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Local tissue response to a C-X-C motif chemokine ligand 12 therapy for fecal incontinence in a rabbit model

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Abstract

This study aimed to determine if local injection of CXCL12 reduces sphincter fibrosis, restores sphincter muscle content, vascularization, and innervation, and recruits progenitor cells in a rabbit model of anal sphincter injury and incontinence. Adult female rabbits were assigned to 3 groups: uninjured/no treatment (control), injured/treated (treated), and injured/no treatment (untreated) (n=4 each). Injured groups were anesthetized and a section of external anal sphincter was removed at the 9:00 o'clock position. The treated sphincters were injected with 200ng of human recombinant CXCL12 six weeks after injury and necropsy was performed six weeks post treatment. The external anal sphincter was removed, fixed, embedded in paraffin, sectioned, and mounted to slides for histologic analysis of collagen and muscle content and fiber characteristics; innervation, vascularization and progenitor cell content. Compared to controls untreated had significantly decreased total skeletal muscle, indistinct muscle layers, and disorganized circumferential and inner longitudinal layers at the injury site. Untreated also had significantly increased collagen fiber density at the injury site compared to treated and controls. Cells staining positive for CD34 within the skeletal muscle layer were increased in treated and untreated compared to controls. Staining density for markers of nerves and vascular endothelium, cells staining positive for CD34 within the mucosa/submucosae, and cells staining positive for

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Author Contributions

Conceived and designed research (G.B. and J.K.W.), performed experiments (H.M.R., S.S.L., A.S.A., N.E., and J.K.W.), analyzed data (H.M.R.), interpreted results of experiments (H.M.R. and J.K.W.), prepared figures (H.M.R.), drafted manuscript (H.M.R.), edited and revised manuscript (H.M.R., S.S.L., G.B., and J.K.W.), approved final version of manuscript (H.M.R., S.S.L., A.S.A., N.E., G.B., and J.K.W.).

Disclosures

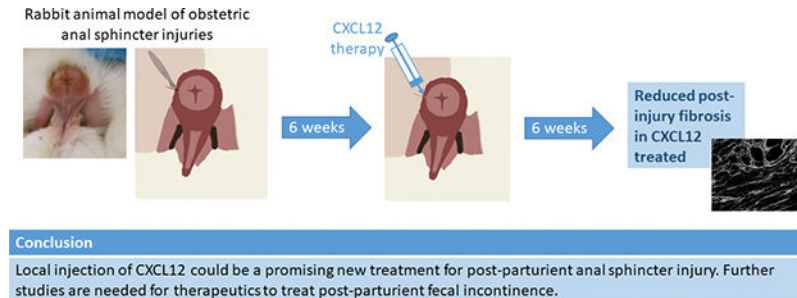
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PAX7 were similar among all groups. Local injection of CXCL12 reduces post-injury fibrosis and results in statistically similar muscle content and organization between treated animals and controls. Further studies are needed for this promising new treatment for post-parturient anal sphincter injury.

Graphical Abstract



Keywords

Obstetric anal sphincter injury; Regenerative medicine; Sphincterotomy; Stromal derived growth factor-1; Picrosirius Red

Introduction

Obstetric anal sphincter injuries (OASIs) are a significant complication of childbirth,(1–3) occur in 2–7% of vaginal births,(4) and are the leading cause of fecal incontinence in women (5). Medical and surgical interventions are utilized but results are suboptimal with 30% of women reporting reduced quality-of-life due to fecal incontinence at long-term follow-up (6). Despite current treatment and prevention practices, subsequent vaginal births further increase women's risk for ongoing fecal incontinence (7).

Regenerative therapies are a promising avenue for intervention. Studies utilizing injected cell therapies or chemokines have been conducted in various animal models and show favorable results (8–12). A chemokine therapy that showed promise for fecal incontinence treatment in a pilot study in rats is monomeric CXCL12 (12). This same chemokine therapy outperformed injected cell therapy for the treatment of urinary sphincter mechanism incontinence (13). CXCL12 is a pleiotropic chemokine that is involved in many processes in the body (14, 15). It exists in multiple isoforms and each isoform is found in differing amounts depending on the tissue type and disease state (14). Further the various isoforms have different affinities for binding receptors, and lead to activation of separate pathways resulting in homeostatic, pro-inflammatory, and anti-inflammatory signaling cascades (14).

The particular monomeric CXCL12 product used in this study has anti-fibrotic and muscle regeneration effects in multiple tissues of the genitourinary tract (12, 13, 16). In an initial pilot study utilizing a rat fecal incontinence model, El Haraki et. al found that administration of this CXCL12 chemokine therapy at six weeks post injury enhanced magnitude of anal sphincter contractions but decreased duration of contraction (12). However they were

unable to correlate changes in function with any histologic changes. Our study builds from the previous pilot study and utilizes a rabbit model as the sphincter injury model. The rabbit model was selected as the myoarchitectural and functional alterations following this sphincter injury have been fully characterized in rabbits (17). Further, larger animal size allows for more specific targeting of anal sphincter injury, and larger tissue sections for histologic assessment. This model is a small focal anal sphincter injury representative of a small tear experienced during parturition. This size of tear would potentially go unnoticed and would not receive a primary repair. However, women often present with fecal incontinence later in life. At this later time point, a site of fibrosis in the anal sphincter could be diagnosed with ultrasound and treated with this proposed focal therapy. In this study we hypothesized that CXCL12 chemokine therapy would 1) reduce sphincter fibrosis, 2) restore sphincter muscle content, vascularization, and innervation, and 3) recruit progenitor stem cells to the injury site in a rabbit model of fecal incontinence.

Materials and Methods

This study was approved by the Wake Forest Institutional Animal Care and Use Committee (IACUC). New Zealand White rabbits (n=12), 4–5m old, and 3.0–3.9kg, were obtained from Envigo (Indianapolis, Indiana, United States) for use in this study. Animals were ordered from the vendor based on their size 3–3.9kg as the purpose of using rabbits was to have a larger animal model for surgical manipulation. They were not selected based on age matching to humans. New Zealand White rabbits reach puberty at around 5 months of age. Sample size was estimated based on skeletal muscle data from the rabbit model characterization by Rajasekaran et al. (uninjured estimated at 72% and untreated at 31% muscle based on Masson's Trichrome stain)(17) and an assumed restoration of sphincter muscle content with treatment of 15% based on prior use of the CXCL12 therapy for muscle regeneration in urethral sphincter mechanism incontinence in a non-human primate model(13). Standard deviation was estimated to be around 15 based on increased variability between animals in treatment effects versus standard deviation of 0-uninjured and 1-untreated injured in study by Rajasekaran et al. We had 80% power to detect a significant effect of treatment on proportion of skeletal muscle if the difference was at least 15% with a sample size of 4 per group.

External Anal Sphincter (EAS) Myotomy

Animals were subjected to either sham (n=4) or EAS myotomy (n=8) surgery as described previously and illustrated in Figure 1 (17). Briefly, animals were sedated with isoflurane via face mask and then intubated and maintained in an anesthetic plane on isoflurane (1–5%) for the duration of the procedure. The anorectal area was clipped and cleaned and a 30 ml saline rectal flush was given to clear the rectum of feces. Lidocaine/prilocaine gel (3 grams topical) was applied to the rectum and pre-operative anti-nausea (famotidine 0.5mg/kg SC), NSAID (0.2mg/kg Meloxicam SC), and opioid (Buprenorphine 0.2mg/kg s.c.) medications administered. The animals were placed in the prone position on the operating table and provided heat supplementation. Following sterile prep of the rectum, a craniocaudal incision, 15 mm long, was made on the skin to expose the EAS muscle. With the use of a sharp dissection knife, a surgical myotomy of EAS was performed at the 9:00 clock position

through the whole thickness of the muscle. The myotomy was 1 mm across and 10 mm long; it extended along the entire craniocaudal direction of the EAS muscle without causing any damage to the underlying mucosa of the anal canal. Skin was sutured back over the myotomy site, an analgesic (bupivacaine 1mg/kg intraincisional) was injected at the incision site, and animals were allowed to recover. Post-operative pain was managed with an NSAID (meloxicam 0.1mg/kg SC or PO for up to 7 days) and Opioid (Buprenorphine 0.2mg/kg SC for up to 3 days). An anti-nausea (famotidine 0.5mg/kg SC or PO) was given any time an NSAID was given.

CXCL12 Cytokine Therapy

Animals with EAS myotomy were subjected to either sham (n=4) or CXCL12 treatment (n=4). Six week post-injury animals were sedated, anesthetized, and prepared for the procedure as in the myotomy procedure. Following sterile prep of the rectum, 200ng of monomeric human recombinant CXCL12 suspended in 0.5mL of saline was injected at the 9:00 clock position of the anal sphincter using a 30 gauge needle. The animals were allowed to recover. Post-operative pain was managed with an NSAID (meloxicam 0.1mg/kg SC or PO for up to 7 days); an anti-nausea (famotidine 0.5mg/kg SC or PO) was given any time an NSAID was given.

External Anal Sphincter Sample Collection

Six weeks post-therapy or sham therapy animals were humanely euthanized with 100mg/kg of intravenous euthanasia solution (Euthasol). External anal sphincter was collected in whole and fixed in 10% neutral buffered formalin. Whole cross section of anus was paraffin embedded, sectioned, and mounted to glass microscope slides for staining and analysis. All analyses were performed at the injury site at the 9:00 clock position of the anal sphincter. The injury and therapy were both targeted to this area and the remainder of the anal sphincter was unaffected (normal).

Histopathologic Analysis

Masson's Trichrome staining: Masson's Trichrome staining was performed. Stained tissue sections (5µm) were cleared with xylene and mounted with Richard-Allan toluene-based mounting medium. Tissue sections were scanned with a 20x objective using a PANNORAMIC 1000 digital slide scanner (3DHISTECH, Budapest, Hungary). Obtained images were viewed using SlideViewer 2.7 (3DHISTECH, Budapest, Hungary) and a field of view capturing the injury site was obtained (Figure 6).

Collagen quantification with picosirius red staining: Picosirius red staining (PSR) was performed as previously described.(18) Stained tissue sections (5µm) were cleared with xylene and mounted with Richard-Allan toluene-based mounting medium. PSR staining was then imaged using Olympus BX63 Automated Fluorescence Microscope fitted with a 20x objective. Fluorescence was detected using a Cy5 filter cube. Images were analyzed using publicly accessible CT FIRE software developed at the University of Wisconsin-Madison. (19) This software detects and quantifies fiber structures in images. For this study, fiber length, width, straightness, and density were measured.

Immunohistochemistry: Formalin fixed paraffin embedded tissue sections (5µm) were deparaffinized and rehydrated using a Leica Auto Stainer XL (Leica Biosystems, Deer Park, Illinois, United States). Antigen retrieval was performed in Dako Target Retrieval Solution, pH 6 [MYH1, UCHL1, PECAM1, MYH1/DES dual stain] or pH 9 [CD34, CD34/Pax7 dual stain] using a Dako PT Link machine (20 min at 95°C, Santa Clara, California, United States). Endogenous peroxidase, pseudoperoxidase, and alkaline phosphatase were inactivated with BLOXALL Endogenous Blocking Solution (10 min 25°C, Vector Laboratories, Newark, California, United States). Non-specific sites were blocked with IHC/ISH Super Blocking 110 (10 min at 25°C, Leica Biosystems, Deer Park, Illinois, United States). For rabbit on rabbit protocol only [CD34, CD34/Pax7 dual stain], tissues were blocked with AffiniPure Fab Fragment Goat Anti-Rabbit IgG H&L diluted in IHC/ISH Super Blocking 110 and incubated overnight at 4°C prior to primary antibody application. All primary antibodies were diluted in IHC/ISH Super Blocking 110 and incubated overnight at 4°C. Secondary antibodies were diluted in Tris-buffered saline containing Tween20 and incubated for 1 h [MYH1, UCHL1, PECAM1, CD34/Pax7 dual stain] or 30 min [MYH1/DES dual stain] at 25°C. For single stains ImmPACT DAB (Vector Laboratories, Newark, California, United States) was used as a peroxidase detection substrate (1 min [MYH1 and UCHL1], 3 min [PECAM1] and 5 min [CD34] at 25°C). For CD34/PAX7 dual stain fluorescent conjugated secondary antibodies were utilized. For MYH1/DES dual stain stains were performed sequentially as single stains with TSA Cyanine 5 Reagent [DES] and TSA Cyanine 3 Reagent [MYH1] (Akoya Biosciences, Marlborough, Massachusetts, USA) were used as a peroxidase detection substrate. Slides were counterstained with hematoxylin or DAPI was included in mounting media for fluorescent stains. Optimization of each antibody included performing negative control stains both without primary antibody and with the same concentration of an isotype-specific immunoglobulin control.

MYH1 was selected to mark skeletal muscle as the external anal sphincter is composed predominantly of type 1 skeletal muscle fibers in humans.(20) A dual immunohistochemistry stain for Myosin, heavy polypeptide 1, skeletal muscle, adult (MYH1) and desmin (DES; general marker for all muscle) confirms that majority of muscle fibers stain positive for MYH1 in rabbits (Figure 5). Ubiquitin carboxy-terminal hydrolase L1, aka PGP9.5 (UCHL1), was used as a general marker for nerve fibers. Platelet/endothelial cell adhesion molecule 1 (PECAM1) was used as a general marker for endothelial cells to identify blood vessels. CD34 molecule (CD34) is a general progenitor cell marker and paired box 7 (PAX7) was used to mark muscle satellite cells.

For all DAB substrate stains, tissue sections were scanned with a 20x objective using a PANNORAMIC 1000 digital slide scanner (3DHISTECH, Budapest, Hungary). Obtained images were viewed using SlideViewer 2.7 (3DHISTECH, Budapest, Hungary) and 10x or 20x field of view were captured for analysis. For fluorescent stains, tissue sections were scanned or single field views captured with a 20x using an Olympus VS120 Slide Scanner (Olympus Life Science, Waltham, Massachusetts, USA).

For assessment of skeletal muscle a single 10x image of MYH1 stain was taken at the 9 o'clock position of the anal sphincter and study group was masked during image assessment.

Images were assessed for presence of distinct muscle layering and scored as: 3) Present, three distinct layers are present (inner layer of longitudinal bundles, a middle layer of major circular and minor longitudinal bundles, and an outer layer of longitudinal bundles), 2) Indistinct, either a single layer is absent or distinct divisions are not discernable, or 1) Absent, no clear divisions are discernable or only a single layer is present. Images were also assessed for muscle fiber organization and each muscle layer was scored as: 4) Organized, $\geq 80\%$ of muscle fibers follow appropriate orientation, 3) Somewhat organized, $\geq 50\%$ of muscle fibers follow appropriate orientation, 2) Disorganized, $<50\%$ of muscle fibers follow appropriate orientation, and 1) Absent. Finally Image J IHC Toolbox(21) was used to isolate DAB stain and threshold feature used to select true stain. Percent area positive for MYH1 stain was then calculated per image. A single 10x image of MYH1 stain on an additional stained section from each animal was taken at the 9 o'clock position of the anal sphincter for a biological replicate. An average of the calculated percent area positive for MYH1 stain was used for analysis.

For assessment of innervation and vascularization a single 10x image of either UCHL1 or PECAM1 immunohistochemical stain was taken at the 9 o'clock position within the skeletal muscle layer. Image J(22) IHC Toolbox(21) was used to isolate DAB stain and threshold feature used to select true stain. Regions of non-specific staining (skeletal muscle and red blood cells) were manually selected and removed from analysis. Percent area positive for UCHL1 or PECAM1 was then calculated per image.

For assessment of progenitor cell recruitment, progenitor cells were defined as CD34 immunopositive. Eight 20x images were captured at the 9 o'clock position of the anal sphincter, four within the mucosa/submucosa, and four within the skeletal muscle for each animal. Four images came from the DAB substrate single stain of CD34 and four images came from the fluorescent conjugated secondary stain of CD34 and PAX7. Positive cells were manually counted per image using Image J cell counter.(22) Fluorescent dual stain images of CD34 and PAX7 were used to determine the percentage of CD34 positive cells dual positive for PAX7.

For assessment of satellite cells, cells were defined as PAX7 immunopositive. Four 20x images were captured at the 9 o'clock position of the anal sphincter, two within the mucosa/submucosa, and two within the skeletal muscle for each animal. Positive cells were manually counted per image using Image J cell counter.(22)

Statistical Analysis

Statistical analyses were performed with Graph Pad Prism 10.1.1. Differences were considered significant at the $P < 0.05$ level. A Shapiro-Wilk test was used to test for normality and transformation was applied to normalize data. Bartlett's test was used to test for homogeneity of variance. For group-wise comparisons at a single time-point, Welch's ANOVA was applied when variance was unequal followed by Dunnett's T3 multiple comparisons test. When variance was equal, group-wise comparisons were made using ordinary one-way ANOVA followed by Tukey's multiple comparisons test. The Kruskal-Wallis test followed by Dunn's multiple comparisons test was applied when data were ordinal or when data could not be normalized through transformation.

Results

CXCL12 chemokine therapy reduces post injury fibrosis.

The anal sphincter was injured at the 9:00 o'clock position and CXCL12 treatment applied only to the injury site, so histologic analysis was performed exclusively in this region. Masson's Trichrome staining was conducted to visualize the ratio of collagen (blue) to muscle (red) at the injury site (Figure 6). PSR staining was conducted (representative images of each group, Figure 2A) and CT FIRE analysis performed. Collagen fiber density is increased in untreated compared to treated and control animals ($p=0.029$ and 0.032 ; Figure 2B). There are no differences in fiber length ($p=0.50$), diameter ($p=0.30$), or straightness ($p=0.20$) between groups.

CXCL12 chemokine therapy may restore muscle content and organization post injury but does not alter post injury innervation and vascularization 12-weeks post injury.

Both overall skeletal muscle content and organization of the muscle fibers in the anal sphincter were assessed at the 9:00 o'clock position using a MYH1 immunohistochemical stain (representative images of each group, Figure 3A). Proportion of skeletal muscle to overall tissue area is decreased in untreated compared to controls ($p=0.0062$, Figure 3B). The anal sphincter should have three distinct layers of skeletal muscle fiber bundles arranged as an outer longitudinal, middle circumferential, and inner longitudinal layer. In untreated animals there is reduced distinction of layering of the anal sphincter skeletal muscle fibers compared to controls ($p=0.012$, Figure 3C). Organization of muscle fiber bundles within each layer was also assessed. Untreated have reduced organization of the circumferential and inner longitudinal layers of skeletal muscle compared to controls ($p=0.019$, $p=0.015$, Figure 3D). Taken together this indicates that untreated animals have reduced skeletal muscle content and impaired skeletal muscle fiber organization, while treated animals' skeletal muscle is statistically similar to controls.

Innervation and vascularization were assessed at the 9:00 o'clock position using UCHL1 and PECAM1 immunohistochemistry. There are no differences between groups ($p=0.97$, $p=0.93$).

Untreated and treated animals have increased CD34+ progenitor cells within the anal sphincter skeletal muscle at 12-weeks post injury.

CD34+ progenitor cell recruitment was assessed with CD34 immunohistochemistry. Density of infiltrating CD34+ progenitor cells is increased in the skeletal muscle layers of untreated and treated compared to control ($p=0.0014$, $p=0.0230$, Figure 4). Positive cells are small, round, with a high nuclear to cytoplasmic ratio consistent with bone marrow derived progenitor cells. There are no differences in infiltrating CD34+ progenitor cells in the mucosa/submucosa between any groups ($p=0.7458$). There are no differences in PAX7+ satellite cells in the skeletal muscle layers between any groups ($p=0.3009$). Only around 28% of infiltrating CD34+ progenitors are PAX7 co-positive (range 0–50%; mean 27.61%). There were no differences in the percentage of CD34+ progenitor cells co-positive for PAX7 between groups ($p=0.2631$).

Discussion

In this study we found that local injection of the injured anal sphincter site with CXCL12 therapy reduced post injury fibrosis and may restore skeletal muscle content and organization in the anal sphincter. We did not find an effect of CXCL12 on innervation, vascularization, or PAX7 positive satellite cells at the single time point evaluated, but we found an increase in CD34+ cells in the skeletal muscle layers of both treated and untreated animals compared to control.

Obstetric anal sphincter injuries are a significant complication of childbirth, are the leading cause of fecal incontinence, and current treatment methods are suboptimal. The CXCL12 treatment utilized in this study involved one injection into the anal sphincter near the site of injury, offering a noninvasive alternative to current surgical interventions. In addition, our study design recapitulates a feasible timeline of clinical treatment, with treatment occurring six weeks after initial injury. Based on work in rat models, this treatment time point is also after initial host expression of CXCL12 has subsided.(23)

A previous pilot study utilizing the same CXCL12 chemokine therapy for fecal incontinence in a rat model did not identify a difference in the ratio of collagen to muscle with assessment of Masson's trichrome stained sections.(12) However, a study utilizing a CXCL12 plasmid treatment in a rat model of fecal incontinence found an increased proportion of muscle and reduced proportion of collagen similar to our study.(24) The conflicting results between these three studies likely relates to the small sample size of the pilot study, with insufficient power to detect a difference. As we utilized a similar sample size in this study, it is also likely that our finding of reduced fibrosis and restored collagen content relates to our differed analysis approach. We utilized PSR staining methods to detect collagen and CT FIRE fiber analysis to specifically evaluate changes in fibrillar collagen architecture. Additionally we utilized MYH1 immunohistochemical stain to detect and evaluate skeletal muscle. These more specific approaches likely allowed for increased sensitivity to identifying the benefits of this therapy. Further our large animal model likely allowed for better visualization and consistency in the injury site as well as targeting of the chemokine injection.

Our finding of increased CD34+ progenitor cells within the skeletal muscle of both the treated and untreated group was interesting. One explanation is that there is delayed/continued attempt at healing in both groups that resulted in delayed/continued recruitment of progenitor cells. Given that satellite cell density is similar among all three groups, our timepoint of evaluation (12 weeks after initial injury, six weeks after treatment) is likely too late to evaluate the regenerative response. A future time course study would provide additional information on the kinetics and mechanism of reduced sphincter fibrosis.

Current medical and surgical interventions are suboptimal with 30% of women reporting reduced quality-of-life due to fecal incontinence at long-term follow-up.(6) Our study identifies a novel treatment prospect for post-parturient anal sphincter injury that reduces local fibrosis and could restore skeletal muscle content. As this was an initial study to determine if there was a beneficial effect of the treatment and if the rabbit provided an

improved model system over rat, only one time point was assessed and animal numbers were limited (n=4 per group). Future studies utilizing a time course approach and larger sample sizes will aide in elucidating the duration of CXCL12 in local tissue, timing of progenitor cell recruitment, and mechanism of reduced fibrosis and restoration of muscle content. Additionally future studies with functional measures will elucidate if the restored muscle content leads to improved sphincter tone and continence. However, despite the small scale of this study it offers promising results, demonstrating reduced post injury fibrosis at end point.

Local injection of CXCL12 cytokine reduces post-injury fibrosis in a rabbit model of anal sphincter injury and fecal incontinence. The larger size of the rabbits aided in targeted injury and treatment. Further studies are needed to explore non-invasive treatment options for post-parturient anal sphincter injury.

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New and Noteworthy

Local injection of CXCL12 cytokine reduces post-injury fibrosis in a rabbit model of anal sphincter injury and fecal incontinence. The larger size of the rabbits aided in targeted injury and treatment. Further studies are needed to explore non-invasive treatment options for post-parturient anal sphincter injury.

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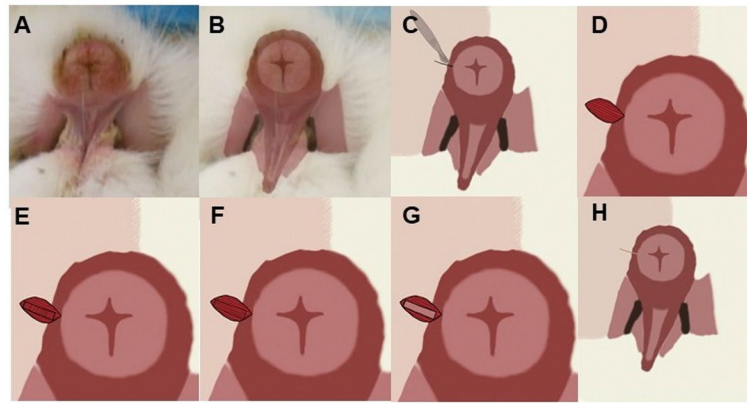


Figure 1.

External Anal Sphincter Myotomy. (A) representative image of female New Zealand White rabbit external genitalia. (B) overlay of female rabbit external genitalia illustrated drawing. Rabbits were sedated with isoflurane via face mask and the intubated and maintained on isoflurane for the duration of the procedure. The anorectal area was clipped and cleaned and 30mL saline rectal flush given to clear the rectum of feces. Lidocaine/prilocaine gel was applied to the rectum and per-operative NSAID and opioid medications administered. (C) a 15mm long incision was made on the skin to (D) expose the external anal sphincter muscle. With a sharp dissecting knife a 10mm by 100mm region of the full thickness (E) outer longitudinal layer, (F) middle major circumferential and minor longitudinal layer, and (G) inner longitudinal layer of external anal sphincter muscle was removed at the 9 o'clock position of the anal sphincter without any damage to the underlying mucosa of the anal canal. The myotomy site was left to heal by second intention. (H) The skin was sutured closed back over the myotomy site and animals were allowed to recover.

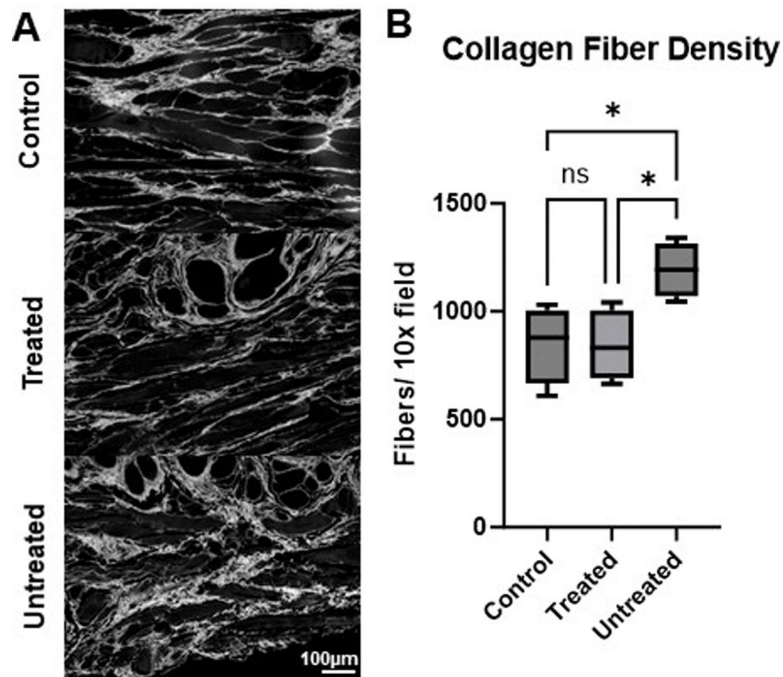
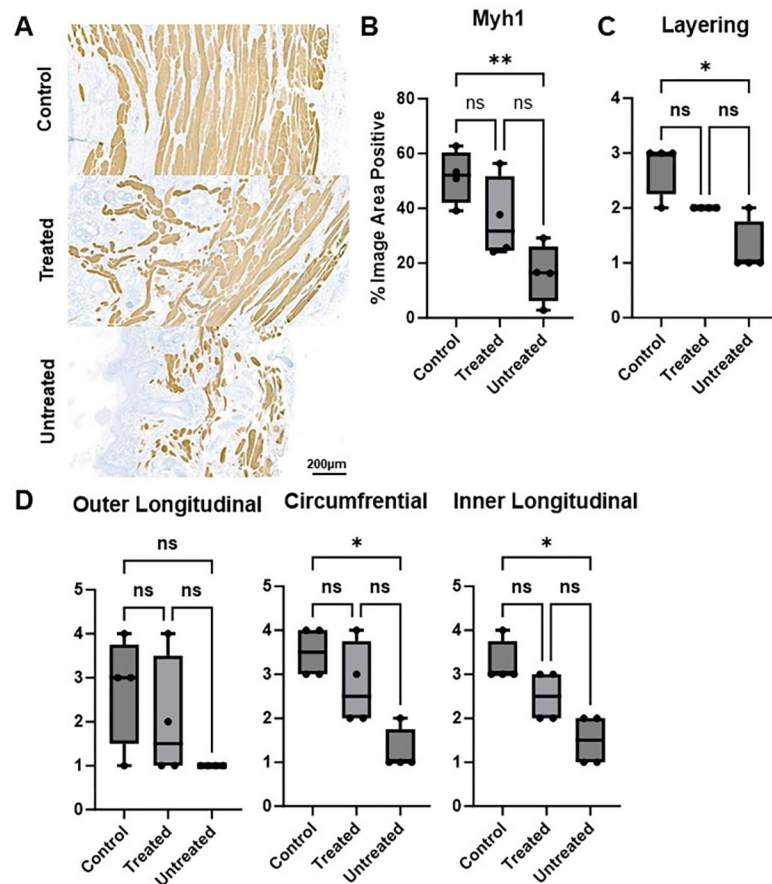


Figure 2.

CXCL12 chemokine therapy reduces post injury fibrosis. Picrosirius red staining was conducted, images were captured using Cy5 Fluorescence and a 20X objective, and CT FIRE analysis performed to assess collagen architecture. (A) A representative image from each group shows collagen architecture. (B) Graph shows collagen fiber density, and results are representative of one image/animal and four animals/group (n=4/group). An ordinary one-way ANOVA followed by Tukey's multiple comparisons test was used to compare groups, (ns) p-value >0.05, (*) p-value <0.05 but >0.01.

**Figure 3.**

CXCL12 chemokine therapy restores muscle content and organization post injury. Immunohistochemical staining for MYH1 was conducted; images were captured in bright field using a 10X objective. (A) A representative image from each group shows skeletal muscle stained positive for MYH1. (B) Graph shows proportion of skeletal muscle to total image area expressed as a percentage. Results are an average of two images/animal and four animals/group (n=4/group). An ordinary one-way ANOVA followed by Tukey's multiple comparisons test was used to compare groups. (C) Images were assessed for presence of distinct muscle layering and scored as: 3) Present, three distinct layers are present (inner layer of longitudinal bundles, a middle layer of major circular and minor longitudinal bundles, and an outer layer of longitudinal bundles), 2) Indistinct, either a single layer is absent or distinct divisions are not discernable, or 1) Absent, no clear divisions are discernable or only a single layer is present. Results are representative of one image/animal and four animals/group (n=4/group). A Kruskal-Wallis test followed by Dunn's multiple comparisons test was applied. (D) Muscle fiber organization for each layer was scored as: 4) Organized, $\geq 80\%$ of muscle fibers follow appropriate orientation, 3) Somewhat organized, $\geq 50\%$ of muscle fibers follow appropriate orientation, 2) Disorganized, $< 50\%$ of muscle fibers follow appropriate orientation, and 1) Absent. A Kruskal-Wallis test followed by Dunn's multiple comparisons test was applied. Results are representative of

one image/animal and four animals/group (n=4/group). (ns) p-value >0.05, (*) p-value <0.05 but >0.01, (**) p-value <0.01.

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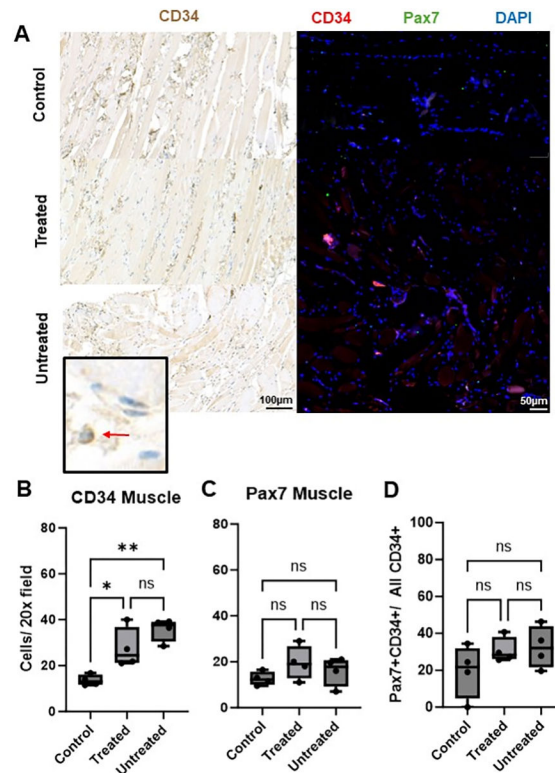


Figure 4.

Untreated animals have increased CD34+ progenitor cells within the anal sphincter skeletal muscle at 12-weeks post injury. Immunohistochemical staining for CD34 was conducted, images were captured in bright field using a 20X objective. (A) A representative image from each group shows CD34 immunopositive cells on a background of light non-specific staining of skeletal muscle. Inlay shows example of positive cell (red arrow) (B) Graph depicts immunopositive cells per 20x field, and results are an average of two images/animal and four animals/group (n=4/group). An ordinary one-way ANOVA followed by Tukey's multiple comparisons test was used to compare groups, (ns) p-value >0.05, (*) p-value <0.05 but >0.01, (**) p-value <0.01.

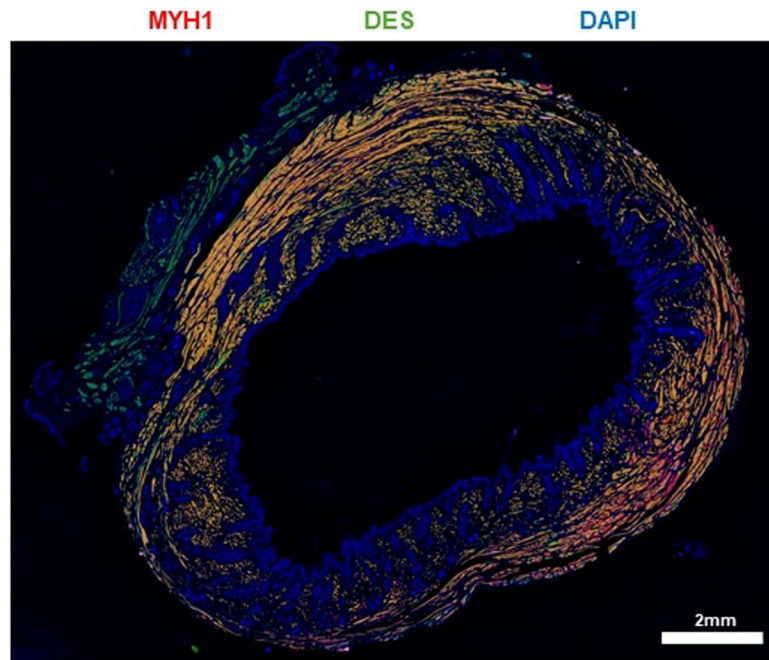


Figure 5. Rabbit anal sphincter is composed predominantly of type 1 skeletal muscle fibers. Immunohistochemical staining for desmin (DES) and Myosin, heavy polypeptide 1 (MYH1) was conducted, image was captured in as a tile scan of the entire tissue using a 20X objective. Image is from a control animal.

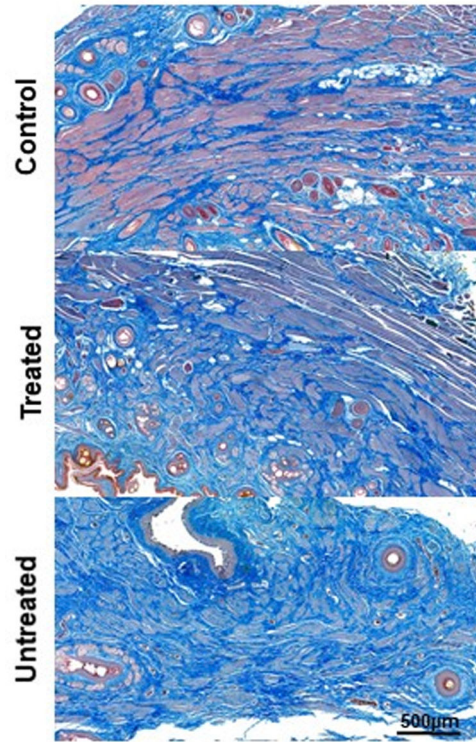


Figure 6.

Ratio of muscle to collagen in rabbit anal sphincter. Masson's Trichrome staining was conducted, images were captured as a tile scan of the entire tissue using a 20X objective and a representative image of each group in the region of injury is depicted.

Table 1:

Antibodies used for immunostaining.

Name(s)	Symbol	Species	Antibody Registry (RRID)	Supplier	Catalog Number	Dilution	Un-masking	Fig #
Myosin, heavy polypeptide 1, skeletal muscle, adult	MYH1	Mouse	AB_2297993	Abcam	ab51263	1:500	pH 6	3, Supp. 1
Ubiquitin carboxy-terminal hydrolase L1 (aka PGP9.5)	UCHL1	Mouse	AB_306343	Abcam	ab8189	1:500	pH 6	-
Platelet/endothelial cell adhesion molecule 1	PECAM1	Mouse	AB_2942096	Biocare Medical	CM 131 A	1:200	pH 6	-
CD34 molecule	CD34	Rabbit	AB_10856746	Bioss	Bs-2038R	1:100	pH 9	4
Paired box 7	PAX7	Mouse	AB_3308859	Novus Biologicals	NBP2-44589-0.1mg	1:200	pH 9	4
Desmin	DES	Mouse	AB_305423	Abcam	ab6322	1:200	pH 6	Supp. 1
Goat Anti-Mouse IgG H&L (HRP) antibody	-	Goat	AB_2755049	Abcam	ab205719	1:250	-	3, Supp. 1
Goat Anti-Rabbit IgG H&L (HRP) antibody	-	Goat	AB_2307391	Jackson ImmunoResearch	111-035-144	1:250	-	4
AffiniPure Fab Fragment Goat Anti-Rabbit IgG H&L	-	Goat	AB_2337925	Jackson ImmunoResearch	111-007-003	1:40	-	4
"Alexa Fluor® 488 AffiniPure Donkey Anti-Rabbit IgG (H+L) (min X Bov, Ck, Gt, GP, Sy Hms, Hrs, Hu, Ms, Rat, Shp Sr Prot)"	-	Donkey	AB_2313584	Jackson ImmunoResearch	711-545-152	1:250	-	4
"Alexa Fluor® 647 AffiniPure Donkey Anti-Mouse IgG (H+L) (min X Bov, Ck, Gt, GP, Sy Hms, Hrs, Hu, Rb, Rat, Shp Sr Prot)"	-	Donkey	AB_2340863	Jackson ImmunoResearch	715-605-151	1:250	-	4
Mouse IgG1 Isotype Control	-	Mouse	AB_3657051	Novus Biologicals	MAB002	1:10-1:400	-	-
Normal Rabbit IgG Control	-	Rabbit	AB_354266	Novus Biologicals	AB-105-C	1:100	-	-
Mouse IgG2a Kappa Isotype Control	-	Mouse	AB_3242234	Novus Biologicals	NBP1-96981-0.5mg	1:500	-	-