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Tissue engineering of the anterior cruciate ligament: a new method using acellularized tendon allografts and autologous fibroblasts

Thomas Tischer · Stephan Vogt · Sebastian Aryee · Erwin Steinhauser · Christopher Adamczyk · Stefan Milz · Vladimir Martinek · Andreas B. Imhoff

Abstract

Introduction The availability of autogenous tendons (middle part of patellar tendon, semitendinosus/gracilis, or quadriceps tendon) for cruciate ligament reconstructions is restricted and related to withdrawal morbidity. Allografts and synthetic ligament materials often show problems regarding long-term stability and immunological reactions. Therefore, the aim of this study was to develop and characterize a new scaffold based on acellular allografts seeded with autologous cells for tissue engineering of the anterior cruciate ligament (ACL).

Materials and methods Semitendinosus tendons of New Zealand White (NZW) rabbits were harvested and acellularized using the detergent sodium dodecyle sulfate (SDS) as the main ingredient. After that, cultured (37°C, 5% CO_2, medium) dermal fibroblasts were injected into the tendons. These constructs were further cultivated for 4, 7, or 14 days under the same culture conditions. Native, acellular, and seeded tendons underwent biomechanical testing (ultimate load to failure [N], stiffness [N/mm], and elongation [%], each $n = 9$] and histological hematoxylin-eosin (H.E.) staining. Detailed immunohistochemical (collagen I, III, IV, VI, pro-collagen I, versican, and vimentin) analyses were conducted to detect changes in the composition and structure of the extracellular matrix (ECM) after acellularization.

Results Histologically, a cell-free, crimped slack tendon structure after acellularization and a good integration of the cells after injection (4, 7, and 14 days) were seen. Metabolic activity of the seeded cells was demonstrated by positive immunohistochemical staining for pro-collagen I, which was negative in nonseeded constructs. Major differences in staining patterns of the various other ECM components were not observed. Biomechanically, the maximum load to failure of these tendons was comparable to native tendons ($P = 0.429$; native 134.5 ± 12.9 N; acellular 118.5 ± 7.3 N; seeded 132.3 ± 5.6 N). Stiffness and elongation were comparable between native and acellular tendons, but differed significantly after seeding ($P < 0.001$).

Conclusion The described method is suitable to make tendons completely cell free without changing their major biomechanical properties. Preservation of the ECM and of the collagen fiber structure by this method should give an ideal environment for autologous cell integration and metabolic activity in contrast to other approaches for tissue acellularization. The cell disruption and extraction of cell detritus should minimize adverse immunogenic reactions.

T. Tischer and S. Vogt contributed equally to this work.

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Keywords Anterior cruciate ligament · Replacement · Autografts · Allografts · Tissue engineering

Introduction

The anterior cruciate ligament (ACL) is often injured by rotation injuries of the knee. Because of limited intrinsic healing capabilities [16], the surgical reconstruction using autologous patellar, semitendinosus/gracilis, or quadriceps tendons is recommended to restore optimal function in these mostly young patients [4, 5]. Major disadvantages are donor side morbidity (persistent patellar pain, insufficiency in strength of knee stabilizing muscles) and—especially in cases of multi-ligament injuries or in cases of revision surgery—limited availability of suitable autologous grafts. To overcome this problem, synthetic, silk, allograft, and xenograft materials have been used in reconstruction surgery of the ACL [2, 11, 19, 24, 25].

Synthetic materials such as polytetrafluoroethylene (Gore-Tex, Elkton, MD, USA) are noninfectious and easy to produce, but may not be biocompatible because of host foreign body responses [22]. Incorporation, remodeling and long-term stability of synthetic materials to date are still inadequate and often lead to late mechanical failure. Most of the synthetic materials implanted so far showed deterioration over time (Gore-Tex, Aramid) [10, 26].

Recently, especially in USA, the use of allografts has become more popular [9, 24, 25]. Allografts possess disadvantages like immunogenic reactions, transmission of infectious diseases (HIV, various types of Hepatitis, and veneral diseases), or slow remodeling and incorporation of the graft. They have been used with variable success in humans for ACL/PCL reconstruction. Additionally, allografts undergo increased elongation and mechanical failure, with a worse outcome in contrast to autografts [14]. The use of xenografts in humans is attractive because of its availability and convenient mechanical characteristics. The obvious disadvantage of this material is its antigenicity [19]. Various techniques have been employed to decrease the antigenicity of allografts and xenografts. Allografts are typically fresh-frozen or lyophilized to decrease antigenicity [17, 31]. Since most antigenicity is mediated by the fibroblasts, another technique to decrease antigenicity is to extract all cellular components [31]. Recently, the use of solvents/detergents [sodium dodecyle sulfate (SDS), Triton-X 100, tri-nitro-butyl-phosphate (TnBP)] to acellularize tendon allografts has been described [7, 8, 15, 30]. This method is currently also investigated for the tissue engineering of cardiac pericardium, oesophagus transplantats, and others [6, 12, 21]. Most important is that this technique allows preserving the collagenous matrix, since matrix destruction would decrease the pre-implant mechanical strength of the graft. Changes in the collagen matrix may also inhibit repopulation and remodeling of the grafts by in-growing fibroblasts. This process is critical if long-term mechanical strength is to be maintained. In addition, other matrix components should be preserved in order to present the in-growing cells a widely natural environment.

The aim of this study is to generate new constructs for tissue engineering of the ACL based on allograft tendon material. Therefore, allograft rabbit semitendinosus tendons were acellularized using SDS and cultivated with autologous dermal fibroblasts in cell culture. Special attention is given to any change in the composition of the extracellular matrix (ECM) during the acellularization process and subsequent cell seeding, which is done by directly injecting the cells into the tendons.

Materials and methods

Tissue processing

Female New Zealand White (NZW) rabbit tendons were investigated [ex-breeders, 1-year old, average body weight (BW) 4,044 g, range from 3,150 to 4,820 g, closed femoral growth plates]. Semitendinosus tendons were acquired from animals used for other experiments. Animals were purchased from a provider of research animals (Charles River, Kissleg, Germany). Semitendinosus tendons ($n = 45$) were harvested using a mini-tendon stripper. All tendons were immediately frozen and then stored at −80°C until further processing.

For acellularization, the tendons were thawed and further processed. Acellularization was accomplished in six steps, using aqua dest for 24 h, 1% SDS solution for 24 h, aqua dest for 24 h, and 70% ethanol for 24 h. Following that, scaffolds were washed extensively in phosphate buffered saline (PBS) (modified from Cartmel et al. [8]). Fibroblasts were extracted from the dermis of rabbits using an explant method [3]. Briefly, small pieces of dermis were put in cell culture dishes (Ø 10 cm) containing Dulbecco's Modified Eagle Medium (DMEM) supplemented with fetal bovine serum (FBS) (5%), L-glutamine (2 mM), and penicillin/streptomycin (100 IU/ml). At an average of 10 days under cell culture conditions (37°C, 5% CO_2), the cells were confluent and split using trypsin/EDTA solution. After another passage, the cells were harvested for seeding of acellularized tendons. Therefore, a cell suspension (1.0×10^6 cells/0.5 ml medium) was prepared. This suspension was injected into the tendons in line with the collagen fibers by a syringe (gauge 17). Multiple injection sites were used. In separate experiments, cells were injected into cell culture dishes using the same syringe and cell adherence was observed, showing that cells were not harmed by this injection. In addition, cell suspension was applied for 4 h to the tendon surface for outer settlement of

the cells, then additional medium was added to the wells. After 48 h, the medium was changed and tendons were cultivated for a total of 4, 7, and 14 days, with changes of medium every third-day.

Biomechanical testing

Nine native tendons, nine acellular tendons, and nine cell seeded (4 days of culture) tendons were tested. Biomechanical testing was done using a universal material test machine (Modell Zwicki 1120, Fa. Zwick, Germany) with specially designed clamps to allow optimal longitudinal application of traction. Testing started immediately after a preload of 2 N was achieved. The load-to-failure test was performed by increasing the tensile loading continuously with a speed of 10 mm/min. Furthermore, the elongation of the tendon until rupture [in %] and the stiffness [N/mm] were recorded. During all steps, the specimens were always kept moist with PBS.

Histology/immunohistochemistry

For histological examination of morphology and cell content, six acellular tendons were used. After fixation in 90% methanol at 4°C for at least 24 h, the specimens were infiltrated overnight in a 5% sucrose solution in PBS at pH 7.4, mounted on chucks in Jung tissue embedding medium (Leica, Wetzlar, Germany), frozen in a HM 500 OMV cryostat (Mikrom, Berlin, Germany), and cryosectioned at 12 μm. The sections were stained with hematoxylin-eosin (H.E.). Three longitudinal sections (one medial, one in the center, and one lateral) of each specimen were examined. Acellularized and seeded tendons were examined 4 ($n = 3$), 7 ($n = 3$), and 14 days ($n = 3$) after cultivation. The same topographical regions as in the previous specimen were used. For comparison, native tendons were also sectioned and investigated ($n = 3$).

For analysis of changes in the ECM, tendons (native, acellular, and seeded at days 4, 7, and 14) were labeled with a panel of monoclonal antibodies directed against various ECM constituents (Table 1) [20]. For a detailed description of the immunohistological staining see Tischer et al. [27]. Briefly, all sections were treated with 0.3% hydrogen peroxide in methanol for 30 min to block endogenous peroxidase activity and nonspecific binding of the primary antibodies was reduced by blocking with normal horse serum for 60 min. The control sections were incubated with normal mouse immunoglobulins (10 μg/ml) or were obtained by omitting the primary antibody and treating the sections with PBS alone. Antibody binding was detected with a Vectastain ABC 'Elite' avidin/biotin kit (Vector Labs, Burlingham, CA, USA) and the sections were counterstained with Mayer's hematoxylin. Staining intensity was graded semiquantitatively (no staining (−), only weak staining (+), and strong staining (++) [20]).

Table 1 Sources and labeling characteristics of the monoclonal antibodies used

Antigen(s) recognized	Antibody	Dilution	Enzyme	Source
Collagen 1	Col1	1:2,000	Ch ABC	Sigma
Pro-collagen 1	M38	1:5	Ch ABC	DSHB
Collagen 3	4H12	1:500	Ch ABC	ICN
Collagen 4	M3F7	1:10	CH ABC	DSHB
Collagen 6	5C6	1:10	CH ABC	DSHB
Versican	12C5	1:5	Ch AC	DSHB
Vimentin	AMF-17b	1:5	PBS	DSHB

Note that collagens and versican antibodies required an enzyme pretreatment either with 0.25 U/ml of chondroitinase (Ch) ABC or AC (both Sigma)

Statistical analysis

For statistical analysis of the biomechanical data (ultimate load to failure, stiffness, elongation), the mean and standard error of the mean (SEM) were calculated for all investigated variables. To test if any statistical differences existed among the three groups, one-way ANOVA for nonparametric, nonmatched samples was used (Kruskal–Wallis). To see if there were any differences between the single groups, the Tukey post-test was used. Differences were considered as statistically significant at $P < 0.05$. All calculations were carried out using GraphPad Prism Version 4.00 for Macintosh (GraphPad Software; San Diego, CA, USA).

Results

Biomechanical testing

During cell culture of the seeded tendons, the tendons began to slightly shorten and appeared a little swollen. However, under mechanical traction, they resumed their normal macroscopic appearance. The failure site of all tendons during biomechanical testing was within the ligamentous part. No disruption occurred at the interface between the clamp and tendon.

There were no statistically significant differences ($P = 0.429$) of the ultimate load to failure between the three constructs (native tendons 134.5 ± 12.9 N; acellular tendons 118.5 ± 7.3 N; seeded tendons 132.3 ± 5.6 N; see Fig. 1). However, a significant difference ($P < 0.001$) in the elongation of the different groups was observed: native tendons 2.47 ± 0.33%; acellular tendons 2.46 ± 0.06%, and seeded tendons 7.50 ± 0.5%. Post-test revealed no difference

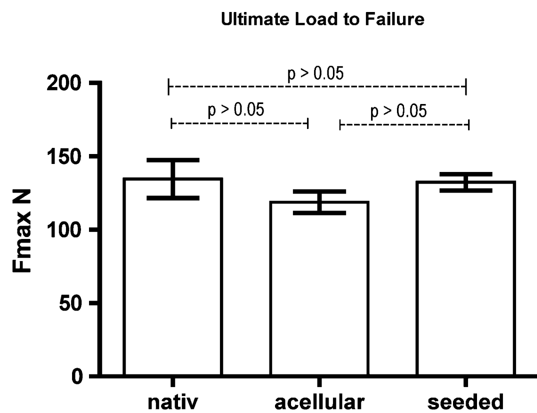

Fig. 1 Graph showing ultimate load of failure in Newton [N] for native, acellular, and seeded tendons. SEM and *P*-values are shown. No statistically significant difference could be detected

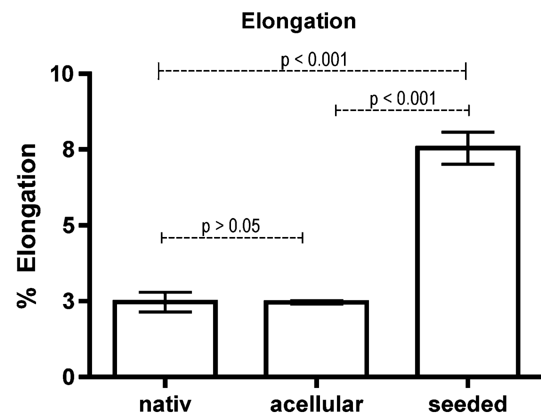

Fig. 2 Graph showing elongation in percent [%] for native, acellular, and seeded tendons. SEM and *P*-values are shown. There were statistically significant differences between the native and seeded and the acellular and seeded tendons

between the native and acellular tendons ($P > 0.05$), but there were statistically significant differences between the native and seeded ($P < 0.001$) and acellular and seeded tendons ($P < 0.001$; see Fig. 2). Accordingly, there were also differences in the stiffness between the different groups ($P < 0.001$): native tendons 58.9 ± 6.57 N/mm; acellular tendons 48.5 ± 3.05 N/mm, and seeded tendons 18.5 ± 1.73 N/mm. There were no significant differences between the native and acellular tendons ($P > 0.05$), but there were statistically significant differences between the native and seeded ($P < 0.001$) and acellular and seeded tendons ($P < 0.001$).

Histology/immunohistochemistry

After the acellularization process all tendons were completely cell free. No cell nuclei could be detected in any of the slides; they were neither detected in the central, medial, or lateral tendon portions (Fig. 3b). More interfascicular spaces were noted after SDS treatment. In contrast, the control tendons showed regular arrangement of fascicles and regular high-density amount of spindle-shaped fibroblasts (Fig. 3a). After cell injection and proliferation for 4, 7, or 14 days, an irregular cell distribution was noted with reduced cell density compared to native tendons (Fig. 3c, d). Since no mechanical stimulation was done, only few cell were spindle shaped. Most cells were round or somewhat flattened. Cells were detected in the center as well as on the surface after 4, 7, and 14 days. Highest cell densities were pronounced at the tendon surface or along inter-fascicular spaces of the tendons.

Immunohistochemical examination of ECM molecules showed no gross change in their occurrence before and after the process of acellularization (Table 2, Fig. 4). However, pro-collagen I, the precursor of collagen I, is clearly detectable in normal tendons, as a sign for ongoing collagen synthesis (Fig. 4c). During the process of acellularization, the collagen I propeptide is washed out together with the cells. After seeding of the scaffold with the fibroblasts, active production of pro-collagen I can be detected around the cells, indicating metabolic activity and active production of collagen I (Fig. 4d). The staining pattern of the tested collagens (collagen 1, 3, 4, and 6) did not show any differences between groups (Fig. 4a, b). Strong staining for collagen I, the main collagen of tendons, was observed in all groups. Strong staining was also observed for collagen III and to a lesser degree for collagen IV (vessel basal lamina) and VI. Staining for vimentin is found in native as well as acellularized tendons, but the staining pattern of vimentin differs between native and acellularized tendons (Fig. 4e, f). During acellularization, the strong, clearly labeled staining pattern blurs somewhat. After seeding, the clear labeling surrounding the cells could be detected again. The staining pattern for versican—one major large proteoglycan expressed by fibroblasts—shows no difference.

Discussion

Acellularized tendons promise advantages compared to allografts or synthetic materials. First, the immunogenicity and antigenicity of the tissue is reduced by extracting the antigenic cells, whereas the only minimal antigenic ECM itself is preserved [1]. Second, by preserving other macromolecules besides the collagenous structure, an ideal environment is given to the cells for incorporation, metabolism, and matrix synthesis. The seeding with autologous cells should fasten the process of tendon incorporation and remodeling. Further, by preserving the natural collagenous structure, the initial biomechanical strength should be preserved.

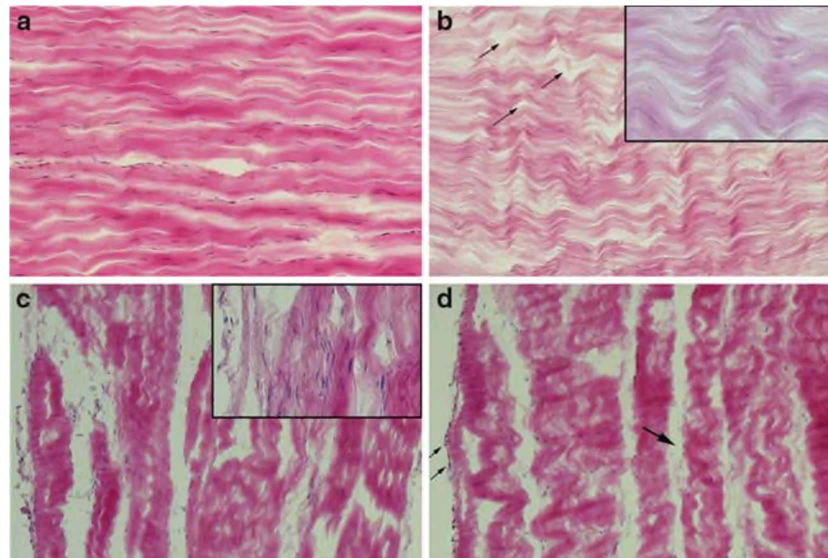

Fig. 3 Hematoxylin-eosin stainings. **a** Showing native tendon with regular cell count and structure (×50). **b** Shows acellularized tendon, no cell nucleoli are visible. Note increased infrafascicular openings (*arrows*, ×50). Inlay shows high-power view from same specimen (×100). **c** Seeded tendons with fibroblasts after 4 days of cell culture showing good cell distribution throughout the tendon (×25). High-Power inlay magnifies the seeded cells (×50). **d** Tendon after 14 days of cell culture (×25). Note especially the dense cell layer around the tendon (*small arrows*). Cells were especially found along the infrafascicular openings (*big arrow*)

Table 2 Immunohistochemical staining in the different groups

	Native $n = 3$	Acellular $n = 3$	Seeded 4 days $n = 3$	Seeded 7 days $n = 3$	Seeded 14 days $n = 3$
Col 1	++	++	++	++	++
Pro-Col 1	+	–	+	+	+
Col 3	++	++	++	++	++
Col 4	+	+	+	+	+
Col 6	++	++	++	++	++
Versican	++	++	++	++	++
Vimentin	+	+	+	+	+

Note especially the occurrence of pro-collagen I and its disappearance during acellularization and reappearance with cell seeding

The biomechanical data between the native and acellular tendon were similar. However, the elongation of seeded tendons was longer. We noticed that the tendons swelled during culturing. Because of only one cycle of loading until failure and no mechanical preconditioning, this could explain the statistically increased elongation together with reduced stiffness after cell culture. But this was not observed after the acellularization process alone. Therefore, extensive pretensioning of the tendons, as done in the operating room, would probably have stretched the tendons to their original length, before biomechanical testing started. But to achieve as reproducible as possible testing conditions for all three different tendon groups, pretensioning of the seeded tendons was not performed.

The question what happens to the ECM with its collagenous structure is important for mechanical stability. Up to date, the composition and structure of the ECM after acellularization have not been examined thoroughly. Here, we could show by immunohistochemical staining that there was no alteration in the collagen I/III staining pattern. Furthermore, by preserving the natural environment, an optimal milieu for in-growth and proliferation of cells is maintained. The biochemical contents of glycosaminoglycans (GAGs) were already found to be similar [7, 30] and also the content of total collagen [30]. In our investigation it has been shown that the acellularization process effectively removed all cells. Further, the staining pattern of the other collagenous molecules, namely collagen IV and VI, vastly remains the same. Pro-collagen I COOH-terminal propeptide is a recognized marker of collagen I turnover in the connective tissue [23]. Its presence in the tissue is limited to a few days and its occurrence in the seeded tendons indicates that the grafted cells are viable and take part in the remodeling process of the ECM by secreting soluble pro-collagen, which after cleavage of the propeptide forms extracellular fibrils [18]. Further, the distribution of macromolecules like versican was unchanged. In contrast to others, who noted some removal of vimentin—an intermediary filament especially characteristic for fibroblasts—in dermis after acellularization [29], we only noticed some decreased and blurred staining pattern, but the staining was clearly detectable. This is most probably due to the fact that during cell removal the intermediate filaments—which connect all

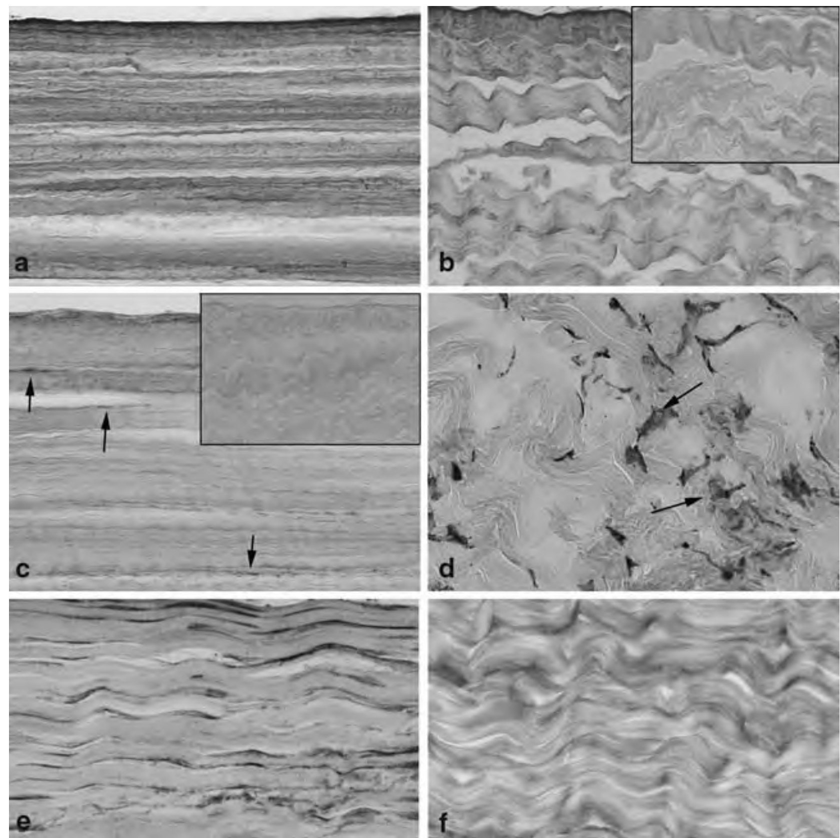

Fig. 4 Immunohistological stainings for **a** collagen I. Native tendons showing strong, regular labeling (×25). **b** Collagen I staining of acellular tendons shows also strong staining (×25). In comparison, the inlay shows the negative control staining, where the primary antibody has been omitted (×25). **c** Staining for pro-collagen I reveals discrete staining in native tendons (*Arrows mark strong areas*). After acellularization, no staining could be detected (*inlay*, ×25). **d** In contrast, after cell culture, staining for pro-collagen I was positive surrounding cells (*arrows*). **e** Vimentin staining is positive in native tendons, typical staining pattern surrounding cells. **f** After acellularization, vimentin staining is still positive but blurred

cells—are disrupted and fragments remain in the tissue, while the rest of the cells are extracted.

The technique of acellularization by using various detergents/solvents like SDS, Triton X-100, or TnBP has already been used, e.g., for the extraction of cells from dermal transplants, cardiac valves, esophagus tissue, or amniotic membranes [6, 13, 21, 29]. Recently, this technique has been introduced for the acellularization of patellar tendon [7] or native ACL [30] for use as ACL replacement. As a result, the patellar ligament could not be processed completely cell free, especially in the fibrocartilage zone at the tendon attachment site there were still intact cells. Therefore, in our experiments, we used semitendinosus tendons, which were totally acellular after described processing. In accordance with the results on patellar tendon and ACL, the mechanical properties of semiteninosus tendons were not influenced by the acellularization process [7, 8, 30]. Since in some studies decreased proliferation after SDS treatment has been noted [7, 15], in our study extreme care was given in prolonging the washing step within PBS. It is well known that remnants of SDS–because of inefficient washing out—could decrease proliferation [8].

Since cells were effectively removed, the scaffold could therefore be used for population with autologous cells. After directly injecting the cells into the tendon, cells survived in this construct as seen by new pro-collagen 1 production. In contrast to others, who only seeded cells on the outer surface and waited for cell migration [7, 15], we used this seeding technique to get the cells directly into the tendon. An obvious disadvantage when using our approach is the cell quantification afterward, since the distribution of the cells is not homogeneous. In areas near the injection canal, not only high cell density was observed, but also areas with low cell densities could be found. As cell source for autologous cells, we decided to use dermal fibroblasts. These cells are easy to obtain, require no complicated surgery, and help saving time and money. It has been shown that dermal fibroblasts survive in the harsh synovial environment of the knee and also have better proliferative capacity than ACL fibroblasts [3]. This was confirmed by van Eijk et al. [28].

In conclusion, we further characterized tissue-engineered constructs on the basis of SDS acellularized tendons. These constructs showed good biomechanical stability with complete removal of cells, preserved ECM, and in-growth of autologous fibroblasts. Further animal testing has to confirm these results. If the animal experiments are successful, tissue-engineered constructs could be generated using a simple dermal biopsy and acellularized tendons as scaffolds.

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