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Angaben zur Veröffentlichung / Publication details:

Sandmann, G. H., S. Eichhorn, Stephan Vogt, C. Adamczyk, S. Aryee, M. Hoberg, S. Milz, A. B. Imhoff, and T. Tischer. 2009. "Generation and characterization of a human acellular meniscus scaffold for tissue engineering." *Journal of Biomedical Materials Research Part A* 91A (2): 567–74. <https://doi.org/10.1002/jbm.a.32269>.



Generation and characterization of a human acellular meniscus scaffold for tissue engineering

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INTRODUCTION

The main function of the human meniscus is load distribution and therefore stress reduction in the knee joint, thereby preventing cartilage damage, as shown by various clinical¹ and experimental studies.^{2,3} In cases where meniscus repair is not possible, meniscus replacement could be warranted to restore normal anatomy in order to prevent early degenerative joint disease following meniscus resection. Nevertheless, there are still unsolved problems.⁴⁻⁶ Several biologic and synthetic materials, such as autologous tendons, submucosa, collagen matrices, or carbon-fiber prostheses were developed for meniscus transplantation,⁷⁻¹³ but only the collagen meniscus implant (CMI), made from bovine Achilles tendons,

is used clinically with varying success rates.¹⁴ Linke et al. demonstrated that the clinical outcome after implantation of a CMI combined with high-tibial osteotomy was not different compared with the control group with high-tibial osteotomy alone after 2 years.¹⁴ In another study, histological findings revealed only remnants of the original CMI, with mostly scar tissue instead of mature meniscus fibrocartilage tissue.¹⁵ To improve long-term stability and integration of the CMI, tissue engineering of the CMI with autologous cells was performed in an animal model. However, the biomechanical characteristics still remained insufficient, leading to premature destruction of the transplanted CMI.¹⁶

Apart from the collagen meniscus implantation, allograft meniscus transplantation is performed in specialized centers for patients with total meniscectomy.¹⁷ Over 20 years ago the first meniscus transplantation was carried out in Munich, Germany.¹⁸ Much progress has been made since then, but the main problems remain similar: slow immuno-reactions

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TABLE I
Protocol Used for the Final Acellularization Process

Day 1	Deionized water
Day 2–14	Sodium-dodecyl sulfate, SDS 2%
Day 15	Deionized water
Day 16	Ethanol 70%
Day 17	Phosphate buffered saline (PBS)

sometimes lead to progressive failure of the transplant.^{5,19–22} Nevertheless, Stone et al. found that allograft meniscus transplants can survive up to 7 years, even in knees with chondromalacia in the compartment of the meniscus transplantation, leading to improved patient satisfaction in terms of pain relief and physical activity.²³ The question of whether the meniscus allograft itself or the accompanying procedures (lavage and debridement of the knee joint) lead to these improvements could not be answered in their study. Further possible disadvantages of allografts are the transmission of infectious diseases (e.g. HIV, hepatitis, venereal diseases), slow graft remodeling, and incorporation into the host tissue.²²

New methods in tissue engineering, for example the acellularization²⁴ of tissues and seeding with autologous cells, have the potential to overcome these problems. To date, many organs and tissues—such as tendons, heart valves, nerves, esophagus etc.^{25–29}—have been acellularized using different methods. The underlying goal remains the same: reducing the antigenicity of tissues by removing the cellular components of the donor while preserving the extracellular matrix and therefore the original biomechanical strength. The aim of our study was to generate an acellular meniscus scaffold for tissue engineering by using a sodium dodecyl sulphate (SDS) based solution. This construct was then characterized biomechanically, histologically, and immunohistochemically.

MATERIALS AND METHODS

All samples were collected from the department of forensic medicine and excluded if there was any sign of degenerative knee disease, or other pathologies that could influence the outcome. All surrounding tissue was removed by sharp dissection. Specimens were stored until further use at -20°C . For complete acellularization of the human meniscus specimens by the detergent SDS, preliminary testing was conducted based on our previous experience with tendons.²⁹ Different testing protocols (SDS concentration of 1, 2, and 5%; incubation time of 7 or 14 days) were used and the degree of acellularization was evaluated histologically by hematoxylin and eosin staining. In pilot studies, six meniscus samples were evaluated to find the optimal SDS concentration and time period to completely acellularize the human meniscus tissue (SDS 2% for 2 weeks). The cell extraction process consists of a multistep procedure. All steps were performed at room temperature

under continuous shaking to enhance diffusion of the chemicals into the samples. At first, the samples were rinsed and placed in deionized water for 24 h. Next, the samples were placed in varying SDS-solutions (1, 2, or 5%) for different time periods, followed by deionized water (24 h), ethanol 70% (24 h), and phosphate buffered saline (PBS) treatment for 24 h to wash out all remnants of SDS. For the final experiments, five medial and five lateral meniscus samples were collected from human cadavers (mean age: 38 ± 6 years, mean weight 73.1 ± 7.4 kg; 3 males, 2 females). All meniscus samples revealed no degenerative changes and were randomized to one of two groups: meniscus samples which did not undergo acellularization or the acellularization group. Each group consisted of 3 medial/2 lateral menisci. The samples in the control/untreated group were put into PBS. Following this acellularization process (Table I), three cylinders were collected for biomechanical testing from each meniscus. The remaining meniscus tissue was fixed in methanol (90%) and prepared for histological investigation.

Biomechanical testing

Acellularized ($n = 5$, 3 cylinders each) or intact menisci ($n = 5$, 3 cylinders each) of medial and lateral menisci of five individuals were used for biomechanical testing. The undersurface of the menisci was oriented perpendicular to the testing device. Three cylinders with a diameter of 5.0 mm and a height of 4.0 mm were punched out of each meniscus. These cylinders were put into a custom-made device and the upper part of the cylinder was shaped to create a surface parallel to the base (Fig. 1). One sample at the time was then put into a custom-designed metallic plate with a circular cavity (diameter 5.0 mm and depth 4.0 mm) to prevent the samples from dislocating during biomechanical testing. The meniscus samples were then

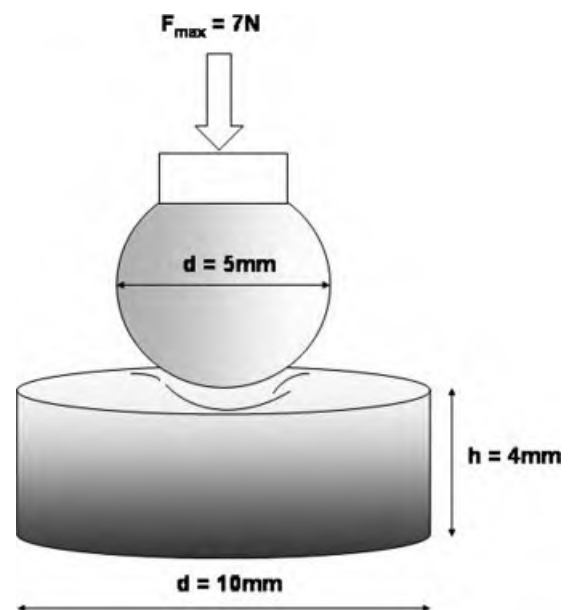


Figure 1. Schematic setup of the ball indentation trial as described in the text.

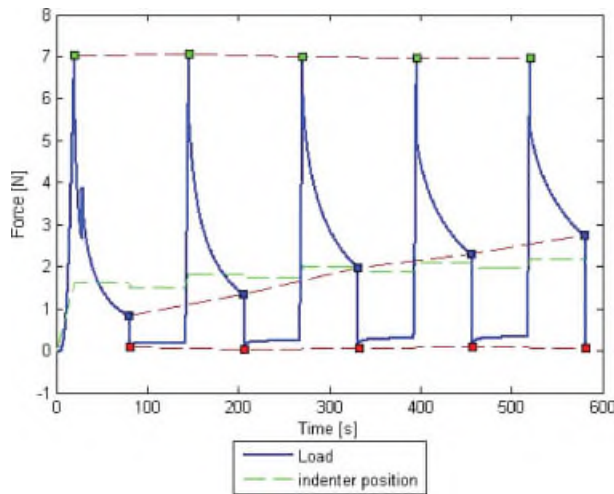


Figure 2. Load curve of a test cycle consisting of five repetitive cycles showing the graphical course of preload, dynamic and static compression and relaxation. Note the linear-elastic slope during dynamic compression. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

tested by a repetitive ball indentation test, as described previously.^{30,31}

The test was performed as a minimally constraint compression-relaxation test with a universal testing machine (Zwicki 1120; Zwick, Ulm, Germany) and a 5 mm steel ball at the tip of the indenter. The testing machine was used with a calibrated load sensor of a maximum of 20 N and an accuracy of 1% (KAP-S; A.S.T, Dresden, Germany). The meniscus samples were kept moist throughout the experiments using physiologic saline solution) and the indenter position was calibrated prior to each test (indenter position zero was at the level of the base of the cavity). A preload of 0.1 N was used and samples were checked intermittently during testing for displacement. The test cycle consisted of four phases: preloading of the sample with 0.1 N; dynamic compression with a constant load velocity of 5 mm/min until 7 N; static compression of the sample for 60 s with a load of 7 N; relaxation of the sample with a constant unload velocity of 1 mm/min until a load of 0.15 N. After an interval of 60 s, the new test cycle started until a total number of five test cycles were reached (Fig. 2). Load, indenter position, and time were displayed by the test software TestXpert (Version 8.1;

Zwick, Ulm, Germany) and three values could be calculated: (1) *Stiffness* determined from the linear elastic slope of the loading curve between 2 and 5 N. High stiffness values indicate high elasticity and vice versa. (2) *Relative sample compression* (indenter position in relation to absolute sample height) at the end of the dynamic compression phase. 'Compression' is an indicator for viscosity and characterizes the ability of a sample to evade the indenter. (3) *Residual force* (load measured at the end of the static compression phase). The 'residual force' is influenced by the ability of tissue to evade the indenter in unconstrained compression (viscosity) as well as by the reset forces present in the tested tissue (elasticity). High residual forces thereby indicate more elastic than viscous properties. The experiments were performed in cyclic loading to simulate physiologic stress. All data were exported from TestXpert to Excel and statistical analysis was performed between groups using the Student's *t*-test.

Histology

For pilot testing, three menisci were obtained, cut in two halves and chemically processed using the different SDS concentrations and time frame as described above. For final histological examination sections of six acellularized menisci (3 medial and 3 lateral) were used, and sections from three intact menisci served as untreated control group. After fixation in 90% methanol in 4°C for 48 h, specimens were infiltrated overnight in PBS with 5% sucrose at pH 7.4 and afterwards mounted on chucks in Jung tissue embedding medium (Leica, Germany), frozen in a HM 500 OMV cryostat (Mikrom, Germany) and cryosectioned at 12 µm. Sections were stained with hematoxylin and eosin. Three transverse sections (one in the anterior horn, one in the middle part and one in the posterior horn) of each specimen were examined for remaining cell nuclei and histological changes after acellularization. Phase-contrast microscopy was performed to observe any changes in collagen bundle orientation. Additionally, the collagen staining pattern was evaluated immunohistochemically using antibodies against collagen I, II, and VI (Table II). The immunohistochemical labeling procedure has been described by Tischer et al.³² In summary, all sections were treated with 0.3% hydrogen peroxide in methanol for 30 min to block endogenous peroxidase activity and non-specific binding of the primary antibodies was reduced by blocking with normal horse serum for 60 min. Control sec-

TABLE II
Sources and Labeling Characteristics of the Monoclonal Antibodies Used

Antigen recognized	Antibody	Dilution	Enzyme	Source	Staining	
					Native	Acellular
Col I	Col1	1:2000	Ch ABC	Sigma	++	++
Col II	CICC	1:6	Ch ABC	DSHB	+	+
Col VI	5C6	1:10	Ch ABC	DSHB	++	++

Note that collagen antibodies required an enzyme pre-treatment with 0.25 Units/mL of hyaluronidase/chondroitinase (Ch) ABC (Sigma). Immunohistochemical labelling results for *intact* and acellular menisci.

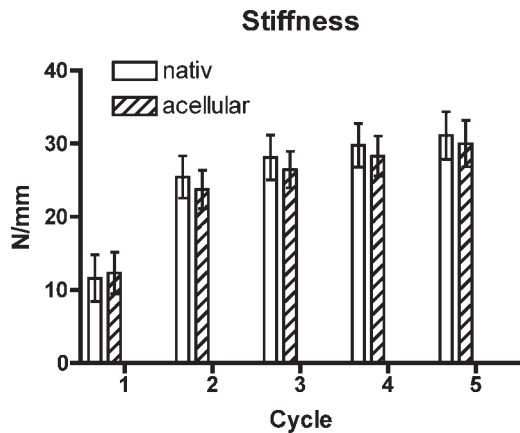


Figure 3. Stiffness of the acellular meniscus graft versus control during load cycles. No statistical significance was found.

tions 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, Burlingame, CA) and sections were counterstained with Mayer's hematoxylin. Staining intensity was graded semiquantitative (no staining (-), weak staining (+), and strong staining (++)).

RESULTS

Biomechanical testing

The mean sample height was 3.9 mm (± 0.2 mm) and all samples could be loaded up to 7 N without signs of plastic deformity. With the ball indentation test we could see no statistically significant differences between the intact and the acellularized menisci in terms of stiffness (N/mm), compression force (N), and residual force (N). Stiffness showed no significant difference during cycle one between intact

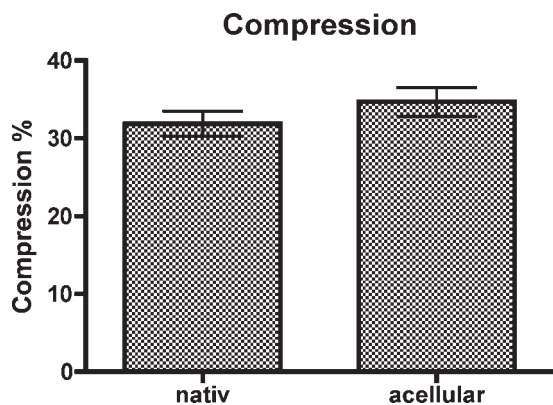


Figure 4. Percentual change of compression intact versus acellular during cycle 5. No statistical difference was found.

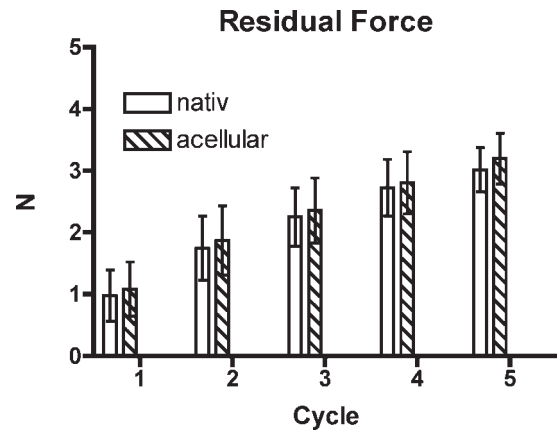


Figure 5. Residual force of the acellular meniscus graft versus control dependent on load cycles. No statistical significance was found.

(mean 11.6 ± 3.2 N/mm) and acellular scaffolds (mean 12.3 ± 2.9 N/mm). Stiffness increased significantly throughout testing by 162% (stiffness of intact meniscus in cycle five: 31.1 ± 3.3 N/mm) and 143% (stiffness of acellular meniscus in cycle 5: 30 ± 3.2 N/mm), respectively ($p < 0.05$). During each testing cycle the differences between the two groups—intact meniscus and acellular scaffold—were not statistically significant (Fig. 3, $p > 0.05$). Mean compression of sample height was 32% ($\pm 7.2\%$) for intact meniscus samples after the fifth cycle. Mean compression of the scaffolds was found to be 35% ($\pm 8.3\%$). Scaffold compression exceeded compression of intact meniscus by 9%, being not statistically significant ($p > 0.05$, Fig. 4). The residual force of the two groups increased after each cycle, but no statistically significant difference could be noticed. Mean initial (after cycle one) residual force for intact menisci and acellularized samples was 1.0 N (± 0.41) versus 1.1 N (± 0.4 N) and 3.0 (± 0.36) versus 3.2 (± 0.41) after five cycles ($p > 0.05$, Fig. 5). Biomechanical results were not significantly different comparing processed medial or processed lateral meniscus samples with the control group.

Histology/immunohistochemistry

Results of the pilot testing showed that complete cell removal could be achieved using SDS 2% for 2 weeks and SDS 5% for 2 weeks as seen by hematoxylin and eosin staining. In contrast, when using a lower SDS concentration (1%), samples still showed nuclei [Fig. 6(c)], but in lower numbers when compared with intact menisci [Fig. 6(a)]. For the following testing procedure SDS 2% for 2 weeks was utilized for acellularization. After treatment with SDS 2% for 2 weeks all samples (both medial and lateral) were identified as acellular by hematoxylin and

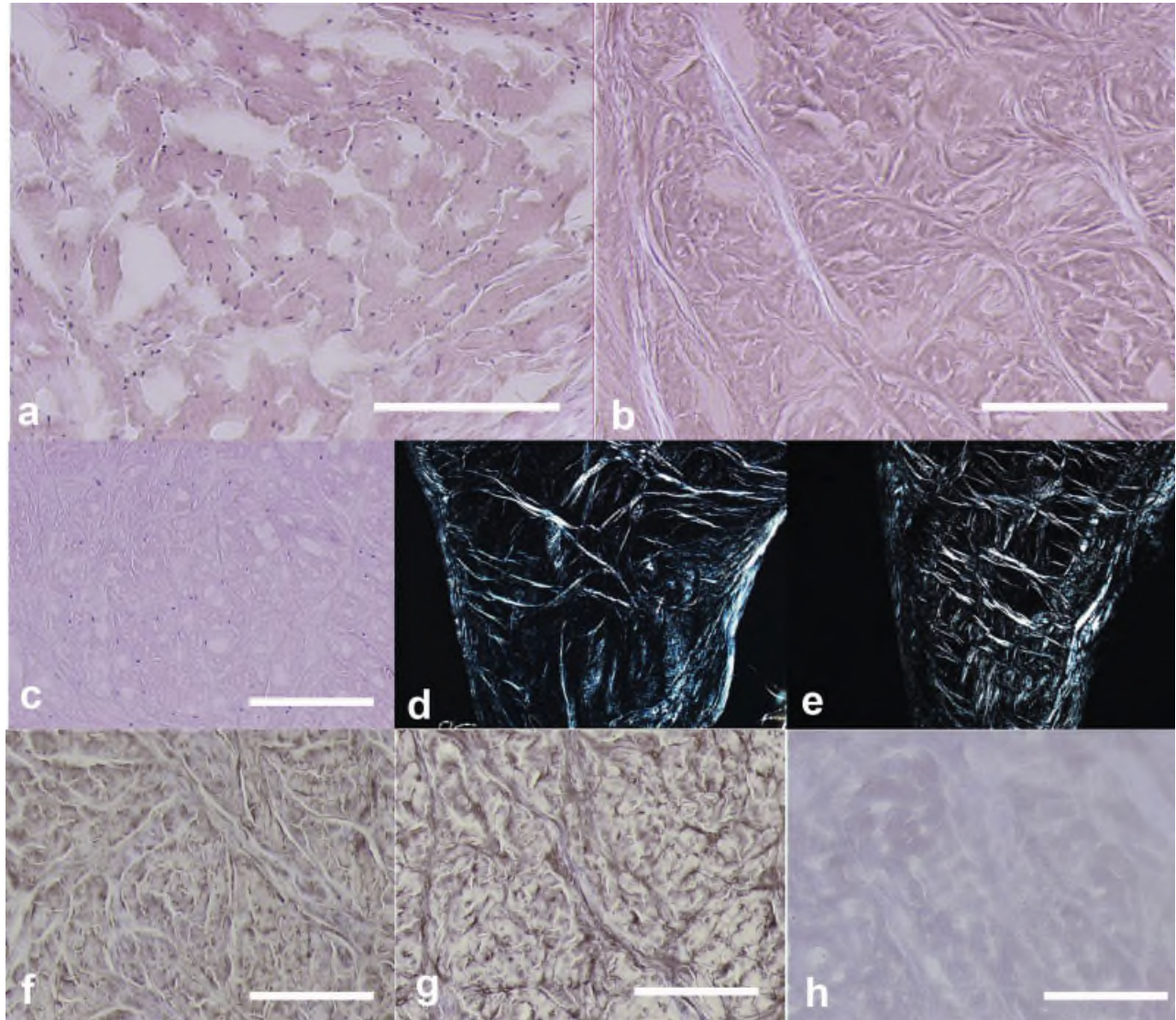


Figure 6. (a) HE staining of intact meniscus (scale bar = 200 μ m) and (b) acellular meniscus (SDS 2% for 2 weeks) showing no discernible nuclei (scale bar = 200 μ m), whereas (c) acellularized specimens with SDS 1% for 24 h shows remaining nuclei in decreased frequency (bar = 200 μ m). No differences in phase contrast microscopy between acellular (d) and (e) intact menisci could be detected. (f) collagen 1 labeling of acellular meniscus (g) labeling for collagen VI (h) control section, here the primary antibody was omitted. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

eosin staining [Fig. 6(b)]. Phase-contrast examinations revealed regular collagen bundle arrangement in the acellular specimens as seen in intact menisci [Fig. 6(d,e)]. Immunohistochemically, no differences in the labeling patterns for collagen I, II, and VI [Fig. 6(f)] were observed when compared with intact menisci. Whereas for collagen I there was strong labeling in the whole meniscus, collagen II was labeled only in the fibrocartilaginous section of both groups. Collagen VI staining was evenly distributed throughout the meniscus tissue, both in acellularized and intact menisci. The immunohistochemical results are summarized in Table II.

DISCUSSION

Over the last decade multiple strategies have been developed to successfully replace damaged meniscus tissue. Tissue Engineering using synthetic constructs like CMI or other materials combined with autologous cell transfer or gene therapy might be one solution. Another approach is the acellularization of allograft tissue to decrease immunogenic reactions by removing all cellular components²⁰ and thereby improve long-term survival and clinical results. Different processing methods can be used for the acellularization of allografts, which all aim to preserve

the extracellular matrix thereby preserving the biomechanical characteristics of the allograft. Additionally, an intact extracellular matrix should provide autologous cells an optimal environment for repopulation. This has already been demonstrated by new synthesis of procollagen I—the precursor to collagen I—in fibroblasts seeded within acellularized tendons.²⁹ Long-term stability should be improved by active remodeling of the graft and ingrowth into the host tissue.

Several nonchemical methods like repetitive freezing/thawing²⁴ and high pressure treatment^{30,33} have been used so far to acellularize allografts. Naal et al.³³ could demonstrate that the high pressure treatment of meniscal cartilage with a pressure up to 600 MPa for 10 min did not negatively affect the biochemical properties. Furthermore, no differences in immunohistochemical staining pattern of the collagen structure (collagens I, II, III) or the proteoglycan composition (versican, aggrecan, link protein) could be noted in comparison to untreated samples. Nevertheless, one disadvantage of repetitive freezing/thawing²² and high-pressure treatment³³ is that it leaves the cell detritus in place, thereby possibly inducing potential immunogenic reactions. To overcome this limitation, different chemical processing methods have been developed. The single use of trypsin has been successfully used to acellularize ovine heart valves, because it cannot digest intact collagen bundles.²¹ Because meniscus tissue is composed mainly of fibrocartilage and collagen with a tightly packed ECM, it is much more difficult to acellularize, and trypsin alone is insufficient. Recently, ovine meniscus was acellularized using a multistep enzymatic process utilizing trypsin, collagenase, and protease.³¹ By using this method, acellular menisci could be generated for the first time. One disadvantage of this procedure is the disruption and partial digestion of the extracellular matrix by these enzymes. The glycosaminoglycans (GAGs), with their crucial role in the regulation of the water content within the meniscus, were partially removed. Biomechanical properties were negatively influenced, as was shown using the same biomechanical test utilized in a previous study.³¹ Different detergent or solvent based solutions may be able to acellularize a meniscus. SDS and other detergents or solvents like Triton-X or Tri-(*n*-butyl) phosphate remove all cells or cellular detritus.³⁴ This has been demonstrated in the anterior cruciate ligament, nerve grafts, and heart valves, but not yet for meniscus tissue.^{25–27,29} Cartmell et al. also noted difficulties with the acellularization of fibrocartilage present at the enthesis of the patella tendon.³⁵ In his observations SDS was more successfully in removing cells than Triton-X or tri-(*n*-butyl) phosphate. With adaptation of the protocol used by Cartmell et al.,³⁶ which

uses higher concentrations of SDS together with a longer incubation time, the acellularization of human meniscus samples was successful in our study. To test the limits of SDS, acellularization (5% SDS for 2 weeks) of hyaline cartilage (with its dense extracellular matrix was also tried) but this process was not successful (data not shown). All three substances were biocompatible and also supported cellular growth to a different degree.³⁵ In comparison to Triton-X and tri-(*n*-butyl) phosphate, SDS was most effective in cell extraction,³⁴ but least supportive of cellular regrowth in the acellularized constructs.^{34,35} Gratzer et al.³⁷ have recently reported negative effects on cellular repopularization of grafts after acellularization with SDS,³ but the relevance of this has yet to be confirmed *in vivo*.

As our results show, the biomechanical properties of the menisci were not adversely affected by this acellularization protocol (Table I). This was tested by measurement of stiffness, compression force and residual force with an indentation method previously published.^{28,29} With the ball indentation experiment we were able to gain more information about the biomechanical properties stiffness, compression and residual force—all important parameters of viscoelasticity (see materials section). The treatment of the meniscus samples affected the biomechanical properties and lead to increased stiffness (143 vs. 162% in the control group), an increase of compression (3%) and residual force (7%) (Figs. 3–5). Increase of stiffness during cyclic loading is probably caused by tissue compression and can be seen in both intact meniscus samples and acellularized meniscus samples. The fact that the increase in acellular meniscus samples is lower than in the control group, shows that the treatment with SDS has no adverse affects. The water content of the chemically processed meniscus samples was not adversely affected, which might be due to the fact that SDS does not digest the GAGs to the same extent as a recently described enzymatic scaffold processing.^{31,35} GAGs have a great effect on water content and their loss might lead to altered load distribution, and in a recent study by Cartmell it has been shown, that the GAG content of tendons was not altered by SDS treatment.³⁵ The residual force and compression force were slightly higher in the acellular meniscus group, demonstrating that elasticity and viscosity were notably higher in the processed group. Though differences are not statistically significant, our results show that the main biomechanical properties are not adversely affected by treatment with SDS which seems to leave the extracellular matrix intact.

Histologically, the degree of acellularization increases with the level of SDS concentration and the incubation time. The time necessary for complete acellularization is dependent on the size of the speci-

men and of the type of tissue being acellularized. This study is the first to test human meniscus tissue as opposed to the generally tested animal specimens (rabbit, rat, pig, dog).^{24,31} Human menisci naturally are much bigger, thus more difficult to penetrate with active solutions. Destructive enzymes such as collagenase may be more likely to digest the outer menisci before the inner parts are sufficiently penetrated and acellularized.

CONCLUSION

In our study, human meniscus samples were successfully acellularized using SDS without negatively affecting the main biomechanical properties. These cell-free constructs could serve as excellent scaffolds with a preserved extracellular matrix maintaining the natural biomechanical properties. Future research is necessary to evaluate the *in vivo* consequences of SDS acellularization.

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