,10–14]. Thus, we have focused on generating a novel therapy for the treatment of SGS using spring-mediated distraction enterogenesis.

Spring-mediated distraction enterogenesis has emerged as a viable solution to lengthen bowel [2,15–19]. Our previous studies have successfully demonstrated intestinal lengthening using springs in numerous animal models [13,16,20,21]. Lengthened segments of bowel have exhibited changes in the mucosa and muscularis layers. Given the increased tissue growth, we hypothesize an increase in mesenteric neovascularization will occur in order to supply increased blood flow and nutrients to the new tissue. This study aims to examine the vasculature associated with the lengthened segment of intestine.

1. Methods

1.1. Animal preparation and surgical procedure

The Administrative Panel on Laboratory Animal Care (protocol 32278) approved all animal surgeries. Female juvenile Yucatan pigs

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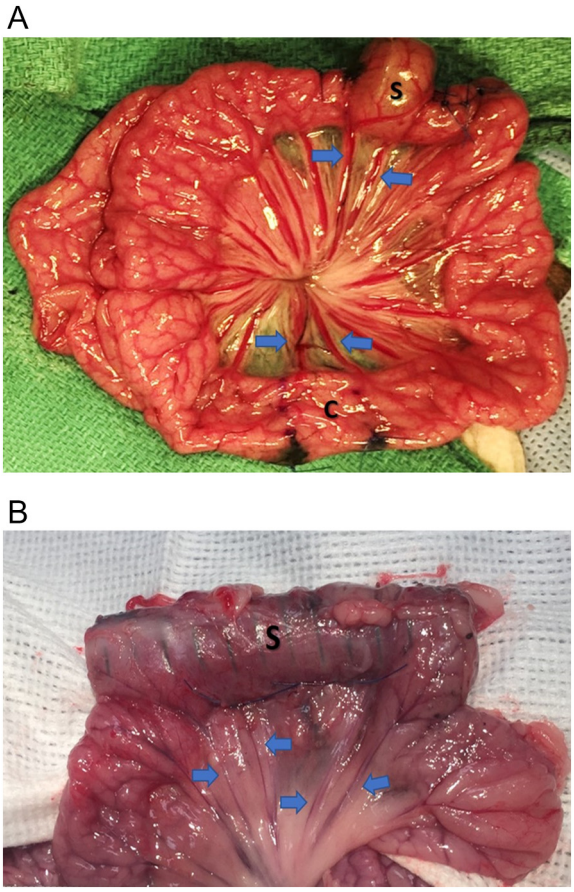

Fig. 1. Macroscopic view of mesenteric vessels. (A) Segment of jejunum from porcine intestine at time of initial surgery demonstrating 2 mesenteric vessels (blue arrows) within the spring segment (S) and 2 mesenteric vessels within the control segment (C). (B) Segment of jejunum from porcine intestine at time of sac demonstrating 4 macroscopic vessels (blue arrows) within the plication sutures of the spring segment (S).

ages 4–6 weeks from S&S Farms (Ramona, CA) were utilized in this study. Gelatin-encapsulated compressed nickel-titanium (nitinol) springs were placed in-continuity in the jejunum as previously described [2,15,16]. Briefly, all pigs were placed under general anesthesia and were prepped in draped in a sterile fashion. A midline laparotomy incision was made and the ligament of Treitz was identified. Encapsulated springs were inserted approximately 60 cm from the ligament of Treitz through an antimesenteric longitudinal incision. The bowel was plicated to 50% of the bowel diameter proximally by two interrupted 4-0 polypropylene sutures and distally by four sutures to ensure the spring remained in place. A control segment of similar length to the spring segment was also marked with 2 4-0 polypropylene sutures. The enterotomy was closed primarily, the bowel was returned to the abdomen, and the abdomen was irrigated and closed with 0-polydioxanon suture. Animals were given a liquid diet postoperatively.

1.2. Gross and histologic evaluation

Pigs were euthanized on postoperative day (POD) 7 and POD 14. Macroscopic blood vessels were counted at the times of surgery and sacrifice within the plication sutures (Fig. 1A and B). For examination of the microscopic blood vessels, we collected the mesentery close to the gut and samples of bowel from both the control segment of bowel and spring segment. Samples were fixed in 10% buffered formalin and imbedded in paraffin. Slices of 8 μm were obtained and adhered on glass slides. Hematoxylin and eosin staining was performed to observe the histology of the mesenteric specimens (Fig. 2A.1 and B.1). Slides were observed under the microscope with cellSens software (Olympus, Tokyo, Japan). Slides were reviewed under 40× and 100× magnification using bright field.

1.3. Immunohistochemical staining and evaluation

Smooth-muscle actin (SMA, Abcam, Cambridge, UK) primary antibody was incubated overnight on permeabilized slides. The following day, a secondary antibody (Life Technologies, Carlsbad, CA) was applied with 4′,6-diamidino-2-phenylindole (DAPI) to counterstain nuclei (Fig. 2A.2 and B.2). The number of vessels seen per high power filed was counted. Using ImageJ software (National Institutes of Health, Bethesda, MD) each mesenteric tissue sample was normalized by measuring the length of the sample to determine the density of blood vessels within the mesentery.

Segments of bowel were prepared as above and analyzed using ImageJ software. Each sample was normalized by measuring the perimeter of the sample to determine the density of blood vessels. Bowel layers were categorized into submucosa, longitudinal intramuscular and intermuscular (between the longitudinal and circular muscle layers) layers (Fig. 3).

1.4. Statistical analysis

Data were expressed as mean values ± standard deviation. Two-tailed paired Student's t-tests were used for statistical analysis of mesenteric blood vessels.

2. Results

All pigs tolerated the procedure well and survived to the study endpoint without complication ($N = 12$). There was minimal change in weight in all animals. Compared to the control segment, spring segments demonstrated average relative lengthening of 314% ± 130% ($p < 0.001$).

2.1. Macroscopic evaluation of mesenteric vasculature

At the time of surgery, spring segments in the jejunum had an average of 1.9 ± 0.7 macroscopic mesenteric blood vessels. Compared to when the animal was sacrificed, there was a significant increase in the number of macroscopic mesenteric blood vessels with an average of 4.7 ± 1.2 blood vessels ($p = 0.001$, Fig. 4).

2.2. Microscopic evaluation of mesenteric vasculature

Control segments of bowel had an average of 2.0 ± 2.1 arterioles and 2.0 ± 1.7 venules per high power field (Fig. 5A). Lengthened spring segments had significantly increased number of microscopic vessels: 3.0 ± 2.7 arterioles and 4.0 ± 2.4 venules ($p = 0.01$, $p = 0.002$, Fig. 5A).

The density of mesenteric vessels was also significantly increased in spring segments after lengthening when compared to the control segment of bowel (Fig. 5B). There were an average of 3.0 ± 3.0 arterioles/mm in the control bowel compared to 7.0 ± 9.0 arterioles/mm in the spring segment ($p = 0.01$). Likewise, there were 4.0 ± 3.0 venules/mm in the control bowel compared to 8.0 ± 8.0 venules/mm in the spring segment ($p = 0.003$). (See Fig. 5B)

2.3. Microscopic evaluation of bowel vasculature

2.3.1. Submucosa layer

Control segments of bowel had an average of 30.5 ± 30.8 arterioles and 62.3 ± 48.5 venules per high power field. In lengthened segments of bowel, there was a statistically significant increase in arterioles to 80.4 ± 53.0 ($p = 0.02$, Fig. 6). The average number of venules in spring lengthened segments of bowel also increased to 93.5 ± 68.7 ($p = 0.10$).

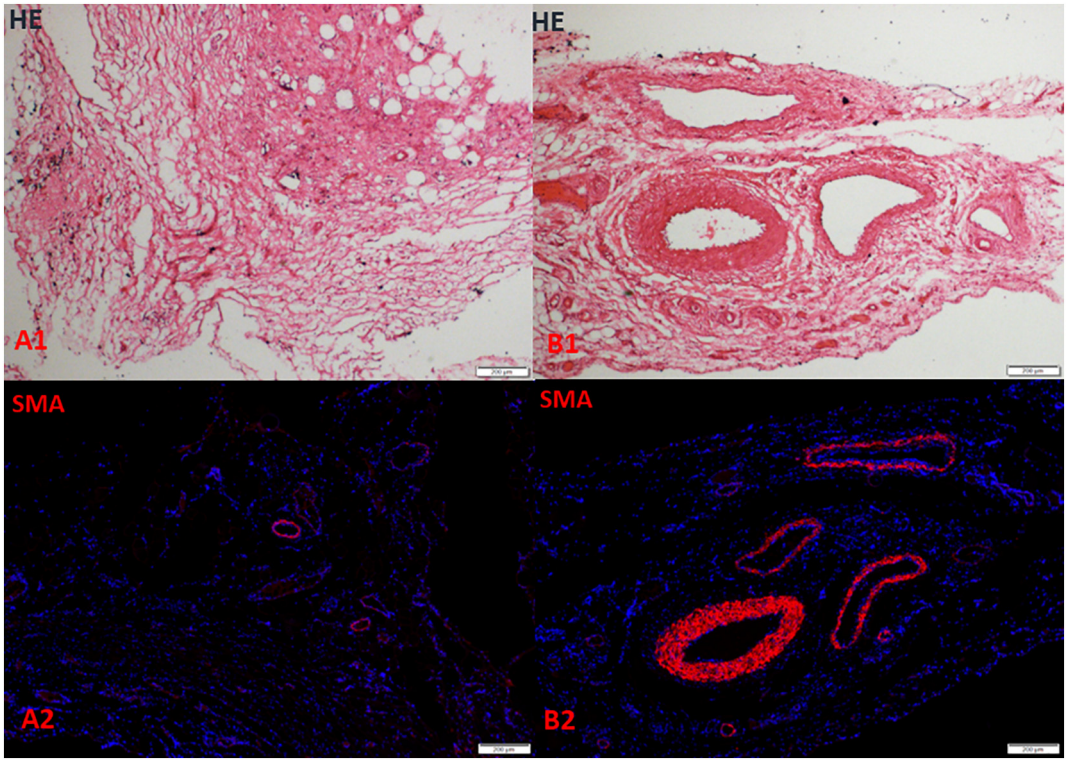

Fig. 2. Microscopic view of mesenteric vessels. Representative cross-sectional light micrographs of H&E and SMA stained immunochemistry of control segment of jejunum at 10× magnification. (A) Segment of control bowel. (B) Spring segment after lengthening, demonstrating 4 microscopic blood vessels. Arterioles are seen with thicker walls, while venules exhibit thinner walls.

Similar to the number of vessels, a statically significant increase in the density of arterioles was seen when comparing control bowel to spring-lengthened bowel (control: 1.12 ± 0.91 arterioles/mm, spring segment: 2.80 ± 1.03 arterioles/mm; p = 0.004, Fig. 7). The density of venules also showed an increase (control: 2.22 ± 1.31 venules/mm, spring segment: 3.05 ± 1.34 venules/mm; p = 0.12).

2.3.2. Longitudinal intramuscular layer

Spring lengthened segments of bowel demonstrated a statistically significant increase in arterioles when compared to control segments of bowel (spring segment: 9.9 ± 9.9 control: 0.4 ± 0.9; p = 0.01, Fig. 6). Likewise there was statistically significant increase in the average number of venules in spring lengthened segments (spring segment:

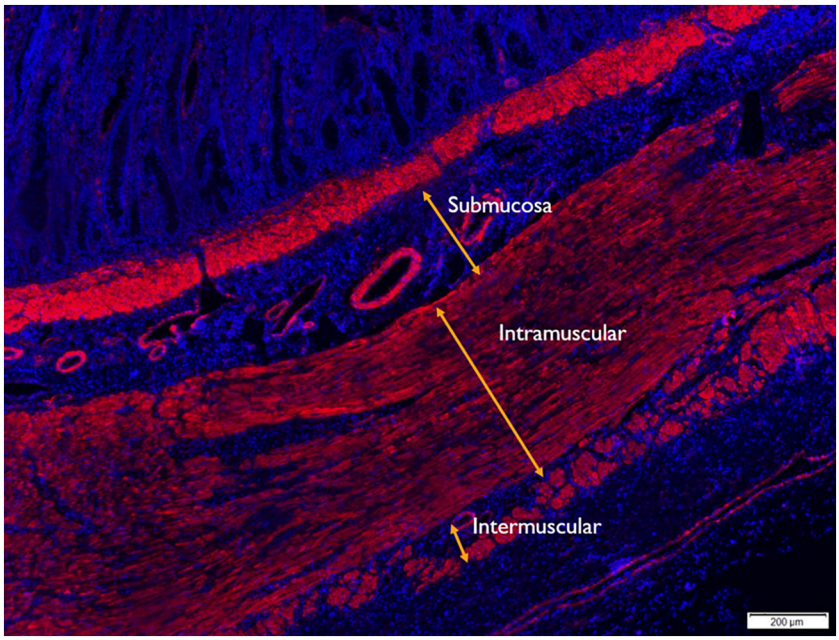

Fig. 3. Microscopic view of vessels within intestines. Immunofluorescence of SMA observed in different layers was used to evaluate microscopic blood vessels within the intestine.

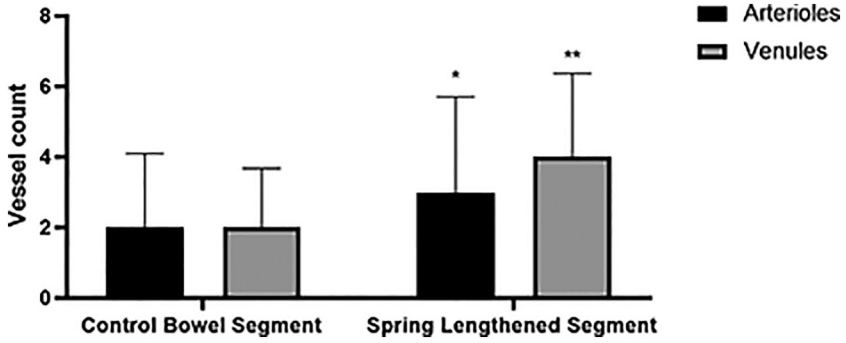

Fig. 4. Quantity of macroscopic mesenteric vessels. Differences in the number of mesenteric vessels for arterioles (mean ± SD: control 2 ± 2.10 vessels, spring 3 ± 2.70 vessels, *p = 0.012) and venules (control 2 ± 1.68 vessels, spring 4 ± 2.37 vessels, **p = 0.002) were significant.

15.6 ± 15.4 control: 3.8 ± 4.9; p = 0.01).

The density of blood vessels was significantly increased in spring segments after lengthening when compared to the control segment of bowel. There were an average of 0.01 ± 0.03 arterioles/mm in the control bowel compared to 0.25 ± 0.23 arterioles/mm in the spring segment (p = 0.005, Fig. 7). Likewise, there were 0.12 ± 0.14 venules/mm in the control bowel compared to 0.41 ± 0.35 venules/mm in the spring segment (p = 0.006).

2.3.3. Intermuscular layer

Control segments of bowel had an average of 27.4 ± 19.6 arterioles and 46.0 ± 25.6 venules. Lengthened spring segments demonstrated an increased number of microscopic vessels: 40.2 ± 24.7 arterioles and 50.8 ± 30.7 venules (p = 0.07, p = 0.55, Fig. 6).

The density of arterioles within bowel was not significantly increased in spring segments after lengthening when compared to the control segment of bowel. There were an average of 0.88 ± 0.68 arterioles/mm in the control bowel compared to 1.12 ± 0.68 arterioles/mm in the spring segment (p = 0.25, Fig. 7). The density of venules did not change as there were 1.44 ± 0.71 venules/mm in the control bowel compared to 1.43 ± 0.87 venules/mm in the spring segment (p = 0.91).

3. Discussion

This study sought to quantify and evaluate mesenteric neovascularization in porcine jejunum that had been lengthened via spring-mediated distraction enterogenesis. We demonstrated an increase in not only the macroscopic number of mesenteric blood vessels, but also microscopic vessels and the density of these vessels within the mesentery. Our group has previously demonstrated consistent increase in bowel length with mucosal thickening when utilizing compressed nitinol springs in a variety of porcine models [2,3,15,16,20]. This study produced similar results to previous works in terms of intestinal lengthening. It is the first study examining the role of blood vessel recruitment in our porcine models after utilizing spring-mediated enterogenesis.

The increase in vasculature that we observed in the mesentery of spring-lengthened segments is likely a result of increased blood flow and nutrients to support its expansion. In concordance with bowel lengthening the mesentery will form new blood vessels to support the demand for additional oxygen and nutrients to the growing tissue. We also demonstrated an increase in both the number of vessels and the density of vessels within the bowel wall of spring-lengthened segments further demonstrating the process of neovascularization in growing tissue. Other studies have found neovascularization of porcine bowel in response to a stretching force. For example, Ralls et al. utilized CT imaging with 3D reconstruction in order to demonstrate increased neovascularization seen in porcine bowel after distraction-induced enterogenesis using a curvilinear hydraulic device [4].

Our method of quantifying mesenteric vasculature relied on a combination of H&E staining and SMA immunochemistry. SMA antibody was utilized owing to its ability to identify newly formed blood vessels. This not only confirmed the presence of vasculature, but helped to differentiate arterioles and venules. A limitation of this technique is the possibility of operator error. The decision to determine which vessels to include in the analysis was based on the premise that arterioles are closely paired with venules. This, in addition to the thickness of the vessel walls helped to distinguish arterioles and venules, but it is possible that not all blood vessels were accounted for in each sample [22].

Angiogenesis plays an important role in tissue regeneration by providing oxygen and nutrients. Substrates such as insulin growth factor (IFG-1) and vascular endothelial growth factor (VEGF) have been implicated in the process of angiogenesis. When IGF-1 binds to IFG-1 tyrosine

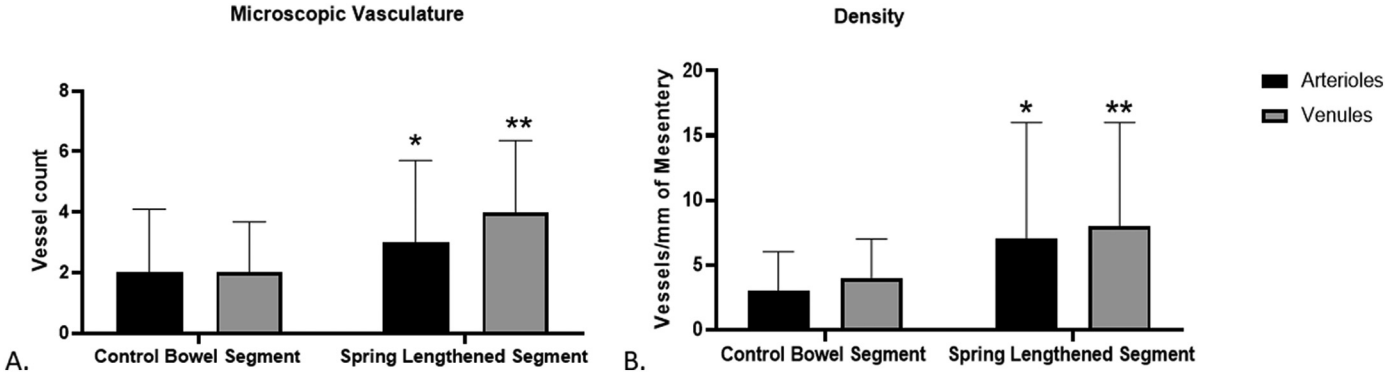

Fig. 5. A. Quantity of microscopic mesenteric vessels. Differences in the number of microscopic mesenteric vessels for arterioles (mean ± SD: control 2.0 ± 2.1, spring 3.0 ± 2.7, *p = 0.01) and venules (control 2.0 ± 1.7, spring 4.0 ± 2.4, **p = 0.002) were significant. B. Density of microscopic mesenteric vessels. Statistical differences were observed in the densivty of mesenteric blood vessels for arterioles (mean±SD: control 3 ± 3 vessels/mm, spring 7 ± 9 vessels/mm, *p = 0.012) and venules (control 4 ± 3 vessels/mm, spring 8 ± 8 vessels/mm, **p = 0.003).

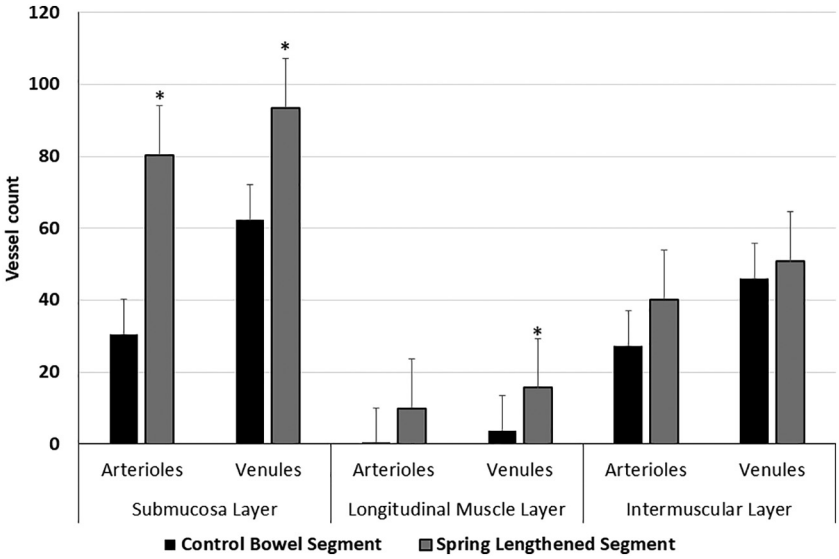

Fig. 6. Quantity of intestinal vessels. Differences in the number of arterioles and venules were seen in the submucosa layer and venules in the longitudinal intramuscular layer (*$p < 0.05$).

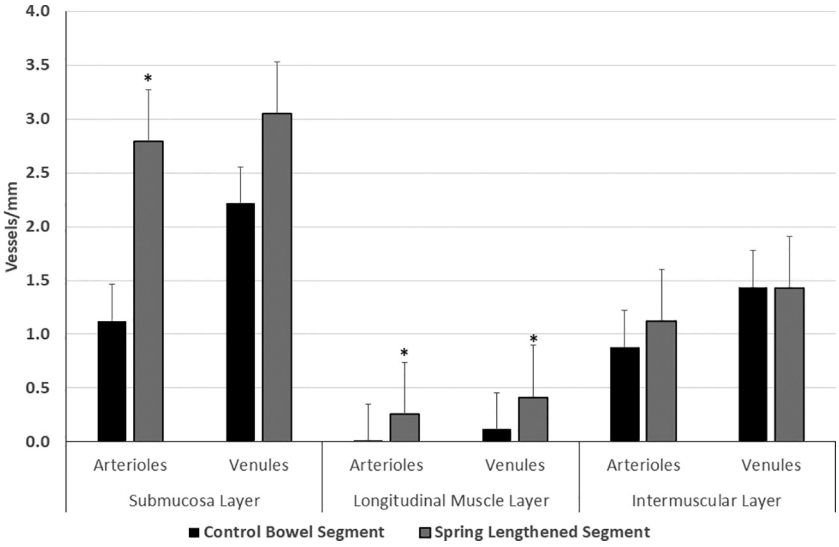

Fig. 7. Density of intestinal vessels. Statistically significant differences in the density of arterioles in the submucosa layer and arterioles and venules in the longitudinal intramuscular layer (*$p < 0.05$) were observed.

kinase receptor mediates numerous intracellular signals. In co-culture, IGF-1 has been shown to stimulate the expression of angiogenesis-related growth factors through P13-kinase/Akt signaling pathway [23]. Dunn et al. reported a 6-fold increase in the levels of IGF-1 in smooth muscle cells of lengthened rat jejunum [14]. Although this effect was likely observed owing to smooth muscle proliferation in jejunal segments, it is unknown if the effects of IGF-1 extended to mesenteric neovascularization. Similarly, VEGF plays a crucial role in angiogenesis. In fact, multiple members of the VEGF family have been implicated in vessel development during embryogenesis and are required for normal intestinal development and angiogenesis [24]. VEGF expression is regulated by hypoxia inducible factor-1 (HIF-1), which is activated by a physiologic hypoxic environment. The demand for oxygen and nutrients could outweigh the supply in growing intestine. In theory, this could prompt the release of HIF-1, activating VEGF. Given the wide range of effects of IGF-1 and VEGF, it is possible they also play a role in the development of increased mesenteric vasculature seen in the lengthened segments of jejunum. Future studies can aim to elucidate the proposed mechanism of angiogenesis in spring-mediated enterogenesis.

4. Conclusion

Spring-mediated distraction enterogenesis is a successful method of jejunal lengthening providing the groundwork for therapeutic intervention in the treatment of SBS. Mesenteric neovascularization occurs in concordance with bowel lengthening likely to provide additional nutrients and blood flow to growing tissue.

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