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RESEARCH ARTICLE

Successful recellularization of human tendon scaffolds using adipose-derived mesenchymal stem cells and collagen gel

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Abstract

The major goal of regenerative medicine is to determine experimental techniques that take maximal advantage of reparative processes that occur naturally in the animal body. Injection of mesenchymal stem cells into the core of a damaged tendon represents such an approach. Decellularization of native tendons as potential targets and seeding protocols are currently under investigation. The aim of our study was to manufacture a recellularized biocompatible scaffold from cadaveric tissue for use in total or partial tendon injuries. Results showed that it was possible to introduce proliferating cells into the core of a decellularized tendon to treat the scaffold with a collagen gel. The method was effective in maintaining scaffold extracellular matrix and for expressing collagen type I and cartilage oligomeric matrix protein by injecting mesenchymal stem cells. Copyright © 2012 John Wiley & Sons, Ltd.

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1. Introduction

The major objective of regenerative medicine is to develop regenerative techniques that take maximal advantage of reparative processes that occur naturally in animals. Regeneration of mammalian tendons is uncommon and epimorphic regeneration tissue repair mechanisms do not occur naturally. Instead, edema and swelling are followed by inflammation response. These processes induce a complex and slow healing process that does not lead to a total functional *restitutio ad integrum* (Liu *et al*., 2011; Liu *et al*., 2008). Tendon pathologies are frequent and debilitating and in the US alone, more than 32 million traumatic and repetitive motion injuries to tendons and ligaments occur each year (Shoen, 2005). For example, hand flexor tendons can undergo two different kinds of failure; rupture due to excessive loads and accidental laceration. Tendons suffer various degrees of injury ranging from relatively mild inflammation to full thickness transaction (Liu *et al*., 2008). Surgical techniques use autogenous tendons (biological grafts) to repair these deficits although tissue defects can lead to patient morbidity and diminished functional performance. However, the mechanical strength of such grafts never returns to complete functional recovery following their insertion and tendon repairs are often weak and susceptible to relapses (Liu *et al*., 2011). Furthermore, the use of biological grafts is limited by availability, possible risk of an adverse immunological reaction and to disease transmission.

Tendon tissue engineering therefore represents a promising approach since its aim is to promote full tendon regeneration rather than simply replacing damaged tendons with partially functional external substitutes. Various natural and synthetic materials have been used to construct extracellular matrices (ECMs) for *in vitro* cell culture and *in vivo* tissue regeneration (Cooper *et al*., 2005; Liu *et al*., 2006; Cao *et al*., 2002; Ouyang *et al*., 2003; Awad *et al*., 2003; Juncosa-Melvin *et al*., 2006; Gentleman *et al*., 2006; Bagnaninchi *et al*., 2007; Funakoshi *et al*.,

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2005a, 2005b; Majima *et al*., 2005). However, there is currently no scaffolding material that simultaneously offers superior biocompatibility, bio-functionality, effective mechanical properties and tractability.

The use of a natural decellularized scaffold from cadaveric tissue could preserve the physiological and mechanical properties and ECM proteins for attachment, migration and proliferation of cells (Gilbert *et al*., 2006). It has been observed that decellularization of natural scaffolds reduces immunogenicity and preserves their histological structures (Hudson *et al*., 2004). Several approaches to decellularization have been investigated including peracetic acid, t-Octyl-phenoxypolyethoxyethanol (Triton X-100), sodium dodecyl sulfate and tri-n-butyl phosphate (TnBP) (Gilbert *et al*., 2006; Woods and Gratzer, 2005; Cartmell and Dunn, 2000; Deeken *et al*., 2011; Cartmell and Dunn, 2004; Harrison and Gratzer, 2005; Ingram *et al*., 2007). Some studies showed that 0.1% (w/v) sodium dodecyl sulfate (SDS) was an effective tendon cell removal agent and tendon fascicles treated with SDS were similar to control values with no alterations in ECM (Ingram *et al*., 2007; Pridgen *et al*., 2011). Previous studies have suggested that different cell types can be used to engineer tendons to repopulate scaffolds such as fibroblasts, tenocytes or adipo-derived MSCs, although some technical problems remain to be solved (Chen *et al*., 2009; Angelidis IK *et al*., 2010; Woon CY *et al*., 2011). Isolation of tenocytes from patients for autologous clinical use required long culture periods and invasive sampling while MSCs showed an easier collection and proliferated faster in cell culture (Kryger *et al*., 2007).

To reseed scaffolds, a tissue-engineered approach has resulted in several treatments including static culture, injection, pulsatile perfusion, centrifugal force and ultrasonication; however, recellularization was not homogeneous in all cases (Ingram *et al*., 2007; Kryger *et al*., 2007; Thevenot *et al*., 2008). Generally, the seeded cells formed only a monolayer over the scaffold surface (Pridgen *et al*., 2011; Kryger *et al*., 2007) or when successful in penetrating the matrix, their number and density was significantly lower than those seen in native tendon (Ingram *et al*., 2007). The aim of the current study was to develop a recellularized biocompatible scaffold from cadaveric tissue for use in total or partial tendon injuries to promote better cellular regeneration. We described that it was possible to introduce proliferating cells in the core of a decellularized tendon for treating the scaffold with a collagen gel.

2. Methods

2.1. Sampling of tendons

Fifty-one human flexor tendons were harvested from body parts removed during surgery for severe hand and digit traumas following ethical guidelines of the General Hospital of Padova, Italy.

2.2. Decellularization of tendons

Thirty-six flexor tendons were used for decellularization, harvested as described above and washed with phosphate buffered saline. Whole tendons were placed for 2 h at 37 °C with agitation in 50 mL of hypotonic Tris buffer (10 mM, pH 8) containing 0.1% (w/v) ethylenediaminetetraacetic acid (EDTA) and two proteases inhibitor: phenylmethylsufonyl fluoride (100 mM) and leupeptin (1 mg/ml). Subsequently, tendons were placed in 0.1% SDS (w/v) in hypotonic buffer for 5 h at 37 °C followed by agitation. Finally, tendons were rinsed 3 x 30 min in PBS at room temperature. After washing, tendons were incubated in 2000 KU DNase (D4263; Sigma-Aldrich, Milan, Italy) in 1 M NaCl to remove residual cell detritus and then shaken for 1 h at room temperature to solubilize nuclear contents and degrade DNA. After two washings with PBS, tendon samples were stored in PBS containing 1% antibiotic solution at 4 °C. After the decellularization protocol, scaffolds were sterilized under UV light overnight.

2.3. Cell culture

Adipose-derived stem cells (ADSCs) were extracted from six human adipose tissue samples obtained from healthy female patients undergoing cosmetic surgery procedures (liposuction) following guidelines from the Clinic of Plastic Surgery, University of Padova, Italy. Cells were cultured as described in a previous study (Martinello *et al*., 2010). Briefly, samples were washed thoroughly with sterile phosphate-buffered saline (PBS) to remove debris and red blood cells. Washed aspirates were treated with type I 40 μg/ml collagenase (Sigma-Aldrich, Milan, Italy) in PBS at 37°C with gentle agitation until complete disaggregation. The collagenase was inactivated with an equal volume of DMEM, 10% fetal bovine serum (FBS) and filtered through a 100-μm mesh filter and centrifuged at 1400 *g* for 20 min. The supernatant was removed and the pellet washed with PBS. After a second centrifugation at 1600 *g* for 10 min, the supernatant was removed and plated onto conventional tissue culture plates in control medium. After three days of incubation, adipose-derived stem cells were developed, recovered and set for amplification and cryopreservation.

2.4. Recellularization

Two seeding methods were performed and 1 x 10^6 cells/scaffold were used for each tendon. First, six recellularized tendon scaffolds were reseeded by injecting a concentrated cell solution in 200 μl of medium into the centre of the scaffold using a 27-guage needle. The scaffold was allowed to incubate for 2 h waiting for cell attachment and then growth medium was added. Second, 200 μl of collagen solution (C34243; Sigma-Aldrich, Milan, Italy) was injected to the core of the decellularized tendon forming a gel at 37°C for 1 h. Next, the cell solution was

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injected into the scaffold. After 1 h, incubation growth medium was added. All cell-scaffold constructs were incubated at 37°C in a humidified tissue culture chamber with 5% CO_2 in medium for seven days before analyses and the medium was changed every three days. Fifteen tendons were recellularized using the second method.

2.5. Histological analysis of the scaffolds

Six human control tendons, six decellularized and 12 recellularized scaffolds (six for the first method and six for the second) were fixed for 24 h in 4% paraformaldehyde solution in PBS (pH 7.4) at 4°C. Samples were washed in distilled water, dehydrated through a graded alcohol series, embedded in paraffin and sectioned at a thickness of 5 μm. Several serial sections were stained with different procedures such as H&E, Masson's trichrome (HT15; Sigma-Aldrich, Milan, Italy) and periodic acid Schiff Alcian blue (PAS-AB). Images were taken with an Olympus BX50 photomicroscope (Polyphoto s.p.a., Milan, Italy)

2.5.1. Immunohistochemistry

Immunohistochemical reactions were performed using the VECTASTAIN® elite ABC Kit Peroxidase (PK-6100, Vector laboratories, Milan, Italy). The primary antisera anti-collagen type 1 antibody (monoclonal antibody anti-COL-1, C2456, Sigma-Aldrich S.r.l, Milan, Italy) and anti-anticartilage oligomeric matrix protein (rabbit polyclonal antibody anti-COMP, ab74524, Abcam®, Cambridge, UK)) were applied overnight at 4°C in a humidified chamber at 1:100 dilution in PBS/Tween20 (0.01%) and secondary antibody biotinylated anti-mouse IgG (BA-9200) and biotinylated anti-rabbit IgG (BA-1000) (both from Vector laboratories, Milan, Italy) were used. After rinsing in PBS buffer, sections were incubated using VECTASTAIN. After washing in PBS, the immunoreactive sites were visualized using a DAB (3,3′-diaminobenzidine tetrahydrochloride) substrate kit (Vector, Italy). To determine structural details, sections were dehydrated, mounted in Eukitt® (Sigma-Aldrich) and examined under an Olympus BX50 photomicroscope (Polyphoto s.p.a., Milan, Italy). The specificity of the immunostaining was verified as follows: 1) by incubating sections with PBS instead of the specific primary antibody; 2) by incubating sections with preimmune serum instead of primary antibody; and 3) by incubating sections with PBS instead of secondary antibodies. Results of these controls were negative (staining was not detected).

2.5.2. DNA analysis

To assess total DNA content of native tendons ($n = 6$), decellularized ($n = 6$) and recellularized scaffolds ($n = 6$) with collagen were pulverized in liquid nitrogen and homogenized in 1 mL DNA buffer (EDTA-Na 0.1M; Tris HCl 0.05M, pH 8; SDS 10%) and 20 μl proteinase K 20mg/ml overnight at 55°C. The following day, 300 μl of NaCl 5M solution was added and samples were stirred for 20 min and centrifuged at 12 000 rpm for 30 min. DNA was precipitated with isopropanol, washed with 75% ethanol and air-dried. The pellet was finally dissolved in ribonuclease-free water and stored at −20°C. DNA content was qualitatively evaluated by amplification reaction using β-actin primers. PCR was conducted using specific primers using Primer Express software (Applied biosystems®, Life Technologies Italia, Monza, Italy) purchased online at http://www.eurofinsdna.com. A β-actin fragment of 185 bp was amplified as follows:

- forward primer: 5′–CCATCTACGAGGGGTACGCCC–3′
- reverse primer: 5′–TGCTCGAAGTCCAGGGCGACGTA–3′

A negative sample control was also added to the same PCR reaction. An annealing temperature of 60°C for 35 cycles was performed. DNA extraction and PCR products were visualized on agarose gels by ultraviolet transillumination.

2.5.3. XTT assay

To determine cellular viability and survival of cells in the scaffold after collagen treatment, we used an XTT-based cell proliferation kit II (Roche Diagnostics S.p.A., Monza, Italy) colorimetric assay after seven days of incubation. For controls, decellularized and recellularized tendons ($n = 3$ for all groups) were incubated in 2 ml yellow XTT solution (Sigma) for 2 h. After incubation, the metabolically active cells developed an orange formazan product and 3 x 100 μl of each sample was quantified using a Spectra Count enzyme linked immune absorbent assay plate reader (PerkinElmer®, Milan, Italy). The amount of orange formazan formed was directly correlated to the number of living cells. Data were expressed as the mean standard deviation. Normality of the data was confirmed using the Kolmogorov-Smirnov test ($\alpha = 5\%$). Statistical analysis was performed using the paired Student t-test (SPSS software, version 11.0, SPSS Inc. Chicago, Illinois, USA). The level of statistical significance was set at $p \leq 0.05$.

3. Results

3.1. Scaffold preparation

Human flexor digitorum tendons were harvested from different donors and decellularized with one unique cycle using a detergent-enzymatic method comprised of one wash with EDTA in hypotonic Tris buffer to lyse cells and release intracellular contents and one wash in SDS to remove cell membranes and cytoplasmic proteins. We noticed a correlation between size of tendon and SDS treatment time. To determine an optimum decellularization method, different size tendons were subjected to

various SDS treatment times (data not shown). Optimal size was found to be 2 x 0.25 cm while 5 h of SDS treatment was the most advantageous incubation period to obtain complete removal of cells and an integral matrix.

Using H&E staining, the control tendon showed a normal distribution of collagen fibres (Figure 1a). The tendon decellularized with 0.1% SDS exhibited complete loss of cells with no substantial changes in ECM structure. Moreover, histological features observed by H&E staining demonstrated that no cell fragments were retained within the tissue and no apparent disruption of overall tissue histoarchitecture was noticed (Figure 1b).

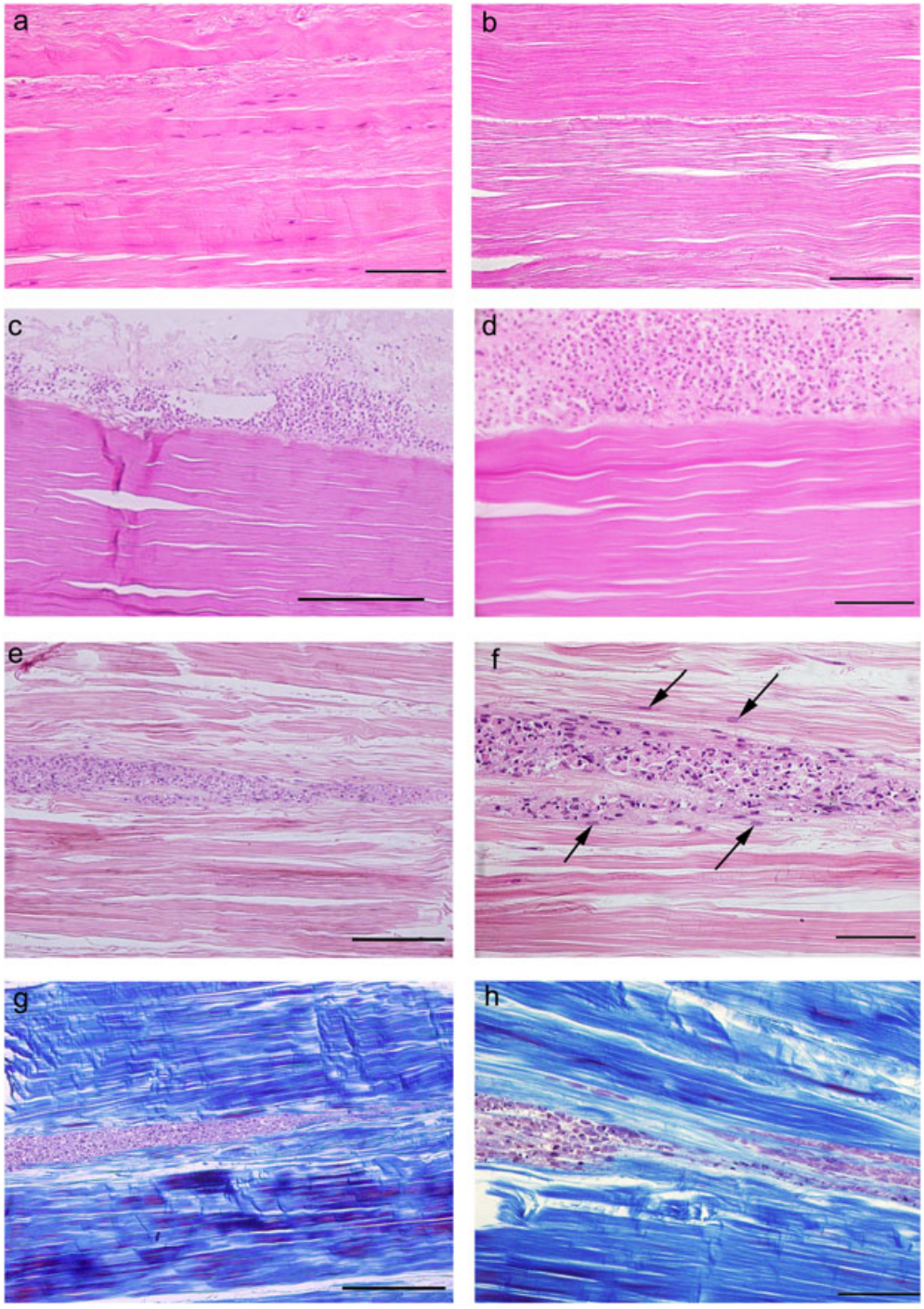

Figure 1. Hematoxylin/eosin (a-f) and Masson trichromic (g-h) stainings of untreated and treated tendons. The first method used provoked removal of cells while only the second method achieved cells to penetrate the scaffold. (a) Untreated tendon, scale bar = 100 μm; (b) acellularized tendon, scale bar = 100vμm; (c, d) tendon recellularized with method 1; cells form layers on the surface of the tendon at the level of peritenonium, scale bars: c = 500 μm, d = 100 μm; (e, f) tendon recellularized with method 2 using collagen; cells gather in the deep portion but some elongated cells (arrows) integrate in the matrix of the scaffold, scale bars: e = 500 μm, f = 100 μm; (g, h) Masson staining confirmed the presence of cells into the scaffold, scale bars: g = 500 μm, h = 100 μm. In H&E, blue staining indicates the nuclei of cells; in Masson trichromic, blue indicates collagen fibres while red indicates cells

3.2. Scaffold seeding

Recellularization of decellularized scaffolds was performed using two slightly different methods (one involving collagen gel and one without). During the seeding step, we used the same cell density of 1 x 10^6 cells/scaffold. The recellularizazion protocol without the use of collagen in which the formation of an external layer of cells was clearly evident as seen in Figures 1c-d. Conversely, the use of collagen in recellularization of the scaffold significantly improved the penetration of injected cells (Figures 1e-f). It can be seen that together with rounded mononuclear cells, there were also flattened cells that aligned with collagen fibres (Figure 1f, arrows). Masson's trichromic staining confirmed that scaffold recellularization using collagen was composed of type I collagen and MSCs diffusing into the matrix (Figures 1g-h). Immunohistochemistry experiments showed a strong reaction for collagen type I especially in elongated cells present at the border with the collagen fibres (Figures 2a-b) while the rounded cells did not present any positive staining (Figure 2b, asterisks). The anti-COMP antibody revealed that all injected cells were positive and expressed this protein at the cytoplasmic level (Figure 2d).

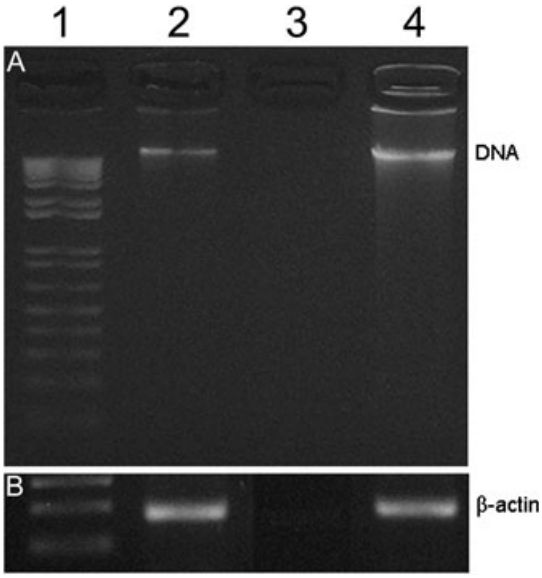

Figure 3. DNA analysis of decellularized and recellularized (with the second method) tendons. (A) Genomic DNA content in native (lane 2), decellularized (lane 3) and recellularized (lane 4) tendons. (B) β-actin housekeeping gene expression in native tendon (lane 2), decellularized (lane 3) and recellularized tendons (lane 4). Lane 1 = Molecular size standard (1Kb Plus DNA Ladder, Invitrogen)

3.3. Valuation of cell presence in the scaffold

DNA assay confirmed that with respect to the native sample, DNA was removed by the decellularization process (Figure 3a) and lack of amplification for the housekeeping gene β-actin (Figure 3b) confirmed that decellularized tendons were DNA depleted. Figure 3 shows that cells were still present following seeding with collagen treatment after seven days of culture. Moreover, DNA was

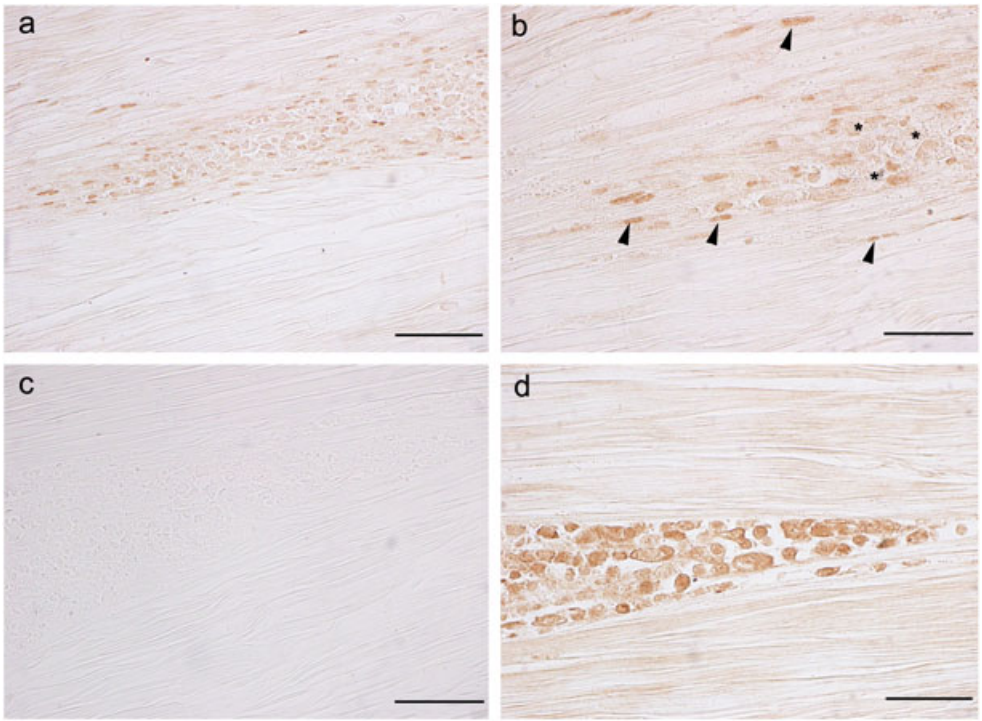

Figure 2. Immunolocalization of collagen I and COMP proteins in recelullarized scaffolds. (a-b) Injected cells that presented an elongated shape were positive to the mAB anti collagen I (arrowheads) while rounded cells were negative (asterisks); scale bars: a = 100 μm, b = 50 μm. (c) Representative tendon were used as negative controls (omitting the primary antibodies), scale bar: c = 100 μm. (d) the anti-COMP polyclonal antibody reacted with all injected cells; the positivity is well evident at the cytoplasm level, scale bar: d = 50 μm.

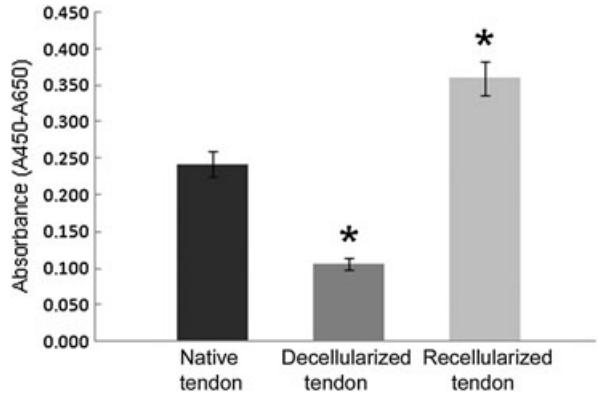

Figure 4. Colorimetric assay to determine the survival of cells. The values shown are the average ± deviation standard of absorbance of native, decellularized and recellularized tendon. The Anova test was used to set statistical significativity (*$p < 0.01$)

present and the β-actin gene was amplified by PCR. To determine the effects of the seeding method with collagen on cell viability, an XTT assay was performed after seven days of incubation. Figure 4 indicates the presence of proliferative cells in native and recellularized tendons. Moreover, recellularized tendons showed a greater viability with respect to native tendons (Figure 4) since the introduced cells presented higher proliferative capacity. The decellularized scaffold showed a background level of absorbance value.

4. Discussion

Biological scaffolds derived from decellularized tissues have been successfully used in both preclinical animal studies and human clinical applications (Ingram *et al*., 2007; Baiguera *et al*., 2010; Baiguera *et al*., 2011; Lu *et al*., 2010; Xu *et al*., 2009). Removal of cells from native tissue offers many advantages with respect to artificial scaffolds including improved biocompatibility, enhanced ability for cellular repopulation and increased biomechanical strength (Liu *et al*., 2008). The use of recellularized tissues appears, therefore, to be an attractive approach for the resolution of frequent and debilitating tendon traumas. Potential applications include hand (extensor and flexor tendon repair) and orthopaedic surgery (ligaments, rotator cuff, Achilles' tendon repair). The unique biomechanical properties of tendons were attributed to the high degree of organization of ECM and for this reason; the goal of any decellularization protocol was to efficiently remove all cellular and nuclear materials, thereby minimizing adverse effects on the composition, biological activity and mechanical integrity of the remaining ECM. Cartmell and Dunn (2000) and recently Deeken *et al*. (2011) demonstrated that tissues treated with TritonX-100 contained many disrupted cells and that there was little damage to the collagen architecture. Cartmell and Dunn (2000) showed that the ionic detergent SDS was more effective in cell removal and did not cause significant denaturation of rat tendon matrix, demonstrating that the most effective decellularization protocol should include a combination of physical, chemical and enzymatic approaches. Indeed, in our study, the decellularization protocol began with the lysis of cell membranes using a hypotonic solution containing EDTA followed by solubilization of cytoplasmic and nuclear components using detergents (SDS 1%). Finally, the removal of cellular debris and depletion of DNA were obtained by treating the tissue with nucleases. These steps were combined with mechanical agitation to increase their effectiveness. Baiguera *et al*. (2010, 2011) proposed bioengineered human tracheas and larynxes obtained from several decellularization cycles of natural tissues; they demonstrated that to obtain complete removal of residual cellular components without disruption of collagen architecture, it is essential to adapt the decellularization treatment to the initial tissue. Previous experiments indicated a correlation between size of tendon and SDS treatment. In the current study, we tested different measures of tendons by changing SDS concentration, incubation time and number of cycles. The protocol presented in the current study is optimal for a specific tendon size of 2 x 0.25 cm. Histological analysis of the decellularized tendons revealed that cells and nuclear components were removed and the integrity of the matrix structure remained preserved. This result was confirmed by DNA analysis.

Some studies have suggested different methods for seeding proliferating cells in tissue scaffolds, stating that the success of tissue engineering products is based on the number of cells found in the scaffold but not on spreading of the cells within the matrix architecture. Ingram *et al*. (2007) indicated that cells formed a monolayer on the surface of the scaffold but after seven days, they were able to penetrate to the centre even tough the matrix had become disorganized and did not resemble native tendon. In 2008, Venot *et al*. investigated cell distribution in a co-polymeric scaffold with an elegant approach and obtained a 3D image after seeding. They concluded that cells tended to reside on the seeded surface and that only a few cells were present in the centre of the construct after injection seeding. Pridgen *et al*. (2011) recently presented a successful decellularized biocompatible human tendon using SDS but the cell attachment was observed only on the tendon surface. Only when the tendon was decellularized with paracetic acid did scaffold porosity improve cell penetration and migration (Woon CY *et al*., 2011). Raghavan *et al*. (2012) demonstrated that both SDS and PAA resulted in ideal treatments for the removal of cellular antigens; as a result, a decreased immune response was observed when the decellularized scaffold was placed into rats.

Our efforts focused on developing and optimizing a successful and functionally robust recellularization protocol for a human tendon scaffold decellularized by SDS. We obtained negative results with a recellularization protocol comprised of injection together with a dynamic seeding method. Cells remained on the surface without penetration. To achieve our aim, we pretreated a tendon scaffold

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with a collagen solution, which at 37°C formed a gel, and only when it injected into the cells. The collagen used was a commercial product isolated from a closed herd and purified using a GMP manufacturing process that included inactivation of any possible prion or viral contamination and therefore could be safely used for human clinical purposes.

Our results demonstrated that the presence of a collagen gel improved penetration of injected cells. The latter presented a rounded morphology and did not express collagen type I. Instead, using the collagen gel, cells that spread towards the tendon matrix aligned with collagen fibres showed an elongated morphology and expressed collagen type I. Both types of cells expressed COMP protein, an abundant non-collagenous pentameric glycoprotein of the tendon, particularly required when the axial alignment of type I collagen fibres is essential for tensile strength of tendons (Posey and Hecht, 2008; Sodersten *et al*., 2005). Effective recellularization was confirmed by the presence of genomic DNA and expression of the housekeeping gene β-actin.

It is important to note that scaffolding materials for tendon tissue engineering require fundamental requirements including biocompatibility before and after degradation as well as bio-functionality or at least the ability to support cell proliferation. The scaffold presented in the current study and recellularized by a collagen gel showed significant biofunctionality as shown by XTT proliferation assay. The recellularized tendon also showed an increased absorbance value since the adipose-derived stem cells used in tendon injection possessed a higher proliferative capacity compared to tenocytes of native tendon (Kryger *et al*., 2007).

Commonly, cell types used in tendon tissue engineering studies are fibroblasts, tenocytes and stem cells of mesenchymal origin (Cao *et al*., 2002; Ouyang *et al*., 2003). Kryger *et al*. (2007) demonstrated that epitenon tenocytes, sheath fibroblasts, BMSCs and ASCs were all possible candidates for use in tendon tissue engineering; however, the authors remarked that adipo-derived MSCs showed higher proliferation at late passages compared to epitenon tenocytes and were easier to harvest. Other studies have suggested that *in vitro* cultured stem cells injected in tendon constructs and exposed to an appropriate environment could be driven towards tenocyte differentiation (Zhang and Chang, 2003; Ge *et al*., 2005). Our data showed that MSCs were able to produce collagen, confirming their contribution to tendon matrix remodelling *in vivo*.

Our data also indicated the feasibility of a strategy for recellularization of scaffolds that could achieve efficient infiltration into the core of the tendon. The collagen gel allowed the injected cells to migrate into the tendon matrix, elongate and flatten as well as express collagen I and COMP. These results provide excellent new opportunities for tendon scaffold engineering for hand reconstruction. Among biologic scaffolds, those derived from decellularized homologous tissues represent an innovative and appropriate choice. Based on the fact that there is no better way to repair "like with like", a decellularized homologous scaffold could be the most suitable structure for mimicking the architecture guiding the regeneration of the original damaged tissue. Simultaneously, the problem related to immunogenicity could be controlled by effective decellularization and recellularization processes.

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