Decellularized porcine Achilles tendon induces anti-inflammatory macrophage phenotype in vitro and tendon repair in vivo

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Decellularized Porcine Achilles Tendon Induces Anti-inflammatory Macrophage Phenotype In Vitro and Tendon Repair In Vivo

Aysegul Dede Eren a, Ravi Sinha b, Egemen Deniz Eren a, Yuan Huipin c, Sultan Gulce-Iza a, Henriette Valster d, Lorenzo Moron b, Jasper Foolen a, Jan de Boer a,∗

a Institute for Complex Molecular Systems, Department of Biomedical Engineering, Eindhoven University of Technology, Eindhoven, the Netherlands
b Maastricht University, MERLIN Institute for Technology Inspired Regenerative Medicine, Complex Tissue Regeneration Department, Maastricht, the Netherlands
c Expand Biotechnology BV, Bilthoven, the Netherlands
d HighTech Contract Manufacturing Medical, Nijmegen, the Netherlands

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ABSTRACT

Decellularized tissues and organs from animal sources are widely used in regenerative medicine and tissue engineering. However, in tendon tissue engineering there is limitation not only in terms of tissue source - allografts and autografts but also standardization of decellularization techniques. The goal of this study is to decellularize porcine Achilles tendon to be used as an off-the-shelf product for tendon reconstruction. We describe a novel, mild decellularization strategy which retains the biochemical and biomechanical characteristics of native tendon upon decellularization. We further show that decellularized tendon sections in vitro induce mesenchymal differentiation in stem cells and anti-inflammatory response in naïve macrophages. Upon implantation in an Achilles tendon defect in rabbits, we observed that decellularized tendons integrated with the host tissue without signs of tissue rejection or encapsulation. Together, we demonstrate that decellularized tendons produced with our new protocol bear a great potential for tendon tissue regeneration.

1. Introduction

Tendon injury is one of the most common types of musculoskeletal injuries, which is associated with intrinsic and extrinsic factors including age, gender, and obesity as well as with sport-related or other rigorous physical activities. Due to its poor blood supply, low cell density, low regenerative capacity, natural tendon healing results in the formation of scar tissue, impaired structural integrity and mechanical strength. As a consequence, the healed tendon is more susceptible to re-injury. Treatment of tendon injury includes physiotherapy, anti-inflammatory drugs, painkillers or surgery yet the net outcome is ineffective as patients suffer from a substantial decrease in personal productivity. In surgery, the damaged tendon is replaced with autografts, allografts, or natural extracellular matrix (ECM) derived grafts. Autografts are limited in number, dimension and sites of harvest, and lead to donor site morbidity. Allografts are limited in terms of available healthy donors as well as the potential of causing an inflammatory response in the host and can be expensive. There are commercial xenogenic scaffolds available, however not yet of tendon origin but instead dermis, intestinal submucosa and peri cardium. The use of these scaffolds are unfortunately not without complications such as rerupture, postoperative edema, aggravated pain and decreased range of motion. In addition, synthetic polymers such as poly(glycerol acid) (PGA), poly(lactic glycolic acid (PLGA) or polysaccharides such as chitosan or various collagen derivatives have been used for tendon reconstructions. However, due to the complex ECM structure, composition, and mechanical properties; no biomaterials have yet progressed beyond in vitro or early in vivo evaluation. Within the last few decades, researchers have applied decellularized tissues for tissue regeneration, due to their inductive nature which makes them a strong candidates for clinical application. Decellularized tissues such as dermis, small intestine, pericardium and heart valves harvested from allogenic or xenogeneic sources are commercially available and have been used in the clinic for various applications including soft tissue regeneration and valve replacements. Similarly, demineralized bone matrix has been used for decades as a bone graft substitute. Although decellularized non-tendon tissues are being used in tear repairs or provide support, decellularized tendons from xenogeneic sources to be used in tendon orthopaedic applications are not available. Therefore, the aim of this study is to develop and validate a protocol towards an...
off-the-shelf product for tendon and ligament reconstruction.

Several protocols describe decellularization of a whole tendon or tendon sections, and involve physical and/or chemical disruption of cellular components and use different tendon sources. Special attention is given on in vitro and in vivo characterization of decellularized tissue in order to prove that material can be used as an off-the-shelf product. ECM structure and composition after decellularization is important and should be similar to the native tissue to retain the mechanical properties typical of tendon tissue. Moreover, decellularized tissue should be cytotocompatible, support the invasion of tissue-resident cells after implantation, and support tenogenic differentiation. Decellularized allogeneic and xenogeneic tissues may even induce an immunogenic response favouring tissue remodelling.

Keane et al. used decellularized porcine small intestine produced with three different methods and investigated host response in terms of macrophage polarization profile. Their results indicate that effective decellularization resulted in M2 macrophages, which is associated with constructive tissue remodelling. Even though challenging, producing decellularized tendons while preserving ECM structure and composition, allowing recellularization in absence of anti-inflammatory response in the host material is a major step towards a clinically applicable tendon graft. Therefore, we formulated a mild but robust decellularization protocol and demonstrated that decellularized tendons were devoid of DNA yet retained important biochemical and biomechanical properties. The decellularized tendons we produced can support in vitro and in vivo cell viability and tissue formation. In addition, the produced decellularized tendons were shown to induce an anti-inflammatory macrophage phenotype.

2. Results

2.1. Gross structure, DNA content and biochemical properties of tendon after decellularization

To evaluate remaining DNA after decellularization, we visualized nuclei in decellularized and native tissue using Hematoxylin & Eosin (H&E) and 4′, 6-diamidino-2-phenylindole staining (DAPI). In native tissue sections, we observed nuclei in both H&E (Fig. 2A) and DAPI stained sections (Fig. 2C). Cells were aligned between the crimp of the collagenous extracellular matrix (ECM) and more densely packed in the endotenon region (Fig. 2A). In decellularized tissue sections, we observed no nuclei with both stains (Fig. 2B, D). Next, we quantified the DNA content of both tissues. Native tendon contained 814 ± 14 ng/mg DNA per tissue dry weight, which was significantly reduced to 34 ± 13 ng/mg (Student t-test, p < 0.0001) per tissue dry weight after decellularization, which is below the threshold set by decellularization criteria. Finally, gel electrophoresis demonstrated that in decellularized samples, DNA fragments were not observable; however, high molecular weight DNA was observed in DNA isolated from native tissue (Supplementary Fig. S1). Together, we conclude that our novel protocol was successful in decellularization of tendon tissue.

Both tissue structure and biochemical composition should be maintained as much as possible to act as a tissue-instructive scaffold. We noted that the pink colour of ECM coming from eosin staining was lighter in decellularized tissue, indicating loss of ECM proteins. Alcian Blue staining showed glycosaminoglycan (GAG) staining in native tendon sections throughout the tissue section, but more pronounced in the endotenon region (Fig. 2E). Decellularization resulted in less prevalent staining of GAGs compared to native tendons sections (Fig. 2F).

Micro Sirius Red Stain was used to stain all collagens in native and decellularized tendon ECM and we observed collagen type I in red, and type III in green under polarized light (Fig. 2G and H). We observed a slight colour difference in collagen I and collagen III between native and decellularized tendon. Next, we quantified total GAG and collagen in native and decellularized tendons. Total GAG content was 6.9 ± 0.6 μg/mg and statistically significantly reduced to 3.2 ± 0.2 μg/mg (p < 0.0001, student t-test) following decellularization, confirming our histological observations. For the total hydroxyproline content, an indicator of collagen content, of native tendons was 33.0 ± 8.7 μg/mg and increased to 40.9 ± 5.9 μg/mg following decellularization, which was however not statistically significantly different (Student t-test, p > 0.05). Here, we conclude that decellularization has no effect on collagen composition and structure in tendon ECM; however, GAG content significantly decreased.

Tendon has a unique tissue structure with bundles of collagen fibres aligned along the tissue forming unique crimp structures. We observed a similar crimp pattern of collagen fibre in both native (Fig. 3A) and decellularized (Fig. 3B) tissues using scanning electron microscopy. At higher magnification, we observed the alignment of collagen fibres along the tissue as shown in Fig. 3C and D. Biochemical and histological examination thus demonstrates mild effects on the biochemical composition of decellularized tendons, but intact structure.

2.2. Mechanical properties of tendon after decellularization

To assess the mechanical properties after decellularization we subjected decellularized and native tendon tissues to uniaxial tensile testing in a biaxial chamber in which samples were incubated in PBS during the whole test period (Supplementary Fig. 2). Both ends of the samples were embedded in epoxy resin to limit slippage from the clamps during tensile testing (S2. C-E). Unfortunately, slippage was still observed at high strains and samples were thus not tested completely until failure. The stress-strain plots revealed a discrepancy (S2. F,G) between the decellularized (DPT) and native (NTP) group, likely due to sample deformation upon re-hydration. Primarily this resulted in varying toe regions (S2. F,G) between samples. Still, all stress-strain curves displayed a linear region, devoid of slippage, the elastic moduli could be quantified. The elastic modulus of the NPT group (22.1 ± 12.0 MPa) was significantly higher (p < 0.05, Welch test) compared to the DPT group (9.2 ± 10.2 MPa) (S2. H).

![Fig. 1. Decellularization protocol. After freeze-thaw cycles, tissues were incubated with Triton X-100 and Benzonase. In between every step, tissues were washed with hypotonic or hypertonic solutions to achieve cell lysis. The final step consisted of a five-day long washing step of tendons in PBS.](image-url)
Fig. 2. Loss of nuclear components and alterations in tendon composition following decellularization. Histological sections of native (A, C, E & G) decellularized (B, D, F & H) were stained with nuclear stainings H&E (A,B) and DAPI (C,D), alcian blue staining (E,F) for GAGs and picro sirius red stain (G,H) for collagen type I and type III. In nuclear stainings, no nuclei are observed on decellularized sections (B, D). In native sections, nuclei are observed in H&E staining (purple) (A) and blue colour in DAPI staining (blue) (C). In alcian blue stain, GAGs are stained in blue and nuclei are stained in pink/red colour. In native tendon (E), GAGs and nucleus staining is visible. However, decellularization results in a visible colour change of GAGs and no nuclei are observed (F). Picro sirius red staining, which dyes type I collagen red and type III in green exhibit a slight colour change after decellularization (G, H). Scale bar indicates 100 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)
2.3. Decellularized tendons are cytocompatible

To assess in vitro cytocompatibility of decellularized tendons, we seeded hMSCs decellularized tendon sections and measured the release of lactose dehydrogenase 24h after seeding (Fig. 4). Cells cultured in tissue culture plastic in basic medium were used as a negative control, and as a positive control, we added lysis buffer to cells cultured on tissue culture plastic. LDH release was significantly different between positive control and negative control, whereas no significant difference between negative control and hMSCs cultured on decellularized tendon sections was observed (Fig. 4A). Here, we conclude that decellularized tendon sections do not have a noticeable cytotoxic effect.

To further investigate cell viability, we cultured hMSCs on decellularized tendon sections for 6h, 1, 3 and 7 days and labelled live cells with calcein and dead cells with ethidium bromide (Fig. 4B). After 6h, we observed attachment of hMSCS to decellularized tendon sections with very few dead cells (Fig. 4B). At later time points, cells displayed a high rate of proliferation and were viable (Fig. 4B). We observed only a few dead cells in all replicates for each time point. Interestingly, calcein stain showed that even at the earliest time point (6h) cells aligned along the tissue (Fig. 4B).

2.4. Decellularized tendon sections induce a tenocyte-like morphology and support differentiation of hMSCs towards tenogenic lineage

To study the effect of decellularized tendon sections on tenogenic differentiation, we investigated morphology and gene expression of hMSCs for 7 days. At day 1, both nuclei (blue) and cytoskeleton (red) of hMSCs started to align between collagen fibres and took the shape of collagen crimps (Fig. 5A). From day 1 to day 4, we observed an increase in the number of cells, while cells retained an elongated shape (Fig. 5A). Nuclear aspect ratio at day 1 (1.67 ± 0.41) significantly increased to 1.91 ± 0.96 at day 4 (p < 0.05, student t-test). At day 7, the surface of decellularized tendon section was covered with elongated hMSCs (Fig. 5A) and nuclear aspect ratio (1.89 ± 0.59) remained similar to day 4. Additionally, the orientation of the cellular stress fiber network of the hMSCs was calculated. At day 1 and day 4, stress fibers displayed a strong anisotropy −18.26 ± 13.62° at day 1 and −20.37 ± 10.74° at day 4 and strictly followed the collagen fiber direction (S3. D,E) At day 7, orientation of the cellular stress fiber network was −6.68 ± 8.75° (S3F).

To investigate the effect of the decellularized tendon on the expression of tenogenic marker genes, hMSCs were cultured on the decellularized tendon and at day 1, day 4 and day 7 qPCR was performed on the following genes: SCX, TNMD, MKX, EGR-1 and COL1A1 (Fig. 5B–F). As control surfaces, tissue culture plastic was left uncoated (TCP) or coated with rat tail collagen type I (CC). Expression of SCX, which is a tenocyte specific transcription factor, was significantly up-regulated on decellularized tendon sections (DTS) compared to TCP conditions at day 1, but similar at day 4 and day 7 (Fig. 5B). Expression of TNMD, which is a mature tenocyte marker, was statistically upregulated at day 1 in DTS conditions compared to TCP and significantly upregulated compared to CC and TCP at day 4 (Fig. 5C). However, at day 7, TNMD expression decreased and was similar to CC and DTS conditions. MKX, a transcription factor which controls tenocyte differentiation and cell homeostasis, was significantly upregulated in CC condition compared to TCP and DPT at day 1 and significantly upregulated compared to CC and TCP at day 4 (Fig. 5C). However, at day 7, TNMD expression decreased and was similar to CC and DTS conditions. MKX, a transcription factor which controls tenocyte differentiation and cell homeostasis, was significantly upregulated in CC condition compared to TCP and DPT at day 1 and significantly upregulated in CC conditions compared to DTS day 7 (Fig. 5D). EGR-1 is a zinc finger transcription factor early growth response 1 and was shown it involve in the differentiation of stem cells towards tenogenic lineage. Expression of EGR-1 (Fig. 5E) was not changed over the 7 days in hMSCs cultured on DTS and reduced in TCP. COL1A1 is one of the main ECM genes expressed in tenocytes, and its expression did not show a significant change for 7 days in DTS condition yet its expression was significantly downregulated compared to the CC condition at day 1 and day 4 (Fig. 5E). Based on these results, we can conclude that culturing hMSCs on DPTs improved tenogenic differentiation, especially when compared to tissue culture plastic at early time points.
2.5. Decellularized tendons induce anti-inflammatory phenotype in macrophages

Assessment of macrophage polarization towards pro- or anti-inflammatory phenotype is another aspect for the characterization of decellularized tendons. We differentiated the THP-1 macrophage cell line into M₀ macrophages. Then, we either exposed M₀ macrophages with IFNγ and LPS to polarize them towards an M₁ (pro-inflammatory) phenotype, or with IL-4 to polarize them into M₂ (anti-inflammatory) phenotype, and used these cells as positive controls for macrophage

![Graph showing LDH cytotoxicity](A) Absorbance measurement of LDH release. hMSCs cultivated on decellularized tendon sections released the same amount of LDH with hMSCs cultivated on tissue culture plate with the basic medium. (B) Fluorescence microscopy images of hMSCs cultured on the 300 μm thick of decellularized tendon sections at 6 h, 1 day, 3 days, and 7 days stained with LIVE/DEAD stain. Live cells stained in green (calcein AM) and dead cells stained in red (EthD-1). At early time points (6 h) cellular attachment started and over 7 days, a high percentage of viable cells with very few dead cells are observed. Scale bar indicates 100 μm (MAX = maximum LDH release, TCP = Tissue culture plate, DTS = Decellularized tendon section. (***indicates a p-value of<0.001). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

![Fluorescence microscopy images](B)

**Fig. 4.** Induction of cell viability of decellularized tendon sections (A) Absorbance measurement of LDH release. hMSCs cultivated on decellularized tendon sections released the same amount of LDH with hMSCs cultivated on tissue culture plate with the basic medium. (B) Fluorescence microscopy images of hMSCs cultured on the 300 μm thick of decellularized tendon sections at 6 h, 1 day, 3 days, and 7 days stained with LIVE/DEAD stain. Live cells stained in green (calcein AM) and dead cells stained in red (EthD-1). At early time points (6 h) cellular attachment started and over 7 days, a high percentage of viable cells with very few dead cells are observed. Scale bar indicates 100 μm (MAX = maximum LDH release, TCP = Tissue culture plate, DTS = Decellularized tendon section. (***indicates a p-value of<0.001). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)
Fig. 5. Promotion of tenogenic phenotype and differentiation on decellularized tendon sections (A) Fluorescence microscopy images of hMSCs cultured on the 300μm thick of decellularized tendon sections at 1 day, 3 days, and 7 days stained with DAPI (nuclei, blue) and Phalloidin-AF488 (F-actin, red). At day 1, nuclei and cytoskeleton of hMSCs adopted the direction of the collagen fibres. At day 4, cells proliferated and aligned between collagen fibres. At day 7, cell kept proliferating and covered the surface of decellularized tendon section. Scale bar indicates 100μm. (B–F) Tendon-related gene expression of hMSCs cultured on TCP, CC and DTS. Expression of SCX was significantly increased at day 1, gradually decreased at DTS condition, and was stable under CC and TCP conditions. (B) Expression of TNMD at day 4 compared to TCP and CC but reduced at day 7. (D) Expression of MKX was significantly higher at day 4 in CC compared to TCP and DTS and showed similar expression profile at day 7. (E) Expression of COL1A1 was significantly higher at day 1 and day 4 in CC condition compared to TCP and DTS. (F) Expression of EGR-1 was highest day 1 and day 4 but reduced at day 7. In DPT and CC conditions, expression of EGR-1 remained same. Relative gene expression was calculated by normalizing the data to TCP day 1, using 18S RNA as a housekeeping gene. (TCP = Tissue culture plate, CC=Collagen coated surface, DTS = Decellularized tendon section). (*indicates a p-value of <0.05, ** indicates a p-value of <0.01). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)
In order to assess the response of M0 macrophages to decellularized tendons, we cultured them on DTSs for 24 h (MDPT) and calculated the fold changes relative to M0 (Fig. 6).

MCP-1, CD68, CCR7, TNF-α and IL-6 were significantly less in both M2, MDPT and M0 groups compared to M1 (Fig. 6A). On the other side, expression of anti-inflammatory markers CD200R1, CD206, CD163 and IL-10 in the MDPT group showed similar expression profile with those in M2 group (Fig. 6B). Thus, decellularized tendon sections induce an anti-inflammatory (M2) phenotype in macrophages.

2.6. Decellularized tendon induces efficient tissue formation in vivo

We investigated the in vivo performance of decellularized tendons (DT) by implanting them into a 10 × 3 mm rabbit patellar tendon defect. As a control group, we implanted autologous tendon (AT) from one knee into the defect that we created (Supplementary Fig. 2). After 6 weeks, we collected tissues and stained them with H&E (Fig. 7A, D), Pico Sirius red (Fig. 7B, E) and Alcian blue (Fig. 7D, F) to histologically examine the response of the host to implants in terms of cellularity and collagen fibre organization. In general, our results indicated no tissue rejection or encapsulation and the interphase between the implant and host tissue was not clear. H&E and Alcian blue staining of DT indicated cellular infiltration from the host (Fig. 7A, C). Based on cell shape, we propose that these cells are tenocytes located between collagen fibres, aligned in the loading direction. In addition, the shape of the cells that repopulated the decellularized tendons, displayed an elongated shape similar to that of the host cells. Moreover, the presence of blood vessels was not observed. Sirius red staining which gives type I collagen a red colour and a green colour to type III collagen under polarized light demonstrated that both at the host (H), DT and AT, the main type of collagen is type I (Fig. 7B, E). Alcian blue staining (Fig. 7C, F) showed GAGs was recovered in decellularized tendon tissue after implantation. To conclude, our results indicate that GAGs that are lost after decellularization are restored, implanted tissue is repopulated with host cells.
3. Discussion

Criteria of biological materials to be used in clinics have been described in a number of official documents. For instance, according to American Food and Drug Administration (FDA) “Regulatory Considerations for Human Cells, Tissues, and Cellular and Tissue-Based Products: Minimal Manipulation and Homologous Use” regulations, transplanted tissue should be able to perform basic functions of replaced tissue. Furthermore, European Union Commission Regulation number 722/2012 states many rules such as reduction, elimination or removal of infectious agents, risk analysis and risk management, slaughtering and processing controls, the contact area, it's the surface type for the use of animal source products as medical devices. fig. 7. In vivo performance of decellularized tendon in rabbit patellar tendon at 6 weeks after surgery. (A–F) Histological evaluations of H&E stained decellularized tendon (DT) with autologous patellar tendon transplantation from the contralateral leg (AT) as control. (A–C) H&E, picro sirius red (collagen staining) and alcian blue staining (GAG staining) of DT implanted tendon show that decellularized tendons are invaded by host (H) tenocytes. Both tenocytes and collagen fibres aligned towards the loading direction (A&C). Pico Sirus red staining is imaged with polarized light under which collagen type I is observed in red, collagen type II is observed in green. Staining results indicate that at the meeting point of host and DT, collagen remodelling is ongoing and collagen type I is dominating the collagenous part of the ECM (B). (D–F) H&E picro sirius red and alcian blue stainings of AT tendon show that implanted tendon is capable of bearing mechanical loads. (D&F). Scale bar indicates 100 μm. Arrows indicate the loading direction, asterisks indicate tenocytes and boxes indicate randomly selected areas to be magnified for viewing cells and suture sites. Dashed circles represent incision sites. (DT = Decellularized tendon, H = Host, AT = Autologous tendon). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)
graft material should induce host cell migration and infiltration, should be sterile and be able to bear the mechanical forces that replaced tissue is exposed to after implantation. For many years, natural ECM derived from different tissues or organs have been successfully used in the clinic. For instance, allogenic bone grafts have been used by surgeons for the past 50 years and the demineralized bone matrix is a readily available and popular form of bone graft substitute. Similarly, in situ tissue engineering approaches have been used in the clinic for the reconstruction of several tissues including the larynx, and for trachea and finger reconstructions. Xenogenic source tissues such as porcine small intestinal submucosa or bovine dermis, but not tendon itself, are used as reinforcements in tendon-related orthopaedics. In this manuscript, we engineered a decellularized porcine tendon and propose that it can be clinically applied for tendon replacement or repair.

Our decellularization protocol, using a combination of physical, chemical and enzymatic methods, enabled decellularization of tendon and did not grossly affect tissue structure although its biochemical composition changed slightly due to the decellularization procedure. Similar results have been described in the literature, where it is stated that decellularization alters both mechanical and biochemical properties of decellularized tissues such as tendons and aortic valves. Based on the results of in vivo experiments, we can claim that there’s no rejection or encapsulation of implanted tissue, and decellularized tissue is re-populated with host cells and biochemical properties are recovered after implantation. In line with this, it has been reported that the in vitro macrophage response to implanted materials is strongly correlated with tissue remodelling outcome. Thus, macrophage polarization assays in the characterization of the materials to be used in clinics is highly convenient to predict the host response to the material. We tested the influence of decellularized tendon sections on macrophage polarization in vitro and we observed that decellularized tendon sections induced an M2 phenotype, which is a regulatory, alternatively activated, anti-inflammatory phenotype which is favourable for tissue remodelling. Supporting this result, Keane et al. showed that decellularized porcine small intestinal ECM showed smaller M1/M2 ratio. This is supported by our histological data in which we observe that GAG staining, which was reduced after decellularization, recovered to normal levels after 6 weeks. The different structure that we observe between host tendon and decellularized tendon might result from the fact that two different animals (porcine versus rabbit) and different tendons (Achilles versus patellar tendon) were used. Another outstanding question regarding the mechanical stability and strength of the decellularized tendon is the durability of the suture points, as one of the main reasons for the failure of current techniques is overloaded sutures. We can claim that, as after 6 weeks of implantation tissue was intact, it will endure longer time points and integrate with the whole tissue, in other words, the interface between the host and implanted tissue can hardly be recognized, which is an indicator for proper integration and likely mechanical integrity.

In order to preserve mechanical and biochemical properties, our decellularization strategy can be optimized in terms of time or methods used such as enzymes or detergents, or the size of the tendon to be decellularized. Not all tendon injuries require a whole tendon replacement; it could be a segmental defect or a tendon tear, hence the graft material needed for repair is different. Another challenge in tendon repair with a graft is that although surgery is an option, there’s no gold standard to be used as xenogenic graft material. Especially, the repair of rotator cuff (RC) injuries is a particular challenge as surgery often includes removal of the biceps tendon that otherwise gets impinged. Dermal grafts are mainly used in RC surgeries for preventing impingement but not for repair. As the risk of injuring rotator cuff tendon increases with ageing and given the current treatment methods, a new strategy can be implemented. Considering this, our method can be implemented to decellularization of bone-tendon grafts for RC reconstructions or decellularization of other connective tissues such as anterior cruciate ligament (ACL) for ACL reconstructions. In parallel to this, our research group also applied the same protocol for bone-patellar tendon-bone (BPTB) decellularization to create a graft material for ACL reconstruction.

In vitro assessment on bioactivity performance of decellularized tendon showed that they were not cytotoxic, allowed attachment and proliferation and induced tenogenic differentiation of hMSCs. However, we did not observe cell infiltration on day 7, which is one of the challenges of recellularization of the decellularized tendon owing to its dense structure. In vivo results showed that within 6 weeks, the decellularized tendon was repopulated with host cells. Repopulation after in vivo implantation can be explained with the exposure the mechanical stimulation. Here, we can speculate that as mechanical forces stimulated tendon fibroblasts, more cells from the host were attracted to the new, empty scaffold. The infiltrated cells had an elongated shape and oriented in the axis of mechanical stimulation. Gene expression results also confirmed that in vitro, decellularized tendons induce an upregulation in the early tenogenic marker SCX and mature tenocyte marker TNMD. Collagen type I is the main collagen type in tendon ECM. Cultivating mesenchymal stem cells on decellularized tendon sections resulted in stable gene expression of collagen type I for 7 days in vitro. This results can be explained by the fact that not only a collagen-rich environment but also an aligned collagen fibre structure in DTS may decrease collagen 1 synthesis. In agreement with these results, Yang et al. demonstrated that expression of COL1A remains similar in a 2D and 3D collagen enriched environments, where as a tendon ECM and non-collagen coated 2D environment results in higher expression. This indicates that decellularized tendons carry enough biochemical and topographical ECM cues to stem cells cultivated on them.

In translation perspective, there are two questions to be addressed before we can use the decellularized tendon clinically: 1) which animal model and which tendon tissue should we use to demonstrate efficacy of the tendon graft? In our study, we selected rabbit patellar tendon owing to its similarity with human patellar tendon in terms of mechanical loading. However, sheep could be a better animal model based on closer biomechanical and mechanical properties to human. Porcine is another candidate as the healing process is similar to humans. 2) What is the method to map the usability of the product and reduce the cost? This requires collaboration and coordination between research laboratories and clinics. Also, targeting the particular problem for each tendon and designing experimental approaches based on that would increase the efficiency, reduce cost and likelihood of failures. In commercialization perspective, tendon grafts are challenging for several reasons. In order to completely replace a ruptured tendon, decellularized tendon graft must be able to bear very large and impulsive forces and stresses during even modest activities of daily living. Considering this, repair of each injury in particular tendon tissue could be unique; hence it is difficult to propose one model of the decellularized tendon as a general graft. However, in terms of augmenting repairs and replacements, it is less challenging as scaffold does not need to resist such large mechanical loads from bone to muscle. Therefore, depending on the need of repairing the injury, our method can be optimized and applied.

While with this study we characterized decellularized tendons extensively for its potential use for tendon tissue repair, we will first focus on pre-clinical efficiency. Production of a pain-free, functional tendon is the prime target, so animal behaviour such as gait pattern in the knees can be monitored right from the moment of implantation. In addition to this, in order to get more insight on the time needed for the graft to fully integrate with the host animal, longer time points such as 2 months, 6 months and 12 months need to be monitored.

4. Conclusion

In this study, we produced a simple but robust protocol to
decellularize porcine Achilles tendon and characterized it in many aspects that can be used not only in the characterization of decellularized tissues but also any biological material. We demonstrate that decellularized tendons support stem proliferation, induce early tenogenic differentiation and induce macrophages towards a tissue remodelling phenotype. Furthermore, in vivo experiments showed that 6 weeks after implantation, decellularized tendons integrate with host tendon without showing signs of tissue rejection or encapsulation. These findings are essential in terms of standardization of not only the decellularized tendons but also tendon-bone or bone-ligament-bone sections in the reconstruction of musculoskeletal defects.

5. Materials and methods

5.1. Tissue harvest and storage

Porcine Achilles tendons were obtained from crossbreeds of Great Yorkshire and Dutch land pigs aged between 6 and 8 months old and between 85 and 95 kg of weight (Compaxo Meat BV, the Netherlands). Achilles’ tendons were transported under conditioned circumstances between 85 and 95 kg of weight (Compaxo Meat B.V., the Netherlands). Yorkshire and Dutch land pigs aged between 6 and 8 months old and 5.2. Preparation of decellularized tendon slices

Frozen tendons were cut into 2–3 cm long sections and freeze-thawed five times in liquid nitrogen and 37°C phosphate buffered saline (PBS) (Sigma-Aldrich), respectively, then incubated for 48 h in distilled water at room temperature. Next, samples were incubated in Tris-EDTA buffer (pH 7.6) containing 1% Triton X-100 for 48 h at room temperature. After another 24 h of wash in distilled water at room temperature, samples were incubated in saline (PBS), respectively, then incubated for 48 h in thawed five times in liquid nitrogen and 37°C phosphate buffered saline and frozen again in liquid nitrogen. Next, 10 mg samples were transferred to a 96-well plate and evaporated under vacuum overnight. Next, 100 μl of the Chloramine T/Oxidation Buffer Mixture was added to each sample and standards and incubated at room temperature for 5 min. Next, 100 μl of the Diluted DMAB Reagent was added to each sample and standards and incubated for 90 min at 60°C. Absorbance was measured at 560 nm. Readings were corrected by subtracting the blank value from all readings. The amount of hydroxyproline present in the samples was calculated based on the standard curve. Data were normalized to scaffold dry weight, obtained prior to digestion.

5.4. Determination of collagen content

The total collagen content of decellularized (N=3) and native samples (N=3) was measured using the Hydroxyproline Assay Kit (Sigma-Aldrich). Briefly, frozen tendons were freeze-dried for at least 24 h at −55°C. Next, samples were cut into a constant weight and digested in 4 M HCl for 4 h at 120°C. 10 μl of each sample was transferred to a 96-well plate and evaporated under vacuum overnight. Next, 100 μl of the Chloramine T/Oxidation Buffer Mixture was added to each sample and standards and incubated at room temperature for 5 min. Next, 100 μl of the Diluted DMAB Reagent was added to each sample and standards and incubated for 90 min at 60°C. Absorbance was measured at 560 nm. Readings were corrected by subtracting the blank value from all readings. The amount of hydroxyproline present in the samples was calculated based on the standard curve. Data were normalized to scaffold dry weight, obtained prior to digestion.

5.5. Determination of glycosaminoglycan content

Glycosaminoglycan content of decellularized (N=3) and native samples (N=3) was measured using the dimethylene blue (DMMB) assay. Briefly, frozen tendons were cut into smaller pieces and freeze-dried for at least 24 h at −55°C. Samples were cut into a constant weight and digested in papain (Sigma-Aldrich) digestion buffer (140 μg per ml in 5 mM L-cysteine hydrochloride (Sigma-Aldrich) plus 5 mM NaN3, EDTA (VWR)) for 16 h at 60°C with agitation. 40 μl of standards (chondroitin sulfate sodium salt from shark cartilage (Sigma, C4384)) and test samples were transferred to 96-well plate and mixed with 150 μl of 1,9-dimethylene blue solution (DMMB) and incubated 2 min at room temperature. Absorbance was measured sequentially at wavelengths 540 nm and 595 nm and the readings were subtracted one another (540–595). Concentrations of the samples were calculated based on the data from the standard curve. Data were normalized to scaffold dry weight, obtained prior to digestion.

5.6. Histology and immunohistochemistry

Decellularized and native samples were fixed in 10% (v/v) neutral buffered formalin (Sigma-Aldrich) for 48 h at room temperature. Next, samples were incubated in 70% ethanol for 1 h, 95% ethanol for another hour and in absolute ethanol for 4 h. Then, samples were twice incubated in Xylene for 1 h. Finally, samples were embedded in Paraplast X-TRA (Sigma Aldrich) at 58°C for 1 h, twice and incubated at room temperature. Embedded samples were sectioned at 10 μm thickness by using a microtome.

5.7. Hematoxylin & eosin and DAPI staining

Sections of native and decellularized tissues were deparaffinized in Histo Clear (Baker), decreasing ethanol series and finally incubated in distilled water for 4 min each. Next, samples were incubated in Weigt’s iron hematoxylin solution (Sigma-Aldrich) for 1 min, washed in tap water and distilled water for 3 min, incubated in eosin (Sigma-Aldrich) for 30 s, and washed again. Finally, samples were dehydrated in increasing series of ethanol, Histo Clear and mounted in DPX mounting medium (Sigma Aldrich). For DAPI staining, after deparaffinization, the samples were incubated with 4',6-diamidino-2-phenylindole dihydrochloride (DAPI) (Sigma-Aldrich) for 5 min, dehydrated and mounted.
5.8. Alcian blue stain

Alcian blue was used to stain glycosaminoglycans. Briefly, sections of native and decellularized tissues were deparaffinized in Histo Clear (Baker), decreasing ethanol series and finally incubated in distilled water for 4 min each. Next, sections were incubated in 3% acetic acid solution (VWR) before they have incubated in 1% Alcian blue solution [Alcian Blue-8GX (Sigma Aldrich) dissolved in 3% Acetic Acid] for 30 min at room temperature. After rinsing in 3% acetic acid, tap water, and distilled water, sections were stained with 0.1% Nuclear Fast Red Solution for 5 min, dehydrated in increasing series of ethanol, Histo Clear (Sigma Aldrich) and mounted in DPX mounting medium (Sigma Aldrich).

5.9. Picro sirius red stain

Picro Sirius Red Stain Kit (Connective Tissue Stain) (Abcam) was used to stain collagen. Briefly, sections of native and decellularized tissues were deparaffinized in Histo Clear (Baker), decreasing ethanol series and finally incubated in distilled water for 4 min each. Next, samples were incubated in Picro-Sirius Red Solution for 1 h. Then, tissue sections were rinsed in 0.5% acetic acid twice and rinsed in absolute ethanol. Finally, sections were dehydrated in increasing series of ethanol, Histo Clear (Sigma Aldrich) and mounted in DPX mounting medium (Sigma Aldrich).

5.10. Scanning electron microscopy

Samples were fixed in 2% glutaraldehyde (Fisher Scientific) in 0.1 M sodium cacodylate trihydrate (Sigma-Aldrich) buffer prepared in PBS pH 7.4 at room temperature for 1 h. Next, samples were washed with 0.1 M sodium cacodylate trihydrate buffer for 3 times 10 min and dehydrated in 25%, 50%, 75%, 90%, and 100% EtOH for 15 min each and incubated in 100% ethanol for additional 15 min. Next, samples were dried in hexamethyldisilazane (HMDS) (Sigma Aldrich) overnight and covered with 5 nm gold-palladium and imaged using a scanning electron microscope (SEM) (FEI Quanta 3D FEG Dual Beam).

5.11. Tensile testing of native and decellularized samples

Native (N = 2) and decellularized (N = 2) tendons were longitudinally cut into 4–6 pieces and imaged using a Nikon SMZ25 stereomicroscope to determine their cross-sectional areas. Tissue ends were embedded in an epoxy resin (Innotec Topfix) to prevent sample slippage from tensile test clamps, often observed due to cross-sectional thinning upon elongation. Upon epoxy curing, samples were incubated in Picro-Sirius Red Solution for 1 h. Then, 50 μL of each sample medium (cells cultured on basic medium as a negative control, cells cultured on decellularized tendon sections and maximum LDH activity group as a positive control) were transferred to a new well plate and mixed with 50 μL of Reaction Mixture, incubated at room temperature for 30 min protected from light. Finally, 50 μL of Stop Solution was added and absorbance was measured at wavelength 490 nm and 680 nm. To determine LDH activity, we subtracted the 680 nm absorbance value (background) from the 490 nm absorbance value.

5.12. Preparation of tendon sections for cell culture

Frozen decellularized tendons were embedded in Optimal Cutting Temperature (OCT, Sakura) compound and fixed to the cutting base plate of a cryotome and longitudinally sectioned into tendon slices with a thickness of 300 μm and washed with PBS, snap-frozen in liquid nitrogen and freeze-dried for at least 24 h. Dry decellularized tendon sections were cut into the size of the surface area of a well plate of interest and incubated in 70% ethanol for 1 h. Remaining ethanol was removed and samples were dried in sterile conditions. Next, samples were washed in sterile PBS for two times, half an hour at room temperature, and one time at 37 °C for half an hour. The day before the experiment started, samples were incubated in culture media, overnight at 37 °C in 5% CO2.

5.13. Cell culture, viability and cytotoxicity assay

Bone marrow aspirate was obtained from a donor (D210, female/74 years old) who were undergoing total hip replacement surgery and had given informed consent. Nucleated cells were counted from the aspirate and seeded at a density of 500 000 cells cm−2 in hMSC proliferation medium containing Minimal Essential Media alpha (α-MEM) containing (Thermo Fisher Scientific) 10% fetal bovine serum (FBS) (Sigma-Alrich), 100 U/ml Penicillin/Streptomycin (Thermo Fisher Scientific), and 20 mM L-Ascorbic acid with an additional 1 ng/ml basic fibroblast growth factor (Neurotech) at 37 °C in a humidified tissue culture chamber with 5% CO2. The hMSCs obtained after the first trypsinization (Trypsin-EDTA (0.05%), Fisher Scientific) were seeded at a density of 2 × 105 cells/cm2 in Minimal Essential Media alpha (α-MEM) containing (Thermo Fisher Scientific) 10% fetal bovine serum (FBS) (Sigma-Alrich), 100 U/ml Penicillin/Streptomycin (Thermo Fisher Scientific), and 20 mM L-Ascorbic acid at 37 °C in a humidified tissue culture chamber with 5% CO2. Cell viability was qualitatively assessed for a week (6 h, 1 day, 4 days and 7 days) with LIVE/DEAD™ Viability/ Cytotoxicity Kit (Thermo Fisher Scientific) assay. Briefly, cell-seeded tendon sections were washed with PBS two times and incubated in 2 μM Calcein AM and 6 μM ethidium homodimer (EthD-1) in serum-free α-MEM with nucleosides and without phenol red (Thermo Fisher Scientific) for 30 min at 37 °C in the dark and washed with the medium twice.

Cytotoxicity assay was performed by measuring lactate dehydrogenase (LDH) release using the Pierce LDH Cytotoxicity Assay Kit (Thermo Fisher Scientific). Briefly, hMSCs were cultured on decellularized tendon sections at a density of 2 × 105 cells/cm2 in Minimal Essential Media alpha (α-MEM) (Thermo Fisher Scientific) containing 10% fetal bovine serum (FBS) (Sigma-Alrich), 100 U/ml Penicillin/Streptomycin (Thermo Fisher Scientific), and 20 mM L-Ascorbic acid (ASAP) at 37 °C in a humidified tissue culture chamber with 5% CO2 for 24h. Then, to measure the maximum LDH activity, 1X Lysis Buffer was added and incubated at 37 °C, 5% CO2 for 45 min. Next, 50 μL of each sample medium (cells cultured on basic medium as a negative control, cells cultured on decellularized tendon sections and maximum LDH activity group as a positive control) were transferred to a new well plate and mixed with 50 μL of Reaction Mixture, incubated at room temperature for 30 min protected from light. Finally, 50 μL of Stop Solution was added and absorbance was measured at wavelength 490 nm and 680 nm. To determine LDH activity, we subtracted the 680 nm absorbance value (background) from the 490 nm absorbance value.

5.14. Collagen coating

Culture plates were coated with rat tail collagen I (Gibco) based on the vendor’s protocol. Briefly, 1 mg/ml of rat tail collagen tail was coated and incubated at 37 °C, 5% CO2 overnight, and wells were washed with PBS three times.

5.15. Macrophage culture and polarization

The human monocytic cell line THP-1 (Sigma-Aldrich) was cultured in Roswell Park Memorial Institute (RPMI) 1640 medium (A10491-0, Gibco), supplemented with FBS (10% v/v, Bovogen) and penicillin/streptomycin (1% P/S v/v; Lonza, Basel, Switzerland, DE17-602E) in 37 °C, 5% CO2. In order to induce macrophage differentiation, 300 000 cells/ml were incubated in culture medium with 50 ng/ml of
with 20 ng/ml IFNγ and 100 ng/ml LPS to promote an M1 phenotype, assessment: RPMI medium supplemented with 10% FBS and 1% P/S
Macrophages were cultured for 24 h following conditions for further washed with PBS and incubated in fresh media for 48 h to acquiesce. 12-myristate13-acetate (PMA). After 24 h, adherent macrophages were consecutive days to prevent infection. Six weeks later, the rabbits were autologous control. Penicillin was intramuscularly injected for 3 con-
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Table 1
Primer sequences of each gene used in this study.

<table>
<thead>
<tr>
<th>Gene name</th>
<th>Forward Primer</th>
<th>Reverse Primer</th>
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<tr>
<td>SCX</td>
<td>ACACCGAGCCCAAACAGA</td>
<td>GCGGTCTTGCTGACACTTTCC</td>
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<tr>
<td>COL1A1</td>
<td>GGGTCTGCTCTCCTTGGAGG</td>
<td>CATATTCTGGAGGCTGTTCCTT</td>
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<tr>
<td>MKX</td>
<td>TCAGGACAAACCTGGGCTTG</td>
<td>ACCTTGTTGCTACGCTTTGAC</td>
</tr>
<tr>
<td>TNMD</td>
<td>ATTCAAGGAGGAAATGGCACTGA</td>
<td>TGGGCTGCTGGCTGAGCAACCC</td>
</tr>
<tr>
<td>EGR-1</td>
<td>GAGCCGCCAAGGAAACAGTTG</td>
<td>TGGAGAATCTGACACTTGT</td>
</tr>
<tr>
<td>MCP-1</td>
<td>CAGCCAGATTCATCTGAGGTT</td>
<td>TGGATCCCTGAAACACTCTCT</td>
</tr>
<tr>
<td>CD68</td>
<td>CTACGTGCCAGAGGAGCAGTG</td>
<td>CGCCCGACTAGCTCAGGATTAG</td>
</tr>
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<td>CCR7</td>
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</tr>
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<td>IL-6</td>
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<tr>
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<td>CD206</td>
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<td>CD163</td>
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<tr>
<td>18S</td>
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<td>AGTTCTCGGCCCTCTTGCTT</td>
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12-myristate13-acetate (PMA). After 24 h, adherent macrophages were washed with PBS and incubated in fresh media for 48 h to acquire. Macrophages were cultured for 24 h in following conditions for further assessment: RPMI medium supplemented with 10% FBS and 1% P/S resulting with RPMI medium supplemented with 10% FBS and 1% P/S with 20 ng/ml IFNγ and 100 ng/ml LPS to promote an M1 phenotype, RPMI medium supplemented with 10% FBS and 1% P/S with 20 ng/ml IL-4 to promote an M2 phenotype and on decellularized tendon sections with RPMI medium supplemented with 10% FBS and 1% P/S resulting in a M0 phenotype.

5.16. RNA isolation and quantitative PCR (RT-qPCR)

RNA of each sample was isolated with the protocol described in the RNeasy Mini Kit (QIAGEN) or TRIzol™ Reagent. Reverse transcription was carried out based on the protocol provided by iScript™ Select cDNA Synthesis Kit (Bio-Rad). qPCR was performed by using iQ SYBR Green Supermix (Bio-Rad). Briefly, iQ SYBR Green Supermix (2x), forward and reverse primers (at 300 nM final concentration), cDNA (100 ng final concentration) and nuclease-free water were mixed for a total of 10 μl reaction volume. qPCR run was performed by using the Bio-Rad CFX manager. The expression of target genes (MCP-1, CD68, CCR, TNF-α, IL-6, CD200R1, CD206, CD163 and IL-10) in macrophage polarization experiments was normalized to glyceraldehyde 3-phosphate dehydrogenase (GAPDH) gene as a housekeeping gene. The expression of target genes (SCX, COL1A1, TNMD, MKX) in tenogenic differentiation was normalized to RNA18S as a housekeeping gene. Relative gene expression was calculated with the ΔΔ Ct formula. Primer sequence of target genes are listed in Table 1.

5.17. Sterilization and in vivo biocompatibility of decellularized samples

Animal experiments were performed in the animal centre of Sichuan University with the permission of the local ethics committee for laboratory animal (Sichuan Laboratory Animal Management Committee) and two adult female New Zealand rabbits were used. The rabbits were anaesthetized with intravenous pentobarbital sodium (30 mg/kg) and surgically operated under general sterile condition. A longitudinal incision was made on the knee and the subcutaneous tissue was divided to expose the patellar tendon. A 10 × 3 mm size defect (length × width) was made in the middle third of the tendon. The implant (10 × 3 mm) was bundled up with 4–0 nylon suture at the two ends and was sutured to the host tendon at the four corners using 4–0 nylon suture. Following the implantation, the wound was closed by 4–0 nylon suture. Using the same implantation procedure, the tendon harvested from one knee was bundled and implanted in the other knee of the sampled animal as an autologous control. Penicillin was intramuscularly injected for 3 consecutive days to prevent infection. Six weeks later, the rabbits were sacrificed by intravenous administration of a lethal dose of pentobarbital sodium and the implants were collected with surrounding tissues. The samples harvested were fixed in 10% formalin and subjected to paraffin embedding for histological evaluation.

5.18. Image analysis

Aspect ratio of the nuclei was calculated by using ImageJ. Briefly, images were thresholded and analysed by using Analyse Particles option for which measurements were selected to Shape description. Directionality of actin fibers was measured by using Directionality section in the Analyse command.

5.19. Statistical analysis

All statistical analysis was performed using GraphPad Prism version 8.0 (GraphPad Software, Inc., San Diego, CA). Student’s t-test, one-way and two-way analysis of variance (ANOVA) was carried out with significance set at p < 0.05 to determine the significance between means. For multiple comparison tests, the Tukey procedure was applied.

Declaration of competing interest

HV is the CEO of HCM-Medical. The other authors do not have any financial interests to declare.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.regen.2020.100027.

References

2. Fenwick SA, Hazleman BL, Riley GP. The vasculature and its role in the damaged and

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