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Tissue-engineered intrasynovial tendons: Optimization of acellularization and seeding

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Abstract—The purpose of this research was to develop a tissue-engineered intrasynovial flexor tendon construct with the use of an acellularized flexor tendon scaffold repopulated with intrasynovial tendon cells. New Zealand white rabbit intrasynovial flexor tendons were acellularized by the following methods: high concentration NaCl + SDS, Trypsin/EDTA, Trypsin/EDTA + Triton X-100, Triton X-100, Triton X-100 + SDS, and freezing at –70 °C followed by Trypsin/EDTA + Triton X-100. Epitenon and endotenon cells were also isolated from rabbit intrasynovial tendons and expanded in culture. Acellularized tendon scaffolds were then reseeded with these cells. A subset of epitenon and endotenon cells was labeled with green and red fluorescent markers, respectively, to further characterize the preferred location of their attachment. Optimal acellularization was achieved by freezing at –70 °C followed by Trypsin/EDTA + Triton X-100. After reseeding, light microscopy of tendon constructs showed attachment of both epitenon and endotenon to the tendon scaffolds, with endotenon cells more likely to be found in the core of the scaffold. An intrasynovial tendon construct was developed with the use of acellularized intrasynovial tendons repopulated with intrasynovial tenocytes. These constructs grossly resemble normal intrasynovial tendons, and cells were found both on the surface and the core of the construct histologically. This new construct represents an important first step in developing a viable tissue-engineered flexor tendon.

Key words: acellularization, endotenon, epitenon, flexor tendon, flexor tendon sheath, hand surgery, intrasynovial tendon, rabbit model, rehabilitation, reseeding, tissue engineering.

INTRODUCTION

Zone II flexor tendon injuries represent a significant challenge for hand surgeons. The superficial and deep flexor tendons in the hand travel though five zones to reach their target insertion site on the distal joints of the fingers. Zone II is located between the palmar crease and the distal portion of the middle phalanx. The flexor tendons travel within a tight fibroosseous sheath and pulley system in Zone II. Injuries in Zone II lead to scarring within this tunnel and significantly limit the range of motion of the affected finger. While techniques for Zone II repair have been refined over the last century, clinical outcomes remain poor for a substantial number of patients. In particular, two main problems exist for Zone II flexor tendon repair: (1) postoperative adhesion, or scarring, and (2) lack of appropriate tendon material for reconstruction. Although not yet used in clinical settings, a number of strategies have been sought to reduce postoperative adhesion formation [1–7]. However, the development of new materials for tendon reconstruction has not been extensively studied.

Abbreviations: CO_2 = carbon dioxide, FDP = flexor digitorum profundus, PBS = phosphate-buffered saline.

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Many have attempted to use synthetic materials to bridge flexor tendon defects. A number of materials have been studied, including Dacron grafts [8], carbon fibers [9], and silastic sheets [10]. However, results for these materials have been poor with regard to healing and mechanical stability [8–10]. It has become evident that synthetic materials are so far unsuitable for tendon reconstruction.

The need for suitable "tendon-like" material has led many to advocate the use of autologous tendon grafts. Currently, autologous donor tendons include the palmaris longus, plantaris, extensor indicis proprius, extensor digiti minimi, flexor digitorum superficialis, and extensor digitorum longus tendons [11–12]. However, use of these tendons may result in significant donor site morbidity as well as limited excursion and poor digital function postoperatively [13]. Poor results have been hypothesized to occur because extrasynovial tendons are used to replace intrasynovial tendon losses. The intrasynovial tendons are lined with only a single layer of epitenon cells. Studies have suggested that intrasynovial tendons with epitenon cells employ an intrinsic mechanism of incorporation into the repair. In contrast, extrasynovial tendons act as a conduit for ingrowth of vessels and cells, primarily through adhesion formation. These differences in healing may account for the superior function of intrasynovial grafts observed in canine studies [14–15]. Recently, intrasynovial donor tendons have been harvested from the flexor digitorum longus tendons of the foot in humans [16]. While results have been favorable, with extensive tendon loss there may not be enough tendon material for reconstruction. Furthermore, donor site morbidity must be considered when harvesting autologous tendons.

Human allografts have also been used to replace tendon losses in Zone II flexor tendon injuries. While initial results were poor, a recent report of two patients receiving allograft reconstruction showed promising outcomes [17]. However, the potential for disease transmission, the need for possible immunosuppression, and bioethical concerns have made the use of allograft tendons less attractive.

Tissue engineering of flexor tendons is the next logical step in providing material for reconstruction. To date, significant research has been conducted in the engineering of bone and cartilage. However, tendon engineering has not been extensively studied. For the engineering of any tissue, two components are required: (1) an extracellular scaffold and (2) viable cells for seeding of the scaffold. The resulting engineered construct should be mechanically durable, nonimmunogenic, and able to retain in vivo sustainability. Experience with heart valve engineering has shown that an allogenic acellularized scaffold repopulated with autologous cells may produce the most reliable construct to meet the above requirements [18]. As for flexor tendon tissue engineering, epitenon and endotenon cells are logical candidates. The function of epitenon cells covering the surface of the tendon is to secrete hyaluronic acid that serves as a lubricant to counteract frictional irritation. The function of endotenon cells is to help to maintain the overall collagen architecture of the tendon. The purpose of this study was to optimize the acellularization and seeding processes for construction of intrasynovial tendons. Specifically, allogenic intrasynovial flexor tendons were acellularized and then the constructs were reseeded with epitenon and endotenon intrasynovial cells expanded in cell culture. Ultimately, these constructs may be used as tendon grafts for reconstructing Zone II tendon defects.

MATERIALS AND METHODS

Acellularization of Allogenic Intrasynovial Tendons

All rabbit laboratory experiments were carried out with strict adherence to the animal care protocols at the Stanford University Hospital and Clinics and the Department of Veterans Affairs Palo Alto Health Care System. Adult male New Zealand white rabbits were euthanized by intravenous injection (n = 10). Using microsurgical technique, the forepaw and hindpaw flexor digitorum profundus (FDP) equivalent was dissected free of surrounding tissues. A 1.5 cm portion of the Zone II FDP tendon was completely excised. Specimens were immediately placed into tissue-preserving solution containing Hank's buffered saline solution enriched with 100 IU/mL penicillin-streptomycin. Tendons for acellularization were then washed with phosphate-buffered saline (PBS). Tendons were then either frozen at –70 °C or acellularized by one of the following protocols: (1) 1.5 M NaCl solution for 24 hours, 48 hours, or 72 hours followed by SDS 0.5 percent solution for 30 minutes; (2) Trypsin 0.05 percent/0.53 mM EDTA for 24 hours, 48 hours, or 72 hours; (3) Trypsin 0.05 percent/0.53 mM EDTA for 24 hours followed by Triton X-100 0.5 percent for 24 hours; (4) Triton X-100 3 percent for 24 hours or 48 hours; or (5) Triton X-100 3 percent for 24 hours followed by SDS 0.5 percent for 24 hours. Frozen tendons were allowed to thaw to room temperature and were then placed into Trypsin 0.05 percent/0.53 mM EDTA for 24 hours followed by

Triton X-100 0.5 percent for 24 hours. Acellularized tendons were then placed in PBS at 4 °C until use. Representative samples of tendon were obtained before and after treatment for light microscopy.

Cell Isolation, Culture, and Seeding

Zone II flexor digitorum profundus tendons were isolated and transected as just described. Intact flexor tendons and tendon sheaths were then separated by dissection under magnification. The intact tendons were treated with 0.25 percent Trypsin at 37 °C for 20 minutes to release the epitenon tenocytes. The epitenon tenocytes were plated and cultured in Ham's F-12 medium. The remaining tendons were treated with 0.5 percent collagenase to release endotenon tenocytes, which were similarly plated and cultured in Ham's F-12 medium. Both cell types were then grown to confluence at 37 °C in a humidified tissue culture chamber with 5 percent carbon dioxide (CO_2). At confluence, cells were passaged by washing with PBS and detached with Trypsin/EDTA.

Cultured tenocytes were then collected and resuspended in rabbit serum at a density of at least 1×10^6 cells/mL. One milliliter of serum with at least 1×10^6 epitenon or endotenon cells was used to reseed each 1.5 cm portion of scaffold. Acellularized tendon scaffolds (–70 °C freezing protocol) were then placed into sterile test tubes and reseeded by one of the following methods ($n = 5$ tendons in each group): (1) static cell-suspension of epitenon cells, (2) static cell-suspension of endotenon cells, or (3) simultaneous static cell-suspension of endotenon and epitenon cells. The cell-scaffold constructs were then incubated at 37 °C in a humidified tissue culture chamber with 5 percent CO_2 to allow attachment of cells. The culture medium was changed every other day during the incubation period. Representative samples of tendon constructs were obtained at 1-, 3-, and 6-week intervals for light microscopy.

Histological Analysis

Harvested tissue was embedded with OCT Compound (Tissue-Tek®, Sakura Finetek U.S.A., Inc; Torrance, California). Frozen sections were cut at 8 μ to 10 μ with a cryostat (Leica CM 1800, International Medical Equipment, Inc; San Marcos, CA). Sections were then mounted onto slides, fixed with acetone for 10 minutes, dried, and then stored at –20 °C until use.

Slides were rinsed with PBS. Harris hematoxylin stain (Sigma-Aldrich; Milwaukee, Wisconsin) was added for 30 seconds, then the slides were rinsed in distilled water. Eosin stain was added for 30 seconds. The slides were dried, mounted with Permount (Fisher), and photographed with a Nikon microscope and digital camera.

Fluorescent Labeling and Microscopy

Epitenon cells were isolated and expanded in tissue culture as previously described. After reaching a density of 1×10^6 cells/mL, cells were passaged by washing with PBS and detached with Trypsin/EDTA. A cell pellet was then obtained by centrifugation. Epitenon cells were then resuspended in prewarmed PBS solution at 37 °C containing the Vybrant® CFDA SE (carboxy-fluorescein diacetate, succinimidyl ester) cell tracer kit probe (Molecular Probes V-12883, Invitgrogen, Life Technologies; Carlsbad, California), at a concentration of 5 μM. The cells were incubated with the probe for 15 minutes at 37 °C then repelleted by centrifugation. Cells were resuspended in the prewarmed medium and incubated for another 30 minutes at the same temperature. Cells were washed with PBS and used for scaffold reseeding as previously described. Scaffolds were studied at 1 week for attachment of cells by fluorescent microscopy with an excitation/emission wavelength of 492/517 nm (green color).

Endotenon cells were treated with Vybrant® CM-Dil (chloromethylbenzamido) cell labeling solution (Molecular Probes V-22888). Cell treatment was as just described. After reseeding, scaffolds were studied at 1 week for attachment of cells by fluorescent microscopy with an excitation/emission wavelength of 553/570 nm (red color).

The slides were mounted on and photographed with a Nikon confocal microscope. Slides were examined for localization of epitenon cells on the tendon surface and endotenon cells in the core of the tendon by the color pattern green versus red.

RESULTS

Acellularization

All acellularized tendons appeared intact and no gross differences were seen between any groups (**Figure 1**). The collagen architecture was undisturbed after all acellularization protocols. Initial attempts at acellularization—using Trypsin/EDTA, Trypsin/EDTA + Triton X-100, Triton X-100, and Triton X-100 + SDS and high-concentration NaCl + SDS—and resulted in persistence of viable cells within the tendon (**Figure 2**). Tendons frozen at –70 °C and then acellularized by Trypsin 0.05 percent/EDTA for

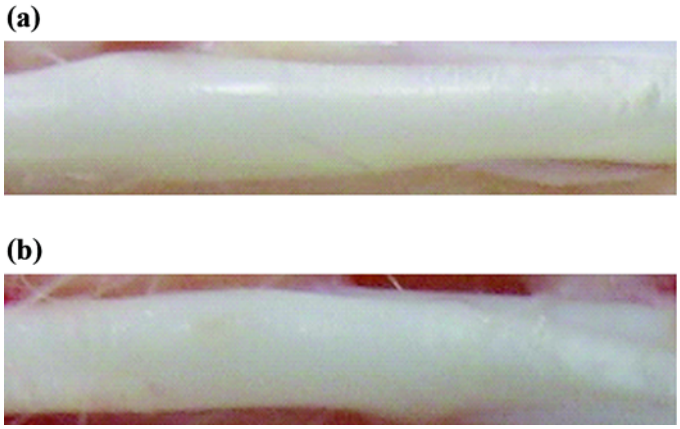

Figure 1.
Gross appearance of **(a)** reseeded tendon construct and **(b)** normal tendon.

24 hours followed by Triton X-100 0.5 percent for 24 hours were completely acellularized based on light microscopy in all representative samples (**Figure 3**). These samples included tendon sectioned from the intact midportion of the acellularized scaffolds.

Cell Isolation, Culture, and Seeding

Acellularized scaffolds statically reseeded with epitenon cells by the suspension method showed a single monolayer of cells at the 1- and 3-week intervals (**Figure 4**). Scaffolds reseeded with endotenon cells showed initial clumping of cells on the outer surface of the scaffolds at 1 week. By 3 weeks, the cells were seen within the substance of the scaffold (**Figure 5**). Similar results were seen at 6 weeks.

Fluorescent Labeling and Microscopy

Both epitenon and endotenon cells were successfully labeled with green and red fluorescent markers, respectively. Tendon constructs reseeded simultaneously with fluorescently labeled epitenon and endotenon cells showed the presence of both cell populations on a single construct. Epitenon cells appeared to attach mainly to the periphery of the tendon. Endotenon cells attached to the periphery, some were also found within the substance of the tendon by 3 weeks (**Figure 6**).

DISCUSSION

Intrasynovial flexor tendons are the optimal tendon grafts for reconstruction of Zone II flexor tendon defects and for cases of hand injuries with extensive tendon loss. The supply of intrasynovial tendon grafts is limited. Tissue engineering of intrasynovial tendons is the next logical step in the evolution of Zone II flexor tendon reconstruction. However, little research has been conducted on the engineering of tendons.

In 1994, Cao et al. reported the use of calf tenocytes to seed polyglycolic acid scaffolds in vitro [19]. The resulting constructs were then implanted in nude mice for further maturation. Histological and biochemical studies performed after implantation showed gross resemblance to normal tendon, with parallel linear organization of collagen fibers and comparable tensile strength. In a more recent study by the same group, adult Leghorn hen tenocytes were used to seed polyglycolic acid scaffolds [20]. The resulting constructs were wrapped in intestinal submucosa, cultured for 1 week, and then implanted into artificially created Zone II flexor tendon defects in hens. Again, analysis showed that implanted tendons resembled normal tendon in both gross and histological appearance. No immune rejection occurred and constructs achieved 80 percent of normal tendon tensile strength after 14 weeks. These studies have been promising for the development of an engineered tendon. However, two areas of concern remain. First, the tendons were engineered using polyglycolic acid as a scaffold. Biomechanical studies performed in these tendons showed that the scaffold material alone was much weaker than normal tendon. At 12 weeks, after ingrowth of tendon had occurred and the scaffold was degraded, the new tendon had strength comparable to that of normal tendon. This lag period is significant when one considers the importance of early motion protocols in the reduction of postoperative adhesions and stiffness. Second, the scaffolds were seeded with tenocytes of unknown origin. As previously discussed, intrasynovial tendons with their lining of epitenon cells have proven superior to extrasynovial tendons with respect to tendon healing. Theoretically, engineering of a tendon construct with both endotenon and epitenon cells would yield improved results.

Zone II flexor tendon defects may be bridged by the use of an allogenic acellularized intrasynovial tendon scaffold repopulated with intrasynovial epitenon and endotenon cells. Studies with human heart valves have shown that allogenic, acellularized valves can be reseeded with human endothelial cells to provide viable engineered constructs [21]. Initial studies in a sheep model have shown that these constructs may provide superior results compared with

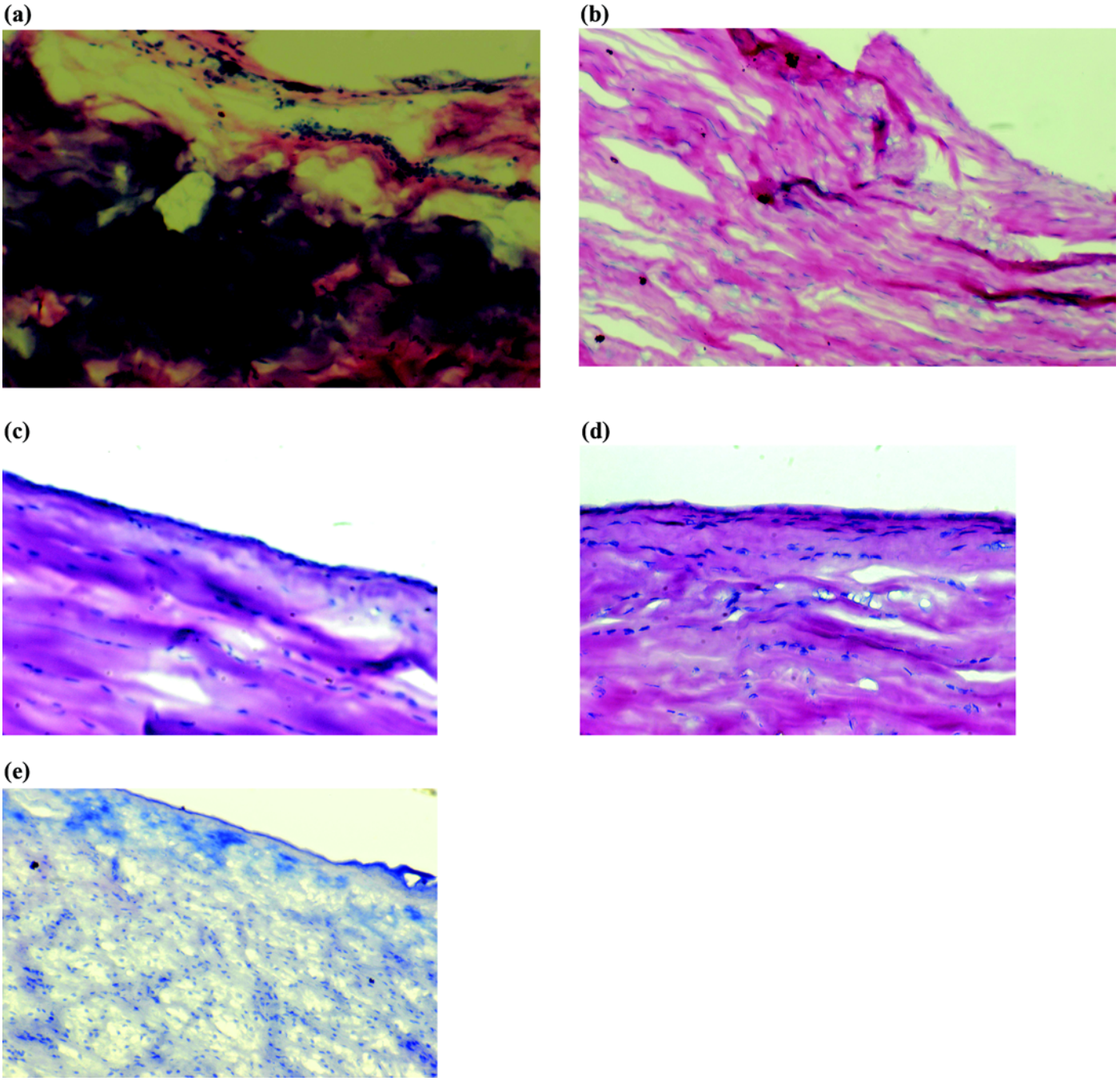

Figure 2.
Acellularization protocols showing retained cells: **(a)** Trypsin 0.05%/EDTA for 72 h (4× magnification); **(b)** Trypsin 0.05%/EDTA for 24 h followed by Triton X-100 0.5% for 24 h (4× magnification); **(c)** Triton X-100 3% for 48 h (10× magnification); **(d)** Triton X-100 3% for 24 h followed by SDS 0.5% for 24 h (10× magnification); and **(e)** 1.5 M NaCl solution for 72 h followed by SDS 0.5% solution for 30 min (4× magnification).

unpopulated scaffolds with respect to tissue reconstitution in vivo [18]. While polymer and xenogenic scaffolds have been used, a number of questions have arisen concerning the viability of the resulting constructs. Polymer scaffolds lack extracellular binding sites for cell adhesion, and studies with heart valves have shown improved cellular adhesion with

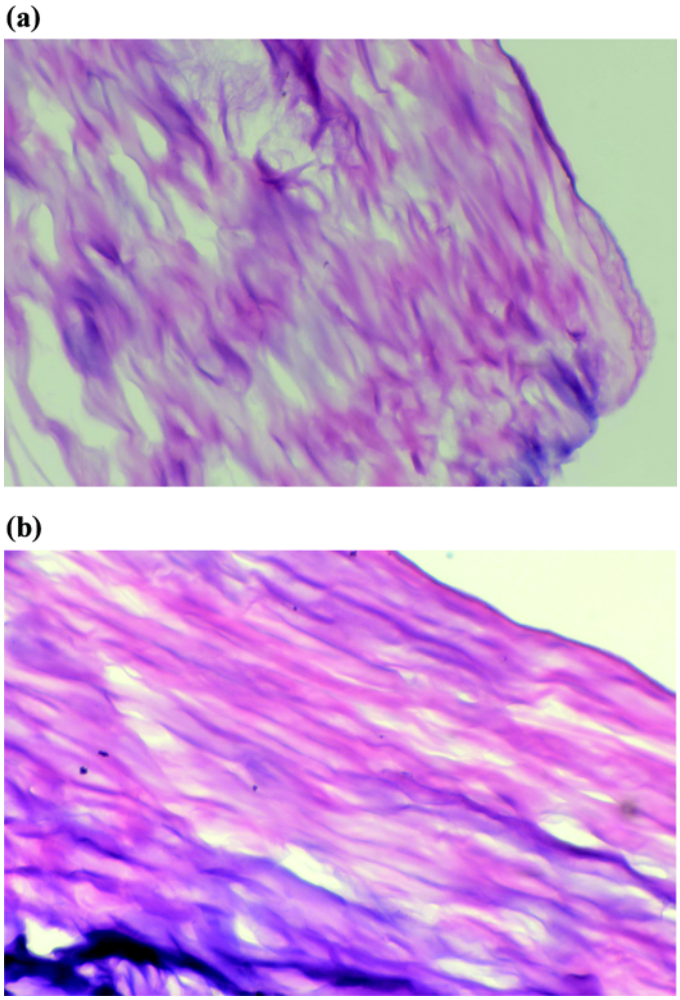

Figure 3.
Tendon acellularized by freezing at –70 °C and then treatment with Trypsin 0.05%/EDTA for 24 h followed by Triton X-100 0.5% for 24 h: **(a)** Tendon end and **(b)** midtendon, both 10× magnification.

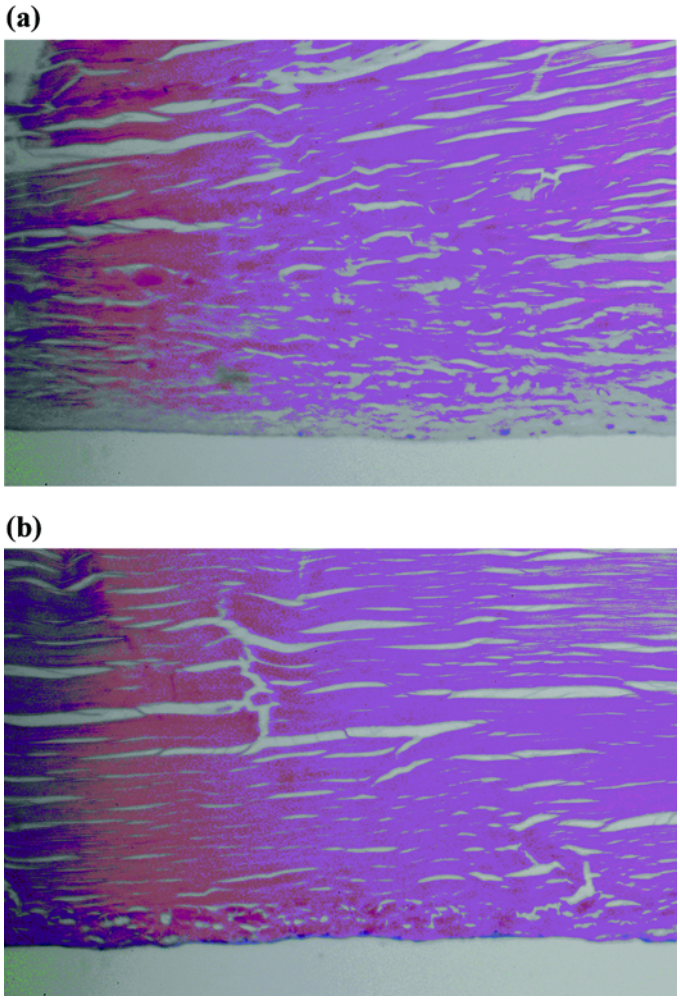

Figure 4.
Acellularized tendon scaffolds reseeded with epitenon cells by suspension method (4× magnification): **(a)** 1 week and **(b)** 3 weeks.

the use of acellularized tissue scaffolds [18,22]. Xenogenic heart valve scaffolds have several drawbacks, including unknown transfer of animal-related infectious diseases and xenogenic rejection patterns [23–24].

In this study, we attempted to determine an optimal protocol for the acellularization and subsequent reseeding of intrasynovial tendons. Based on our results, the protocol consisting of freezing at –70 °C and subsequent treatment with Trypsin 0.05 percent/EDTA for 24 hours followed by Triton X-100 0.5 percent for 24 hours was superior with respect to acellularization of intrasynovial tendon. All other methods of chemical acellularization proved unsuccessful. The freezing and subsequent thawing of the tendon likely allows for expansion of the dense collagen matrix, thereby enabling the detergent to enter the substance of the tendon more effectively. However, the collagen architecture under light microscopy did appear to remain intact. Recent preliminary studies in our laboratory showed that the tensile strength of the acellularized scaffolds is comparable to normal intrasynovial tendons.

Fluorescent labeling of cells proved that both cell types attached with simultaneous seeding of both epitenon and endotenon cells. Furthermore, the endotenon cells appeared to have slightly better penetration into the core substance of the construct.

In the future, adding growth factors or adhesion proteins to manipulate the seeding process may improve the spatial orientation of the epitenon and endotenon cells.

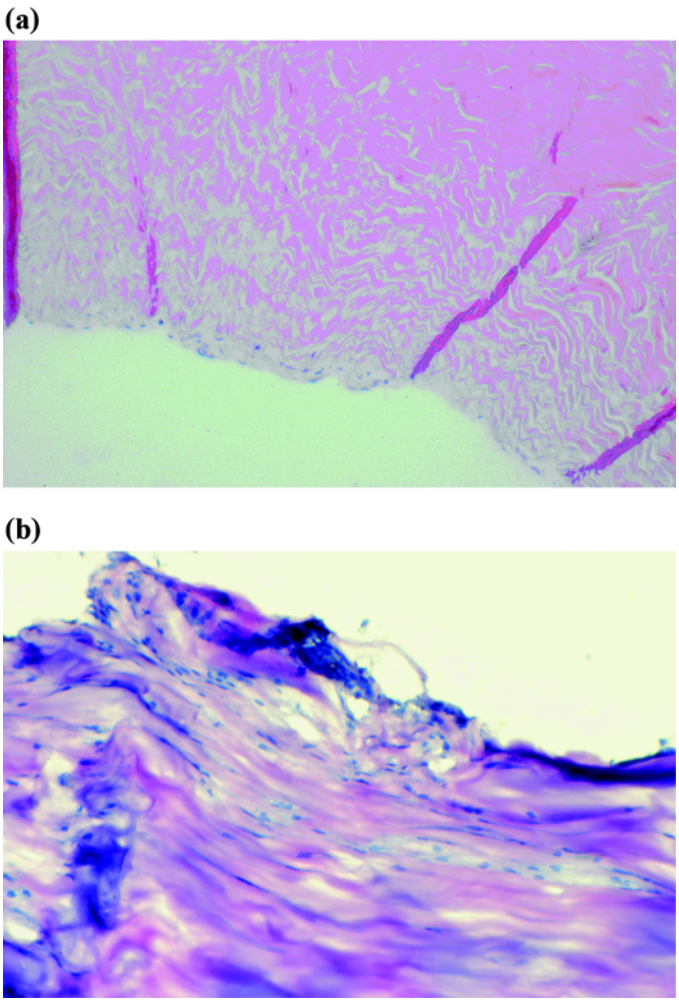

Figure 5.
Acellularized tendon scaffolds reseeded with endotenon cells by suspension method (4× magnification): **(a)** 1 week and **(b)** 3 weeks.

Furthermore, a bioreactor may be used to simulate the mechanical stress of muscle contraction. In terms of producing enough cells for reseeding, we have not encountered this problem in the current study. Studies with heart valve engineering have shown improved seeding of scaffolds in bioreactors that mimic physiological flow and pressure conditions [25]. Also important to note is that the cells used for reseeding in this study were allogenic, not autologous. When producing constructs for implantation, the use of autologous cells is imperative, thereby rendering the constructs nonimmunogenic. Other proliferative cell lines are also being studied, including sheath cells, skin fibroblasts, bone marrow stem cells, and adipoderived stem cells as alternate sources for scaffold seeding [26].

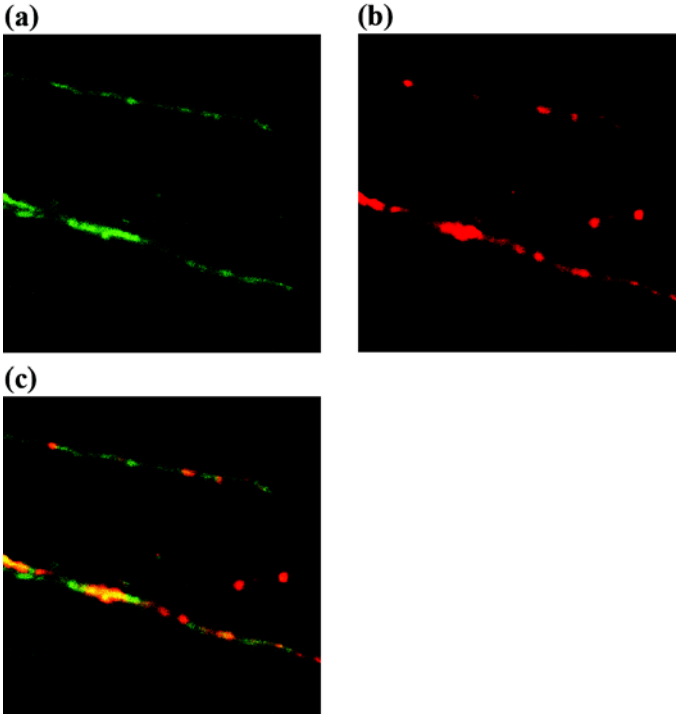

Figure 6.
Fluorescently labeled cells: **(a)** epitenon cells (green), **(b)** endotenon cells (red), and **(c)** double-staining showing both populations present on single tendon construct. Note increased density of endotenon cells within substance of construct.

CONCLUSIONS

A protocol has been developed for the acellularization and subsequent reseeding of intrasynovial flexor tendons. With the possibility of decreased adhesion formation and improved in vivo reconstitution, these tendon constructs may eventually be used to reconstruct Zone II flexor tendon losses. The constructs may also be applicable to other areas of hand and reconstructive surgery in which durable and sustainable tendon material is required.

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Analysis and interpretation of data: A. Y. Zhang, S. J. Bates, E. Morrow.
Drafting of manuscript: A. Y. Zhang, S. J. Bates.

Critical revision of manuscript for important intellectual content: A. Y. Zhang, S. J. Bates.

Statistical analysis: S. J. Bates, E. Morrow.

Obtained funding: J. Chang.

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Study supervision: J. Chang.

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