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The effects of percutaneous ultrasound tenotomy on chronic tendinopathy in a rabbit Achilles' tendon model

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Abstract

Objective: Chronic tendinopathy is debilitating and significantly limits physical function and quality-of-life. For chronic tendinopathy recalcitrant to conservative approaches, percutaneous ultrasound tenotomy (PUT) has become a common minimally-invasive therapy. To clarify the histopathological effects of PUT, we assessed the cellular and tissue-level effects of PUT in an animal model of chronic tendinopathy.

Design: This within-subject randomized-controlled study treated the bilateral Achilles' tendons of 10 skeletally-mature, male New Zealand white rabbits with Kartogenin (KGN) beads to mimic chronic tendinopathy and allowed free cage activity for six weeks. One randomly-selected Achilles' tendon per rabbit underwent sonographically-guided PUT, using 60 s of ultrasound energy. Six weeks post-procedure, the bilateral Achilles' tendons were harvested and histologically graded.

Results: Treated and untreated sides were compared using paired t-tests and mixed models. There were no statistically-significant differences in collagen arrangement, cellularity, or ground substance deposition (all $p>0.16$). Bonar scores for the most superficial level (95% CI) and the whole tendon (95% CI) were 1.7 (0.6, 2.8) and 0.9 (0.0, 1.9) points lower on the PUT side compared with the untreated side ($p=0.037$ and $p=0.059$ respectively).

Conclusion: These findings provide evidence that PUT stimulates Histopathologic improvement in collagen formation and organization in the setting of chronic tendinopathy.

Keywords: Kartogenin, tendons, tendinopathy, tenotomy

Introduction

Chronic tendinopathy is one of the most frequent causes of musculoskeletal pain and functional limitations worldwide. In the United States alone, this condition is responsible for approximately 30 million visits to the physician each year [1, 2]. In most cases, conservative measures to treat tendinopathy are successful in resolving symptoms. However, when such measures fail, these cases contribute to considerable suffering, loss of productivity, and consumption of health care resources.

In the past, chronic tendinopathy was thought to be an inflammatory condition and was termed 'tendonitis.' Recent evidence has proven it to be a primarily non-inflammatory process, better characterized as a degenerative condition, therefore termed 'tendinitis or 'tendinopathy' [3, 5]. chronic symptoms are associated with tendon degeneration from repetitive micro trauma, cellular apoptosis, and auto phatic cell death. In some cases, such degenerative changes result in an inability to restore normal tendon structure and function. A hallmark pathologic feature is angiofibroblastic hyperplasia, which is characterized by an increase in fibroblasts and ground substance, thin and disorganized collagen, fiber kinking, vascular hyperplasia, damaged extracellular and intracellular matrix, and increased interfibrillar glycosaminoglycan (GAG) content. Tenocytes morphology changes from elongated to round. [6] There is also a lack of inflammatory cells, a poor healing response, and collagen degeneration with fiber disorientation. In patients with chronic tendinopathy, clinicians may observe tendon thickening with disrupted tendon architecture evident as hypo echogenicity on ultrasound [7]. Patients with acute tendinopathy may have resolution of symptoms with conservative measures, such as rest, stretching, eccentric strengthening, bracing, and analgesics.

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However, in approximately 20% of patients, symptoms fail to resolve with these conservative treatments [8]. Refractory tendinopathy is frequently treated with more invasive approaches, such as corticosteroid injections or surgery. However, recent evidence supports that corticosteroids may be more harmful than helpful, particularly for certain tendons [8]. Adverse effects with corticosteroid injections include tendon atrophy, decreased tendon strength, and tendon ruptures [9]. There is little evidence regarding precisely how open surgical tenotomy alters tendinopathy tissue [10]. Surgical treatment also may lead to complications such has wound infections, seromas, hematomas, nerve damage or tendon rupture [11]. Percutaneous treatment of tendons with ultrasonic energy as a viable treatment modality has been an effective and safe alternative to more invasive interventions [2]. The technology used in developing the percutaneous ultrasound tenotomy (PUT) tool was originally used for phacoemulsification of cataracts. Using this minimally invasive sonographically-guided ultrasonic debridement tool, the diseased tissue is debrided, lavage and aspirated with a single needle-shaped instrument, with the goal of removing tendinopathy tissue and creating a healthy bed for healing. The removal of the necrotic tissue with PUT improves conditions for a normal healing response, potentially conferring an advantage over percutaneous needle tenotomy. In 2013, the U.S. Food and Drug Administration approved PUT for the treatment of recalcitrant tendinopathy and since has been used for over 100,000 treatments of tendinopathy about the ankle, knee, hip, shoulder, and elbow as well as plantar fasciopathies [2, 10, 12, 18]. Over the last few years, studies have emerged to further support PUT as a viable treatment option for refractory cases of chronic tendinopathy. PUT has provided pain relief one week post-procedure with sustained pain relief, functional improvement, and reduced tendon thickness up to three-year follow-up [17]. Furthermore, the procedure is well tolerated with a reported success rate of 80 to 90%, with a very low rate of complications [2]. When compared to more invasive treatment techniques, such as surgical open tenotomy and debridement procedures, PUT has equivalent or better clinical results [17], with a more rapid onset of healing [19]. Although there is evidence that PUT is clinically effective, there remains a need to understand the biological effects of this treatment to inform dosing and post-procedural recommendations for recovery. To better understand the biological processes involved in tendon healing, this study tested the hypothesis that, in a model of bilateral chronic tendinopathy, PUT would restore the cellular and tissue-level characteristics of tendon in comparison with the untreated contralateral side.

Methods
Study design
A within-subject randomized controlled study was conducted to characterize the effect of percutaneous ultrasound tenotomy (PUT) in an animal model of chronic tendinopathy. One Achilles' tendon of each rabbit was randomized to undergo PUT six weeks following initiation of a chronic tendinopathy using a Kartogenin bead, with the contralateral limb serving as the control. Summative Bonar scores were compared between injured rabbit tendons treated with PUT vs. contralateral injured tendons that were not treated. Bonar scoring is a histopathological system used to grade levels of tendon tissue based on morphological factors such as cellularity and vascularity. Researchers involved in assessing outcome measures were blinded to which Achilles' tendons were treated with PUT.

Animal model
The experimental use of rabbits was ethically reviewed and approved by the IACUC of the corresponding author's institution (Protocol # 2018-245). Ten skeletally mature, male New Zealand white rabbits were enrolled. Male rabbits were included to minimize the confounding effects that female hormones, such as estrogen, play in tendon healing [20]. Use of a within-subject, paired controlled design allowed this study to control for subject-level factors (e.g. physical activity, health, and systemic factors), maximized efficiency and statistical power, and minimized the number of rabbits necessary to address the specific aims [21]. Usually in patients, Achilles' tendon loading is restricted with a walking boot for two weeks following unilateral PUT. However, using a within-subject control model of bilateral chronic tendinopathy, this was not feasible. Therefore, a cage size was selected to minimize tendon loading during the initial two weeks after the procedure and to allow for healing, while permitting limited loading through cage mobility with cardboard for enrichment. A clinical veterinarian provided animal housing and pre- and post-surgical care.

Kartogenin (KGN) bead-induced injury
Kartogenin (KGN) is a small compound known for its ability to promote chondrocyte differentiation and proliferation of stem/progenitor cells. Yuan *et al.* [6] Used the method of injecting KGN beads into rat Achilles tendons to establish a model of over-use tendinopathy comparable to an existing model resulting from extensive treadmill running. Kartogenin micro beads are a combination of KGN, dimethyl sulfoxide (DSMO) and distilled water. This solution is then added to alginate to create a suspension. A gel is formed once this solution is added to 2% calcium chloride. Each drop contains 5 µL of KGN-DMSO-alginate, which is the equivalent of 300 µg KGN per bead in 600 µg alginate, which were air dried for greater than 48 hours prior to use. After a two-week acclimation period to the environment, a clinical veterinarian anesthetized rabbits using ketamine, xylazine and isoflurane. Following anesthesia, an Integra Miltex 2 mm Disposable Biopsy Punch tool (Integra Life Sciences Services, York, PA, USA) was used to create a 200 µm biopsy punch and remove a cylinder of Achilles' tendon tissue 1 cm above each calcaneal tuberosity (Figure 1). The tissue removal created a repository for the KGN beads to be placed into the central region of the bilateral Achilles' (common calcaneal) tendons, 1 cm above the calcaneal tuberosity of both the right and left limbs of each rabbit under sterile conditions [15]. All rabbits were allowed to return to free cage activity for six weeks to allow chronic tendinopathy to be modeled. Veterinary technicians confirmed normal physical activity of each rabbit daily.

Percutaneous ultrasound tenotomy treatment
At six weeks following bilateral KGN bead placement, animals were anesthetized, and one randomly selected calcaneal tendon of each rabbit (randomization.com) was percutaneous treated with a sterile TX-1 instrument, (Tenex Health, Inc., Lake Forrest, CA), under sonography guidance using a consistent dose of ultrasonic energy. The skin was shaved to permit sonography guidance with a Philips CX-50 ultrasound scanner and a 15 MHz ip compact footprint short linear array transducer. A stab incision was made with an 11-blade to create access for the TX-1 tip, which was localized to

the area of tendinopathy. This tip has an approximately 16-gauge outer and 18-gauge inner double-lumen needle and uses ultrasonic energy to rapidly oscillate the hollow tip, which emulsifies and aspirates the degenerative tendon tissue. The area was treated on the medium setting for 60 s in each subject to treat the hypo echoic region of tendinopathy (Figure 2). After instrument removal, excess fluid was manually removed, and the wound was closed with acrylate skin adhesive. Following the PUT procedure, all rabbits returned to free cage activity for six weeks, with veterinary technicians confirming normal physical activity of each rabbit daily. Rabbits were euthanized after an additional six weeks, and the Achilles tendons (PUT-treated tendons and contralateral untreated control tendons) were harvested for analyses.

Histology
The rabbits were preserved on ice, in compliance with institutional Environmental Health and Safety policy for shipping biologic material, during transport. Tendon tissue was fixed in 10% formalin and processed for paraffin embedding and sectioning.
Hematoxylin and eosin (H&E) stains were used to evaluate structural changes. Hyper cellularity, cell morphology, collagen disorganization, tendon vascularity, calcification, and fatty change were evaluated on H&E-stained tendons. Alcian blue staining was utilized to grade cartilage matrices and ground substance.
Using Bonar scoring, a semi-quantitative histopathological scale, a pathologist scored the general structural changes including cell morphology, collagen, cellularity, and vascularity – along with the presence of calcification and fatty changes [22]. As Maffulli et al. [23] Similarly defined, the Bonar scoring system designates a score of 0 as a normal appearance and 3 as abnormal. The total sum for a single tendon sample with four sub-categories, therefore, can range from 0 to 12, with a higher score corresponding with a more pathological appearance.

Statistical analytic methods
Investigators for a prior study with a similar design reported differences of 3.75±0.50 in an untreated rabbit tendinopathy, 1.25±0.46 for a PUT-treated rabbit tendinopathy, yielding an effect size of 5.19. A sample size of three pairs of rabbit tendons would be necessary at a power of 0.98 and a two-tailed alpha level of 0.05 to detect an effect size at least this large. Given that we planned to use a Kartogenin model and assess over six weeks instead of three, this study aimed to detect an effect size as small as 1.4, which would require nine pairs of tendons, and this was increased to N=10 in case of 10% drop-out or missing data. For this within-subject, paired, controlled study, paired t-tests were used to compare outcomes between untreated (KGN-only) and treated (KGN+PUT) tendons. Significance was defined at an alpha level of 0.05. The normality of the data distribution was analyzed with the Kolmogorov-Smirnov test ($p>0.05$). Pathologic scoring was available for three levels of tissue depth within each tendon, so analyses were stratified by level. A summative Bonar score (primary outcome) was also calculated for combined tendon levels. To analyze all three levels of tissue depth on each side, a mixed linear model was constructed with subject as a random factor, with side and level within each subject as repeated factors and a covariance matrix that accounted for paired observations within subjects in estimating least squared means (LS Mean) and standard errors (SE). No adjustment was necessary. Statistical analyses

were completed using SAS version 9.4 (SAS, Cary, NC).

Results
Of the 10 rabbits in the study sample, one rabbit was not included in analyses due to its tendons being sectioned transversely rather than longitudinally. The remaining nine rabbits contributed bilateral calcaneal tendons that were sectioned longitudinally (n=18). No adverse events occurred. Untreated tendons demonstrated cartilage development in the region of the KGN bead insertion, providing evidence that the model was effective at replicating conditions of chronic tendinopathy (Figure 3A). In the most superficial layer, Bonar score was a mean (95%CI) of 1.7 (0.6, 2.8) points lower on the PUT-treated side than on the untreated side (p=0.037). Significant improvement was not detected at the middle (p=0.856) or deepest levels (p=0.152). As shown in Table 1, evaluating all tissue depths in a single mixed model revealed that the Bonar score for the PUT-treated tendons had an LS Mean of 10.5 (95% CI 9.7, 11.3), and the control tendons had a Bonar score of 11.5 (10.9, 12.1). The calculated difference was 0.9 (0.0, 1.9) points lower in the PUT treated group (p=0.059). Likewise, as evidenced by histo chemical staining results, whereas healed tissue of untreated tendons exhibited areas of disorganized tenocytes and vascular infiltration (Figure 4), treated tendons healed well with organized areas of high cell density (Figure 3B,C).

Discussion
The results of this within-subject, paired, controlled study of nine rabbits demonstrated the cellular and tissue-level effectiveness of PUT treatment compared to the natural course of chronic tendinopathy in a model of Achilles' tendinopathy. Treatment with PUT demonstrated evidence of improvement in fatty changes and collagen degeneration in treated tendons when compared to untreated tendons. The tendons treated with PUT exhibited a significantly lower total Bonar score for the most superficial layer of the tendon and confidence limits for analysis of all three tendon depths combined were also suggestive of significant improvement in tendon pathology in the treated limbs.
Despite widespread use of PUT for treatment of chronic tendinopathy, there have been a relatively small number of published studies and those generally reported on clinical outcomes of PUT. There are very few studies that have characterized the histopathological effects of PUT. As reported by Baria et al. [24] examination of the structural effects of PUT on diseased tendon revealed that the procedure successfully debrides tendon tissue while sparing neighboring areas. Although Baria et al. comparably assessed the histological structural effect of PUT on tendons, their findings may not have adequately modeled in-vivo post-PUT tendon behavior found in humans diagnosed with chronic tendinopathy. Rather than using an animal model, their study was performed on cadaveric knee specimen that had not undergone degeneration from chronic tendon overuse or overloading. An animal model was necessary in our study to accurately replicate the complex features of chronic tendinopathy, as well as the healing process following treatment, in a controlled, reproducible manner [25]. A rabbit model of chronic tendinopathy is useful for evaluating the tissue effects of PUT in that a rabbit is the smallest animal that could be obtained that has calcaneal tendons large enough to use the TX-1 instrument.
In the previous rabbit Achilles' model pilot study by Kamineni et al. [15] Histopathological examination similarly revealed that treated Achilles' tendons had better

histopathological scores than non-treated tendons (*p*<0.001). Although Kamineni *et al.* utilized the Movin scoring system to classify histopathological findings of tendinopathy and our study used Bonar scoring, as reported by Maffulli *et al.* [23] the two scoring systems are comparable and only differ in that hyalinization grading is not included in the Bonar score. According to Maffulli *et al.* [23] hyalinization is rare and is not easily reproducible in tendon samples. Following PUT treatment, Western blot analysis of injured tendon samples in the Kamineni *et al.* study indicated increased expression of collagen type I, III, and X to more normal qualitative and semi-quantitative levels. However, in contrast with our study, Kamineni *et al.* included female rabbits and used a between-subject study design rather than a within-subject, paired controlled design. Our study aimed to minimize the confounding effects of female hormones and inter-subject differences between rabbits [20]. To address the limitation of insufficient follow-up time after treatment, as discussed in Kamineni *et al.*, our sample of rabbits received twice the amount of time for activity pre- and post-PUT treatment, as well as for the length of the procedure itself. While Kamineni *et al.* treated the tendons at three weeks following collagenase injection and evaluated histopathological findings at three weeks post-PUT, we conducted PUT six weeks following KGN injection to more accurately model chronic tendinopathy and studied its effects an additional six weeks later. Doing so provided the tendons a longer duration to heal, potentially modeling the outcomes of PUT more characteristic of the effect expected in clinical treatments. Additionally, while rabbits were injected with collagenase in the Kamineni *et al.* study to mimic the features of diffuse inflammation and hyper vascularity associated with tendinopathy [26], that model likely generates a different tendon injury than occurs in chronic tendinopathy. For this reason, rabbits in our study were injected with KGN beads to generate more localized effects that better model tendon pathology seen with chronic overloading or overuse [6]. To the best of our knowledge, similar studies with these experimental conditions had not been reported at the time of conducing this study. As our study and previous studies have demonstrated, the regenerative physiological response promoted by PUT leads to more complete healing of tendon tissue compared to untreated tendons. Thus, by debriding and emulsifying diseased tendon tissue, PUT may create a clean bed for healing and collagen formation [27]. It could also be possible that by producing a small incision in the tendon, PUT stimulates a healing process at the injection site. In a study by Petrou *et al.* [28] Lymphocytes were found at the borders of the hole site following partial Achilles tenotomy, suggesting an inflammatory response to the surgical intervention. The same inflammatory process characterized by angiogenesis and the presence of fibroblast precursors could have occurred as a result of penetration by the PUT instrument. However, this does not seem likely given our finding of improved collagen organization and signs of healing following PUT. Nevertheless, the potential must be considered and there is a need to further evaluate the mechanisms by which PUT

induces tissue regeneration for each type of tissue within the damage tendon, a task that could not be completed with our study sample. Strengths of this study included the within-subject design that minimized variability between intervention and control tendons and blinding such that the investigator who performed the treatments was not involved in the assessment of histological outcomes, thus blinding the outcome assessments and statistical analyses.

Once traditionally classified as an inflammatory condition and treated with corticosteroid injections, chronic tendinopathy is now understood to be a degenerative condition marked by histologic patterns of angiofibroblastic hyperplasia [29, 30]. Current literature has supported the clinical effectiveness of PUT and, as of 2019, over 100,000 PUT treatments have been completed for patients with chronic tendinopathy. However, there remains a need to characterize the mechanism of effect of PUT for tendon biological responses that may account for clinical responses. Thus, while small, this study advanced knowledge regarding use of PUT for chronic tendinopathy. Further investigation of the histopathological changes that occur following PUT could help establish appropriate patient criteria for treatment, dosing and timing of interventions, and post-procedural rehabilitation recommendations.

Limitations
There were several limitations to this small initial study that was powered based upon the available budget-grant funding was sufficient to include 10 rabbits. While 10 was the minimum sample size for statistical power in analyzing differences of interest, tendons for one rabbit could not be used due to being sectioned transversely, leaving only nine pairs of tendons for analyses and limiting statistical power. A study with a larger number of rabbits would be advantageous to increase the statistical power to detect more subtle findings. A significant difference was not found when analyzing individual subcomponents of the Bonar score, which could be related to an insufficient statistical power. Second, cage size could have influenced activity level of the rabbits and affected tendon healing, since rabbits were allowed free activity following procedures. However, the same cages were used for all rabbits and following both the initiation of the bilateral tendinopathy and the unilateral treatment, reducing bias based on time or subjects. Third, although the KGN bead stayed within the tendons and there was evidence of chronic tendinopathy on sonography, we did not inspect the tendons prior to dissection to compare normal and abnormal tendons. This could have impacted the significance of our histopathological findings and their correlation with the effects of chronic tendinopathy and PUT treatment, if for example the bead fell out of the tendon or tendinopathy was not present on the untreated side. Thus, this factor should be confirmed in future research. Finally, when tendons were sectioned, the technician did not mark the treatment location on the tissue, and this led to some initial difficulty in identifying the location of the KGN beads and PUT treatments.

Table 1; Average (±SD) Bonar scores of treated and untreated tendons

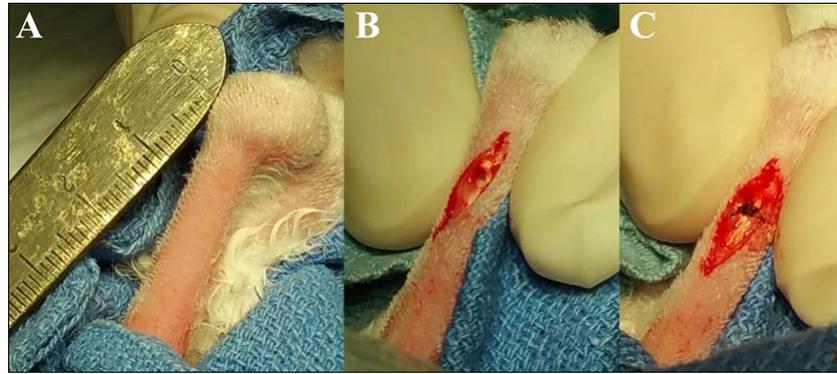| Average overall Bonar score (by tissue depth)	Treated Tendon	Untreated Tendon	p-value
Superficial	9.4 ± 2.2	11.1 ± 1.5	0.037
Intermediate	11.4 ± 1.8	11.3 ± 1.7	0.856
Deep	10.8 ± 1.5	12.0 ± 1.5	0.152
Average overall Bonar score			
All tissue depths	10.5 ± 2.0	11.5 ± 1.5	0.059

Fig 1: KGN bead-induced injury (A) Measurement with ruler 1 cm above calcaneal tuberosity (B) Post-biopsy punch (C) Suture following KGN bead insertion

Fig 2: TX-1 instrument (top) and tendon following PUT. The 32-mm long, approximately 16-gauge outer and 18-gauge inner double-lumen instrument was placed with sonographic guidance. Ultrasonic energy rapidly oscillated the hollow tip, which emulsified and aspirated the degenerative tendon tissue, with minimal disruption of healthy tendon tissue. The tendon following treatment with PUT (bottom).

Fig 3: (A) Alcian blue staining of healed, untreated Achilles' tendon section with hematoxylin and eosin, demonstrating cartilage development in the region of the KGN bead insertion. Alcian blue staining of healed, PUT-treated Achilles' tendon displaying (B) organized tenocytes also seen at (C) higher magnification of healed region
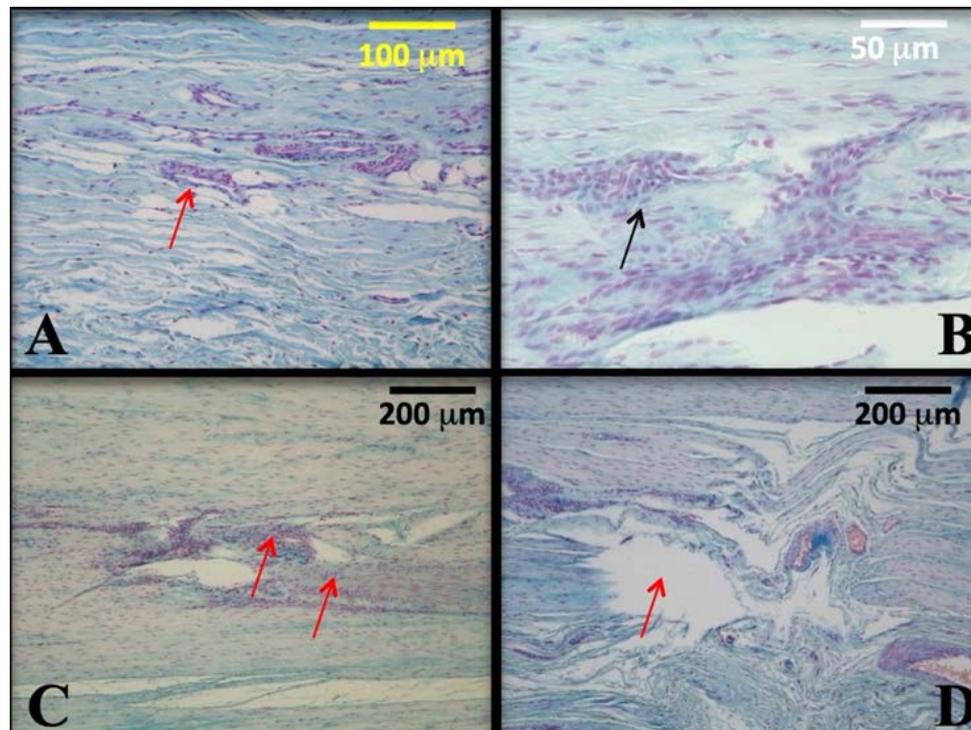
Fig 4: Alcian blue staining of healed, untreated Achilles' tendon marked by areas of high cell density with (B) disorganized tenocytes and (A, C) vascular infiltration. (D) Wound from the KGN bead insertion.

Conclusion
There was significant improvement in tendinopathy among rabbits treated with PUT, as exhibited by lower Bonar scores. This study generated evidence that supports that PUT stimulates beneficial responses in diseased tendon tissue and promotes healing. PUT remains a promising treatment option for chronic tendinopathy in patients whose symptoms have not resolved from more conservative methods, such as rest and stretching. Further research with greater statistical power is needed to better characterize cellular and tissue responses, as well as post-treatment protection regimens to advance understanding of PUT outcomes for chronic tendinopathy.

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