Deferoxamine-Soaked Suture Improves Angiogenesis and Repair Potential After Acute Injury of the Chicken Achilles Tendon

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Background: A major obstacle to the treatment of soft tissue injuries is the hypovascular nature of the tissues. Deferoxamine (DFO) has been shown to stimulate angiogenesis by limiting the degradation of intracellular hypoxia-inducible factor 1–alpha.

Hypothesis: DFO-saturated suture would induce angiogenesis and improve the markers of early healing in an Achilles tendon repair model.

Study Design: Controlled laboratory study.

Methods: Broiler hens were randomly assigned to the control (CTL) group or DFO group ($n = 9$ per group). The right Achilles tendon was partially transected at its middle third. The defect was surgically repaired using 3-0 Vicryl suture soaked in either sterile water (CTL group) or 324 mM DFO solution (DFO group). All animals were euthanized 2 weeks after the injury, and the tendon was harvested. Half of the tendon was used to evaluate angiogenesis via hemoglobin content and tissue repair via DNA content and proteoglycan (PG) content. The other half of the tendon was sectioned and stained with hematoxylin and eosin, safranin O, and lectin to evaluate vessel density.

Results: Hemoglobin content (percentage of wet tissue weight) was significantly increased in the DFO group compared with the CTL group (0.081 ± 0.012 vs 0.063 ± 0.016, respectively; $P = .046$). DNA content (percentage of wet tissue weight) was also significantly increased in the DFO group compared with the CTL group (0.31 ± 0.05 vs 0.23 ± 0.03, respectively; $P = .024$). PG content (percentage of wet tissue weight) was significantly decreased in the DFO group compared with the CTL group (0.26 ± 0.02 vs 0.33 ± 0.08, respectively; $P = .035$). Total chondroid area (number of vessels per mm^2 of tissue area evaluated) was significantly decreased in the DFO group compared with the CTL group (17.2 ± 6.6 vs 24.6 ± 5.1, respectively; $P = .038$). Articular zone vessel density (vessels/mm^2) was significantly increased in the DFO group compared with the CTL group (7.1 ± 2.5 vs 2.1 ± 0.9, respectively; $P = .026$).

Conclusion: The significant increase in hemoglobin content as well as articular zone vessel density in the DFO group compared with the CTL group is evidence of increased angiogenesis in the fibrocartilaginous region of the tendon exposed to DFO. The DFO group also displayed a significantly greater level of DNA and significantly lower level of PG, suggesting enhanced early healing by fibrous tissue formation.

Clinical Relevance: Stimulating angiogenesis by DFO-saturated suture may be clinically useful to improve healing of poorly vascularized tissues.

Keywords: growth factors/healing enhancement; biology of tendon healing of cartilage; Achilles tendon; shoulder; rotator cuff; tendinosis

Soft tissue injuries involving dense collagenous tissues such as ligaments, tendons, and menisci have been shown to account for over half of all reported musculoskeletal injuries worldwide, and tendons are involved in as many as 50% of all sports-related injuries.[23,36] Common injuries involving such tissues include rotator cuff injuries, Achilles tendon ruptures, and meniscal tears. Unfortunately, such injuries rarely heal to the full functional strength of the previously uninjured tissue.[15,24] For example, the final tensile strength of a healed tendon is reduced by 30%.[31] Rotator cuff repairs fail to heal in 20% to 95% of patients and, when they heal, maintain only 30% of their original

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functional strength.[8,40] It is also well established that meniscal tears fail to heal when the tear occurs in the avascular inner two-thirds of the meniscus.[26]

Normal soft tissue healing typically occurs in 4 stages: bleeding, inflammation, scar formation, and scar remodeling. Early healing in acutely disrupted soft tissues is highly dependent on having an adequate vascular supply. Neovascularization allows for an exchange of nutrients, cellular factors, cytokines, and growth factors essential for the subsequent repair and remodeling of acutely injured tissues.[13]

Fibrocartilage comprises the enthesis of the rotator cuff, the midportion (in tendinopathic tendons prone to ruptures) and insertion of the Achilles tendon, and the entire meniscus.[6,26,35,40] The hypovascular nature of these tissues poses a challenge to healing. Inducing angiogenesis and therefore blood supply in these tissues after an acute injury has the potential to augment healing by overcoming one of the major obstacles limiting their native ability to heal.[1,7,21,27]

Deferoxamine (DFO), an iron-chelating agent, has been shown in many studies to stimulate angiogenic pathways by limiting the degradation of intracellular hypoxia-inducible factor 1–alpha (HIF-1α).[2,28,32] HIF-1α exerts its angiogenic effects by activating numerous transcription factors typical of the hypoxic state.[9] Its angiogenic effects are a result of several angiogenic factors, such as vascular endothelial growth factor (VEGF), erythropoietin, and nitric oxide, being transcribed and regulated in a coordinated manner.[3,17] DFO also stimulates VEGF-independent signaling through the upregulation of cyclooxygenase-2, an important regulator of angiogenesis, endothelial cell proliferation, and migration.[2,30]

Past studies have investigated incorporating exogenous factors into a suture coating to promote biological healing at a repair site.[4] A variety of different angiogenic promotors including butyric acid, platelet-derived growth factor, VEGF, and basic fibroblast growth factor mimetics have been incorporated in suture coatings and have been shown to promote tendon healing or vascularity in various animal models.[5,7,21,27] In addition, other studies have coated sutures with adhesion substrates to improve the biological acceptance of sutures and to promote healing.[23,38]

The objective of this study was to test the angiogenic properties of DFO-saturated suture in a chicken Achilles tendon repair model. This study aimed to test the hypothesis that the local delivery of DFO via suture to the injured fibrocartilaginous region of the Achilles tendon would improve angiogenesis and the markers of early healing in the acute phase.

METHODS

All procedures described in this study were approved by the local institutional animal care and use committee. The primary author (W.M.E.) performed all surgical procedures and was blinded to the treatment group at the time of the procedure and throughout the experiment. Eighteen broiler hens, 22 weeks of age, were given an identifying number and assigned at random into either the control (CTL) or DFO group (n = 9 per group). All hens underwent the same surgery: Anesthesia was achieved by masked induction with 2% isoflurane, after which the hen was intubated and maintained on 2% isoflurane throughout the procedure. After the hen was anesthetized, the operative (right) leg was deplumed to the hip. The leg was then prepared 3 times with alternating iodine and ethanol swabs in a concentric circular pattern. The leg was draped in a sterile fashion, and a 3-cm longitudinal incision was made midline over the posterior aspect of the right hock joint. The paratenon overlaying the Achilles tendon was incised to expose the fibrocartilaginous region of the tendon (Figure 1A). Using a No. 15 blade scalpel (Aspen Surgical Products), a 4 mm–wide partial transection of the Achilles tendon was made in the midportion of the tendon, leaving intact tendon fibers on both sides of the defect. The partial transection was then repaired using either water- or DFO-soaked suture. Sutures were prepared and provided during the procedure by a surgical assistant so that the surgeon remained blinded. A locking cruciate stitch was used in all animals to repair the defect (Figure 1B), with the distance from each cruciate stitch to the transection site being approximately 5 mm. The skin and paratenon were then closed with 3-0 Prolene sutures (Ethicon). Fourteen days after surgery, all animals were euthanized by decapitation under isoflurane anesthesia. The operative tendon was carefully harvested and split longitudinally (in the sagittal plane). Each tendon remained labeled with the unique numerical identifier to keep the primary author blinded.

Suture Preparation

All sutures for both the CTL and DFO groups were prepared 1 hour before use in survival surgery. All procedures in the preparation of sutures were performed under sterile conditions. The sutures (3-0 Vicryl; Ethicon) were removed from their packaging and placed into a sterilized 10-mL vial. DFO

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Ethical approval for this study was obtained from the University of North Carolina at Chapel Hill Institutional Animal Care and Use Committee.

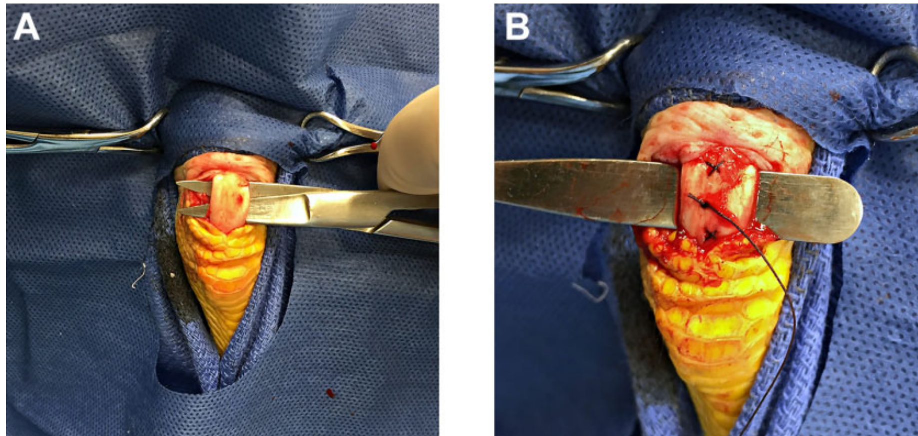

Figure 1. Images of various stages of survival surgery displaying (A) the exposed fibrocartilaginous region of the Achilles tendon and (B) the final repair of the defect using a locking cruciate stitch pattern. (B) A single loop of suture was used in the repair with the knot tied at the center stitch at the transection site.

solution was prepared by reconstituting 500 mg of pharmaceutical-grade DFO (Hospira) in 2 mL of sterile water.

Then, depending on the group, either 2 mL of sterile water (CTL group) or 2 mL of 324 mM DFO solution (DFO group) was added into the vial with the suture. The cap to the vial was closed, and the contents were manually shaken and allowed to sit for 1 hour before use in surgery.

Assays

All assays were performed by the primary author in a blinded fashion by having each tendon labeled only with the respective numerical identifier. One longitudinal half of the tendon was used to evaluate angiogenesis indirectly by measuring the tissue's hemoglobin content as a percentage of wet tissue weight on the day of euthanasia.[39] The tissue's DNA content and proteoglycan (PG) content, both as a percentage of wet tissue weight, were also evaluated as measures of tissue repair on the homogenized tissue after freezing samples at –80°C. Samples were frozen with liquid nitrogen, and a tissue pulverizer (Cellcrusher) was used to homogenize the tissue. Homogenate from each sample was then centrifuged for 5 minutes at 13,000 rpm with 1 mL of 0.2% Triton X-100 cell lysis buffer (Sigma-Aldrich). A portion of the supernatant was then taken for spectrophotometric analysis using a commercial hemoglobin assay (QuantiChrom Hemoglobin Assay Kit; BioAssay Systems) and expressed as a percentage of tissue weight.[16] Analysis for DNA content was performed using a fluorometric assay as previously described by Kim et al.[19] Analysis for PG content was performed by colorimetric assay as previously described by Farndale et al.[11]

Histology

The remaining longitudinal half of the tendon was used for the histological evaluation. Each tendon sample was fixed in neutral buffered formalin for 48 hours. Samples were then paraffin embedded and sectioned. Sectioning was performed in the sagittal plane of the tissue, with sections being taken from the cut surface of the tendon at 2 depths spaced 200 μm apart. Three individual stains were performed on samples for each tendon. Standard hematoxylin and eosin as well as safranin O staining were performed based on a standard protocol. Lectin staining using *Sambucus nigra* was performed to evaluate vessel density per the protocol described by Nanka et al.[25]

Chondroid Area

Digital slide images were uploaded into the laboratory database for further evaluation. In a blinded fashion, all safranin O slides were digitally saved as an RGB image. A consistent set of segmentation values were then used to threshold the red chondroid regions within the tendon (fibrocartilage) using the red color plane of the image with image analysis software (ImageJ 1.51 H; National Institutes of Health). The percentage of total tissue area stained with red within the safranin O section was calculated using the image analysis software.

Vessel Counts

In a blinded manner, lectin slides were analyzed by the author (P.S.W.) using digital image analysis software (ImageScope; Leica Biosystems). Specimens were digitally marked in a longitudinal fashion to separate the fibrocartilaginous (articular) half and tendinous (superficial) half of the tendon. The region of interest was 5 mm in length, extending from the repair site in both the proximal and distal directions for both halves of the tendon for the 2 sections of each specimen. The total vessel number was counted at 10× magnification for each region of interest and was divided by the area of each region of interest to calculate the vessel density (number of vessels per mm^2 of tissue area). Vessel densities for the regions of interest of the articular and superficial halves of the tendon were

averaged separately. In addition, the overall average vessel density across the total tissue area was calculated.

Elution Assay

To evaluate the release of DFO from the suture, 3-0 Vicryl suture segments 15 cm in length were prepared with DFO as above. Sutures saturated in DFO or water (n = 3 per group) were placed in sterile water in a 1.5-mL micro centrifuge tube under agitation at 37°C, and the bathing solution was removed and replaced with fresh water at 1, 3, and 24 hours. Then, 250 μL of the elution sample was added to 250 μL of $FeCl_3$ (1000 μg/dL), and subsequently, a commercial iron assay (QuantiChrom Iron Assay Kit; BioAssay Systems) was run on the combined sample to characterize the drop in iron concentration associated with the presence of DFO in the elution sample.

Immediate Tensile Test of DFO Suture

Sutures were soaked in DFO solution as above or in sterile water (n = 6 per group) for 1 hour. The suture was tied as a loop around two 10-mm stainless pins interfaced to a materials testing system (8500PLUS; Instron). The loop was tied using a surgeon's knot, followed by 6 single throws, and was pretensioned to 1 N, giving an approximate gauge length of 40 mm. The loop was then stretched at 10 mm/min until failure, with the load and displacement data recorded at 20 Hz and the ultimate tensile load evaluated.

Statistical Analysis

All comparisons were made by the Student unpaired *t* test. In cases in which equal variance criteria were not met, the Mann-Whitney rank-sum test was used for statistical comparisons. The Grubbs test for outliers was used on all data sets, and outliers were excluded from analysis. Three hens (1 DFO, 2 CTL) were dropped from analysis because of a complete tendon rupture during the survival period. One hen (DFO group) was dropped from analysis because of improper suture preparation. The final group size was 7 each (n = 7).

RESULTS

Assays

Hemoglobin content as a percentage of wet tissue weight was significantly increased in the DFO group compared with the CTL group (0.081 ± 0.012 vs 0.063 ± 0.016, respectively; P = .046) (Figure 2). DNA content as a percentage of wet tissue weight was significantly increased in the DFO group compared with the CTL group (Table 1). PG content as a percentage of wet tissue weight was significantly decreased in the DFO group compared with the CTL group (Table 1).

Chondroid Area

To measure the extent of chondroid, or fibrocartilaginous, tissue present in the repaired tendons, the percentage of

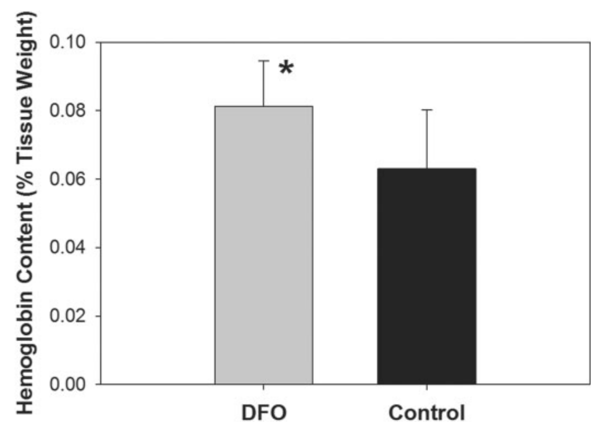

Figure 2. Results of the hemoglobin assay demonstrating the significant increase in hemoglobin content in the deferoxamine (DFO) group as a percentage of wet tissue weight. *Significantly different from the control group (P = .046).

TABLE 1
Analysis of DNA Content, PG Content, and Chondroid Area[a]

	Deferoxamine Group	Control Group	*P* Value
DNA content (% wet tissue weight)	0.31 ± 0.05	0.23 ± 0.03	.024
PG content (% wet tissue weight)	0.26 ± 0.02	0.33 ± 0.08	.035
Chondroid area (% total tissue area)	17.2 ± 6.6	24.6 ± 5.1	.038

[a]Data are presented as mean ± SD. PG, proteoglycan.

total area stained with safranin O was measured in the safranin O–stained slides. The DFO group showed a statistically significant decrease in the total area stained compared with the CTL group. The DFO group had significantly less PG content than the CTL group (Table 1).

Vessel Counts

Lectin-stained slides were used to calculate vessel density as a measure of angiogenesis in the articular and superficial zones of the tendon. Articular zone vessel density (vessels/mm^2) was significantly increased in the DFO group compared with the CTL group (7.1 ± 2.5 vs 2.1 ± 0.9, respectively; P = .026). Superficial zone vessel density was not significantly different between the DFO and CTL groups (15.0 ± 3.7 vs 12.2 ± 5.3, respectively; P = .30). Total vessel density was also not significantly different between the DFO and CTL groups (11.6 ± 4.2 vs 7.5 ± 3.3, respectively; P = .065) (Figure 3).

Histology

Representative images are displayed in Figure 4 for lectin-stained samples that were used for vessel counts. These

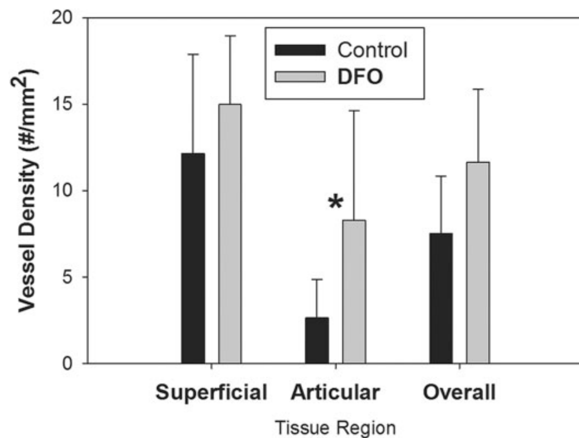

Figure 3. Results of the vessel density evaluation displaying values by region of interest. Articular zone density shows a significant difference between the deferoxamine (DFO) and control groups. *Significantly different from control group ($P = .026$).

images demonstrate increased lectin staining of vessels in the articular region for the DFO group compared with the CTL group. Representative hematoxylin and eosin and safranin O images used for both qualitative analysis and quantitative analysis (chondroid area in safranin O) are also displayed in Figure 4. These images demonstrate decreased PG staining (red zone) in the safranin O sections for the DFO group compared with the CTL group.

Elution Assay

The iron concentrations for the water- and DFO-soaked sutures were the following: water (1 hour: 564 ± 83 μg/dL; 3 hours: 573 ± 145 μg/dL; 24 hours: 537 ± 85 μg/dL) and DFO (1 hour: 92 ± 9 μg/dL; 3 hours: 511 ± 91 μg/dL; 24 hours: 494 ± 73 μg/dL). The iron concentration at 1 hour was found to be significantly decreased ($P < .001$) for the DFO-soaked suture compared with the water-soaked suture, indicating the release of DFO into the elution medium. However, the iron concentrations of the 2 groups did not differ at 3 or 24 hours, suggesting no further release of DFO.

Immediate Tensile Test

The ultimate tensile load of the DFO-soaked suture (53.4 ± 4.0) was found to be significantly greater ($P < .01$) than the water-soaked suture (46.7 ± 2.5), although the percentage improvement in load was minor.

DISCUSSION

This study was undertaken to test the hypothesis that DFO-saturated suture could enhance angiogenesis and the early markers of healing during the acute phase when used to repair a partial transection in the hypovascular fibrocartilaginous region of the chicken Achilles tendon. One of the major findings of this study was an improvement in vascularity with the DFO suture. The significant increase in hemoglobin content in the DFO group compared with the CTL group is evidence of increased angiogenesis in the fibrocartilaginous tissue exposed to DFO. Articular zone vessel density was significantly increased in the DFO group compared with the CTL group. The significant increase in vascularity within this zone indicates that DFO was especially successful in increasing angiogenesis in the fibrocartilaginous region. Superficial zone vessel density was not significantly different between the 2 groups. This may be a result of such tissue being more vascular in its native state, and thus, a change may be harder to detect because of variability in the native vascularity. While total vessel density was not significantly improved in the DFO group, the difference did approach significance ($P = .065$). This study may have been underpowered to detect a difference in total vessel density. The improvements observed in vascularity in our study correlate with past tendon healing studies that have used the local delivery of growth factors or angiogenic promotors to increase vascularity at injury sites.[21,22,33,37] Furthermore, our findings correlate with past studies that have used locally delivered DFO in alternative injury models to promote increased vascularity.[10,29]

Additional findings of the study were the increase in cellularity of the tissue and fibrous composition with the DFO treatment. A DNA content assay was used to evaluate cellularity, and a PG content assay and safranin O area analysis were performed to assess composition. The DFO group displayed a significantly higher level of DNA and significantly lower level of PG when compared with the CTL group. The finding of increased vascularity with DFO-treated sutures correlates with past studies that have used butyric acid sutures to increase tendon vascularity and also found increased cellularity by DNA content.[33] Safranin O analysis also demonstrated a decrease in the total chondroid area in the DFO group compared with the CTL group. The decrease in chondroid area as well as PG content in the DFO-treated sutures indicates that the healing tissue in the treated group was less cartilaginous in nature and more fibrous/vascular. Because DNA is a marker of cellularity, and chondroid tissue has a lower cell density than fibrous tissue, the results of the DNA and PG assays support increased fibrous tissue repair in the DFO group.

Taken in sum, we conjecture that by increasing angiogenesis at the repair site, we might improve tissue oxygenation and enable increased fibrous as opposed to chondroid repair. This idea tends to be supported by studies showing that fibroblasts undergo chondroid differentiation under hypoxic conditions.[14] Studies have also shown that the midportion of the human Achilles tendon undergoes fibrocartilaginous changes because of its hypovascular and hypoxic state.[6] We theorize that by improving angiogenesis in the fibrocartilaginous portion of the healing tendon, tissue oxygenation was improved, and chondroid tissue was diminished, allowing for a more robust fibrous healing response. Because chondroid tissue does not provide much tensile strength, pushing

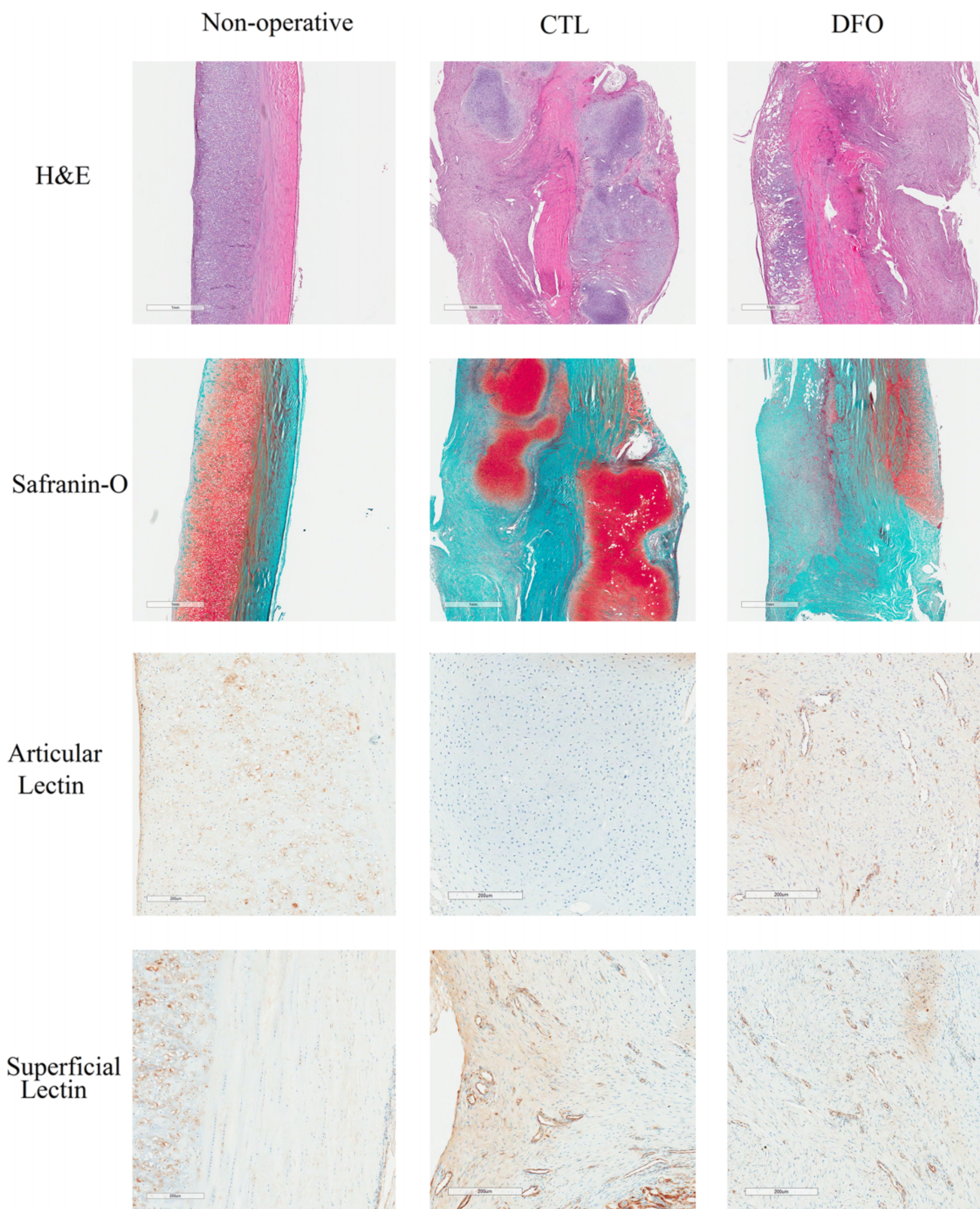

Figure 4. Representative histology for hematoxylin and eosin (H&E) (2× magnification), safranin O (2× magnification), and lectin (10× magnification). Scale bar is 1 mm in H&E and safranin O images. Scale bar is 0.20 mm in the lectin images. CTL, control group; DFO, deferoxamine group.

the repair process in the fibrous direction may accelerate recovery of the tendon's tensile strength. Similar effects were observed in a study of butyric acid–coated suture. In that study, by increasing angiogenesis, the authors showed that suture coated with butyric acid could improve the tensile strength of repairs in a rabbit

Achilles tendon model.[20] Our study data support the hypotheses that (1) locally delivered DFO increases angiogenesis and improves the healing potential in hypovascular fibrocartilaginous regions of the tendon and (2) suture can be used as an appropriate vehicle to deliver DFO locally.

A potential weakness of this study is the use of a newly established animal model. The Achilles tendon of the chicken acts as a gliding tendon as it passes over the hock joint. This region is exposed to increased compressive forces, thus causing fibrocartilaginous tissue to form on the articular surface of the overlying tendon.[12,34] This dense region of hypovascular fibrocartilage represents a ready model for testing the healing capacity of such fibrocartilage. Even though this is a new animal model, the chicken model is useful because hens have been shown to naturally develop tendinopathies, including spontaneous ruptures of the Achilles tendon.[18,20] Additionally, it has been shown that the Achilles tendon of the chicken has a similar fibrocartilaginous makeup to human Achilles tendinopathy specimens.[6,12] Other potential advantages of this Achilles tendon model include the larger size of the tendon (compared with rodents), bipedal loading, and the lower cost to purchase and house animals.

Another potential weakness of this study is the smaller sample size, although our study was powered to detect a 50% improvement in hemoglobin content that was in fact detected in the articular region. The use of absorbable polyglactin suture for the delivery of DFO might be considered a limitation of the study. Braided polyglactin suture was chosen because preliminary testing showed that it had a good ability to absorb the DFO solution with our soaking approach; however, other sutures may also be suitable with alternative coating methods. Our study focused on the acute phase of healing, and further studies at more progressed healing time points are required. Our study utilized an acute injury model, and it is important to consider that our findings may differ from those of a chronic injury model. A strength of this study is the novel use of a readily available drug, DFO, to improve angiogenesis.

CONCLUSION

This study found that DFO-saturated suture enhanced vascularity of the fibrocartilaginous region of the healing Achilles tendon during the acute phase. Results also indicated that DFO-saturated suture increased cellularity and the fibrous tissue makeup of the healing tendon. This study shows promising data to support the novel use of DFO delivered locally by a cost-effective and easily obtained source, suture, to improve healing for some of sports medicine's most common injuries.

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