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Tissue engineering of the anterior cruciate ligament and meniscus using acellularized scaffolds

Gunther H. Sandmann and Thomas Tischer
 Department of Orthopaedic and Trauma Surgery
 Technische Universität München
 Germany

Abstract

Injuries to the anterior cruciate ligament (ACL) and the meniscus are very common. Both the ACL and the meniscus play a crucial role in the complex biomechanics of the knee and operative treatment is often necessary. For example, in the US approximately 200,000 ACL reconstructions are performed annually. Usually, autogenous tendons like the mid- third of the patellar tendon or the hamstring tendons (semitendinosus/gracilis) are used. Nevertheless, the availability of autogenous tendons for ACL reconstruction is limited as well as the possibilities of meniscus repair. Ligament and meniscus allografts often show problems regarding long-term stability, immunological reactions and possible transmission of infectious diseases, whereas synthetic materials can cause foreign body reactions and often lack good initial biomechanical stability or fail to maintain long-term stability.

The idea of acellularizing ligaments and meniscus cartilage has arisen to remove the immunogenic cells to reduce the adverse immunological reactions. By preserving the extracellular matrix (e. g. collagen, glycosaminoglycans, proteoglycans), the main biomechanical properties are preserved and the so obtained scaffolds provide a natural environment for the ingrowing cells. The acellular scaffolds can be used as a scaffold for *in vivo* repopulation or can be seeded with autologous cells *in vitro* before implantation using different cell sources. By this, further improvement of the biochemical and biomechanical properties as well as the remodelling of the graft could be improved.

In the meantime, a variety of different methods has been described to acellularize tissues reaching from repetitive freezing/ thawing over the use of hydrostatic high pressure to different chemical methods. Only by chemical means are the cells actually removed. In terms of chemical acellularization, SDS (sodium dodecyl sulphate), Triton-X or TnbP (tri-nitro-butyl-phosphate) are the most popular substances, with SDS being the strongest cell removal agent for tendon and meniscus tissue. As *in vitro* tests have shown, tissue processing using SDS does not influence the biomechanical properties, while all cells - being responsible for the immunological effects - are removed. However, more research using *in vivo* animal experiments has to be performed before application in humans is possible.

This tissue engineering strategy might be suitable to satisfy the increasing demand for tissue engineered tissue coming along with the increased patients' demands in the future.

1. Introduction

Injuries to the ACL and the meniscus are very common and negatively influence the complex biomechanics of the knee joint. Due to the poor vascularity, both structures show limited healing capacity and surgical repair is often necessary (Arnozcky 1983, Arnozcky 1983). In the US approximately 200,000 ACL reconstructions are performed per year and the number is still growing. The golden standard is the reconstruction of the injured ACL by using autogenous tendons like the hamstring tendons (semitendinosus/gracilis) or the middle- third of the patellar or quadriceps tendon. These methods have been used for several years now and show reliable results in the long- term. However, the problem of development of osteoarthritis in the long term is not solved yet (Lohmander 2007, Messmer 1999). So far no study exists proving a chondro-protective effect after ACL reconstruction. The reported outcome 32 years after ACL replacement is unacceptable in about 50% of cases. In accompanying meniscus lesions the outcome is even worse (Taylor 2009). Whether this is mainly due to cartilage injury at the time of accident or due to altered joint biomechanics is not known yet. So far, there exists no clear consensus, which patients benefit from ACL replacement in the long run. New techniques of ACL reconstruction like the anatomic double-bundle reconstruction (anteromedial and posterolateral bundle) and new methods of fixation might improve the outcome in the future (Kondo 2008, Zantop 2008, Brucker 2006).

Though the ACL reconstruction using autogenous tendons is currently the gold standard, some problems still remain. There is on the one hand the problem of donor site morbidity with persisting anterior knee pain and the inability to kneel, especially after the use of patellar tendon transplants (Kartus 2001). Muscle strength deficits have been found after ACL reconstruction with patellar and hamstring grafts (Aune et al. 2001; Beard et al. 2001), especially the harvesting of hamstring grafts has been found to cause muscular weakness at high knee flexion angles (Makihara et al., 2006; Nakamura et al., 2002; Tashiro et al., 2003). Furthermore the availability of autogenous tendons is limited. In multi-ligamentous knee injuries the use of allografts is often required. Different allograft ligaments may be used: bone-patella-tendon-bone, hamstring, achilles or tibialis anterior/posterior tendons. However, no differences between the used allograft tissue have been reported, whereas significant differences exist, which sterilization processes were used (Carey et al. 2009; Krych et al. 2007). In a recent systematic review comparing ACL replacement using autografts with allografts, similar medium term results were reported in non-randomized studies (Carey et al., 2009). Still, immunogenic reactions may cause degenerative alterations and the potential risk of disease transmission remains. Using standard sterilization procedures like lyophilisation (Crawford et. al, 2004) or gamma irradiation (Fideler et al. 1995; Gibbons et al. 1991) retroviruses (i.e. HIV) can hardly be destroyed.

Apart from the ligaments, the meniscus plays a crucial role in the complex biomechanics of the knee, as it works as a shock absorber and transforms joint pressure in circular meniscus tension. This is mediated by the complex structure (mainly its circular collagen network) of the meniscus. Unfortunately its healing capacities are very poor. As soon as a radial tear destroys the circular collagen network or the horns are ruptured, the meniscus loses all biomechanical function. This leads to joint overload and might end in an overuse of the

cartilage and an early onset of osteoarthritis (Lohmander et al. 2007; Allaire et al. 2008). Many improvements have been made to reconstruct the injured meniscus, but due to its biomechanical and anatomical specialities this is still impossible in many cases.

The transplantation of meniscus allografts is a viable option but has its own problems as well (Packer et al. 2009). On the one hand there is the problem that there are too little donor organs as the meniscus of elderly people has usually degenerative alterations and can thereby not be used. Apart from the lack of organs it is difficult to get a suitable transplant fitting in size. In a recent work Van Thiel et al. 2009 could show that the fitting size of the meniscus is mainly correlated with height, weight and gender of the donor and recipient. Apart from that, the fixation is technically demanding. Because of this, the transplantation of meniscus is reserved to specialized centers. Unfortunately, long-term results still show problems due to immunogenic and degenerative alterations. Verdonk et al. (2006) examined the long-term results of meniscus transplantation clinically and radiologically and they could show, that the meniscus transplantation lead to an improvement of function and relief of pain. Nevertheless, substantial disability and knee pain were present at the follow up after 10 years. On MRI the meniscus transplants had signs of graft extrusion and an increased signal intensity indicating degeneration. The failure rate was 18% with conversion to a total arthroplasty (Verdonk 2006). Wirth et al. (2002) found similar results but could show, that deep frozen meniscal grafts were superior compared to lyophilized meniscal grafts at a follow up of 3 and 14 years. The latter even showed similar results to the control group after meniscectomy. If those problems could be solved in the future, the meniscus transplantation might gain much popularity over time.

As an alternative to allografts the use of synthetic tendon constructs (e.g. silk, Gore Tex, LAD) or meniscus prostheses (CMI®, Actifit® etc.) have emerged (Martinek 2006; van Tienen et al. 2009; Zaffagnini et al., 2008). Most of the prostheses used for ACL reconstruction have failed as they showed an adverse effect on the cartilage and caused in some cases a marked synovialitis. Though their biomechanical Properties were excellent initially, most of the prostheses had the problem of degradation, tunnel widening and high failure rates after several years (Muren et al., 2005; Roolker et al., 2000). Compared to the biomechanical properties of ligaments, meniscus tissue is even more complex and not surprising, the middle to long term results of the artificial meniscus constructs show different results from one study to the other, but in general do not reach the anatomic, biomechanical results (Buma et al., 2007). So far, artificial constructs (neither ACL nor meniscus) do not reach the biomechanical properties of autologous tissue and are therefore not an ideal long-term alternative.

To improve the long-term outcome of ligament and meniscus allografts, new strategies are needed. Recently the use of extracellular matrix (ECM) scaffolds by means of acellularization of allograft tissue has become popular (Badylak et al., 2007, Badylak et al., 2009). In the following we will depict the different means of acellularization, describe our used method and give future prospects in the field of tissue engineering concerning ligament and meniscus tissue.

2. Acellularization methods

Recently, biologic scaffold materials composed of extracellular matrix are widely used in surgical procedures for the reconstruction of numerous tissues and organs and their use in

orthopaedic research surgery has emerged over the last years. As scaffolds allograft or possibly xenograft tendons/meniscus are used, which are acellularized by different methods.

The basic requirements for optimal acellularization methods are:

1. removal of all cellular components as the cells (especially fibroblasts) and macrophages seem to play a crucial role in the cell-mediated immunogenic reactions, i.e. host versus graft reaction (Badylak 2008).
2. preserving the extracellular matrix which is responsible for biomechanical stability and preserving chemoattractants and providing cell attachment sites for ingrowing cells (Reing 2009).

The ECM has chemotactic factors that attract cells (Beattie et al., 2009) and supportive function for the diffusion of nutrients from the blood to the cells. Most important, the biomechanical properties are mediated by the extracellular matrix. For example, the tensile strength of tendons is mediated by its collagen I fibrils, whereas the hyaline cartilage properties are maintained by the interplay of a collagen II network with large proteoglycans (aggrecan) and negatively charged glycosaminoglycans (Tischer et al., 2002). Finally, the process of repopulation is dependent on an intact matrix and the used method should not be cytotoxic to allow cellular ingrowth. The repopulation can then happen *in vitro* before implantation or *in vivo*.

Therefore different methods have been described for the acellularization of tissues and every method has its own advantages and disadvantages. Generally, two methods can be distinguished: first methods which destroy all cells, but leave cellular components and debris and second methods which remove all the cellular components from the tissue by chemical means. In the following section, the most common methods are now discussed in more detail.

2.1. The Gamma irradiation

The gamma irradiation is widely used and one of the methods with the longest experience. It is very effective in destroying cells and most of the pathogenic germs. Still the gamma irradiation faces a dilemma, as doses of 3.5 Mrad (35kGy) are needed to destroy the DNA of the HIV. However, using this amount of energy weakens the transplant, leading to loss of biomechanical stability, making it unsuitable for implantation (Fideler BM et al., 1995). In most cases irradiation doses of 1 to 2.5 Mrad (10- 25kGy) are used with a slight remaining risk of disease transmission (Buck et al., 1989). In a study by Curran it could be shown that irradiation of allografts with a dose of 20kGy lead to minor biomechanical properties compared to the untreated control group. Elongation was more than 27% higher and the load to failure was diminished as well (Curran et al., 2009). In a prospective randomized trial by Sun (Sun et al., 2009) the clinical outcome after ACL replacement with autograft patellar-tendon-bone and irradiated as well as non-irradiated allograft (all used allografts had been frozen) was examined. Whereas the non-irradiated allografts showed similar results to the autografts, the clinical outcome of the irradiated allografts was inferior in the clinical scores and the subjective instability was much more common in this group of patients. Taking that into account the irradiation can no longer be recommended as a

sterilization procedure as it weakens the tissue and thereby negatively influences the biomechanical properties without reliably destroying retroviruses like HIV.

2.2. Lyophilization/cryopreservation

Lyophilization means the preparation of tissue by repetitive freezing and thawing. This leads to the destruction of cells, but as with irradiation the cellular components mainly remain within the extracellular matrix. The lyophilization process has been widely used and has been proven as a simple and cheap method and is therefore most commonly used (Sass et al., 2009; Busam et al., 2007; Lewis et al., 2008). Much work has been performed on the effects of cryopreservation on tendon allografts, but the potential adverse effects on mechanical properties and histological changes by ice crystal formation (Mahirogullari 2007). In a work by Park 2008 the effects of cryopreservation or heating on the mechanical properties and histomorphology of rat bone-patellar tendon-bones (BTBs) were investigated. BTBs were processed by cryopreservation at -80°C for 3 weeks, or heating at $+80^{\circ}\text{C}$ for 10 min. Tensile testing and histomorphological examinations were performed. The cryopreservation of tendons showed less influences on their mechanical properties. When cryopreserved BTBs were fixed in frozen state by the freeze-substitution method, many widened interfibrillar spaces were observed. These results suggest that the collagen fibres of cryopreserved tendons were histomorphologically affected by ice crystals. The cryopreservation does not only affect the cellular components, but also the collagen matrix, which might have effects on the biomechanical properties as well.

However, a new work by Gelber et al. (2009) showed that the ultra structure of the meniscus is not affected by cryopreservation. Therefore, an allograft stored in that way would not alter its biomechanical properties, although its cellular viability is highly unpredictable. As the immunogenicity is cell-mediated, this imponderability makes the cryopreservation not the method of choice for tissue engineering applications, especially as the fibril diameters in frozen menisci show a thinner diameter and had a higher degree of disarray. These alterations of the collagen network can partially explain the pathological changes found in shrunken menisci after transplantation (Gelber et al., 2008).

2.3. High pressure treatment

The treatment of tissue with hydrostatic high pressure has emerged over the last years. By this method tissue is exposed to hydrostatic high pressure of up to 600MPa for 10 minutes. Diehl et al. (2006) could show that this treatment had no adverse effect on the biomechanical properties of the treated tissue. The cells were destroyed by this treatment and similar to the lyophilization the cellular components remain in the extracellular matrix and might thereby still be immunogenic. Apart from the use in tissues, high hydrostatic pressure (HHP) is widely used in the food processing industry, for example to inactivate vegetative microorganisms in meat products, milk and juice, thereby avoiding the addition of any chemical preservatives. Besides this HHP is also an attractive novel approach to effectively kill tumor cells in bone, cartilage or tendon *ex vivo* while leaving the tissues' mechanical properties unimpaired, thus allowing possible reimplantation of the resected tissue explants (Diehl et al., 2006, Diehl et al., 2008). However, human studies on this topic are still missing.

2.4. Enzymatic treatment

Acellularization using enzymatic treatment uses various enzymes like trypsin, collagenase and others to open up the ECM and remove cellular components. For example Maier et al. described an enzymatic way of acellularization of ovine meniscus tissue (Maier et al. 2007). Forty-one menisci were used to establish their own protocol. They used distilled water, trypsin and collagenase A to acellularize meniscus samples and could show that the biomechanical and the biochemical properties were left almost unaffected. In a recently published work by Vavken et al. 2009, they could demonstrate the effectiveness of enzymatic acellularization of the ACL. Therefore, they used a combination of Trypsin, EDTA and PBS and could show that the samples could be acellularized by this enzymatic treatment. The glycosaminoglycan content was negatively affected as well.

Taking everything into account, we believe that enzymatic treatment and especially the combination of collagenase and trypsin has adverse effects on the extracellular matrix and the collagen network. These enzymes can actually destroy the ECM and this could cause problems in reseeding the acellularized samples or even the biomechanical results. Future research would be needed to prove the safety of these methods.

2.5. Chemical acellularisation by solvents/detergents

A newer promising approach is the extraction of cells by chemical solvents/detergents. The most widely used chemicals are sodium dodecyl sulphate (SDS, an ionic detergent), Triton-X (a non-ionic detergent) and tri-nitro-butyl phosphate (TnBP). They have been described to acellularize tissue and successful application was performed on small intestine (Badylak et al., 1995), the urinary bladder (Chen et al., 1999), the liver (Brown et al., 2006), arterial vasculature (Wallis et al., 2003), dermis (Armour et al., 2006), heart valves (Cebotari et al., 2006) and oesophagus transplants (Bhrany et al. 2006; Gilbert et al., 2006) among others. In orthopaedic surgery there have been experiments using Graft jacket® in irreparable rotator cuff defects for tendon augmentation (Barber et al., 2006; Coons et al., 2006). Acellularization of tendons (Tischer et al., 2007; Gilbert et al., 2006) and meniscal samples (Sandmann et al. 2008; Stapleton et al., 2008) has caused further interest in this technique.

Bolland et al. aimed to produce a natural, acellular matrix from porcine bladder tissue for use as a scaffold in developing a tissue-engineered bladder replacement. They used full-thickness, intact porcine bladders, which were acellularised by distention and immersion in hypotonic buffer containing 0.1% (w/v) SDS and nuclease enzymes. Histological analysis of the resultant matrices showed that they were completely acellular whereas the major structural proteins had been retained. Intracellularly, some poorly soluble proteins remained. The amount of DNA per mg dry weight of fresh porcine bladder was 2.8 (+/-0.1) µg/mg compared to 0.1 (+/-0.1) µg/mg in acellularised bladder. In biomechanical testing the tensile testing indicated that acellularisation did not significantly compromise the tensile strength of the tissue. Cytotoxicity assays using porcine smooth muscle cell cultures excluded the presence of soluble toxins in the biomaterial (Bolland et al., 2007). A newer work by Gilbert et al. has shown that the presence of remaining intracellular DNA might not have as adverse effects as at first suspected. They examined the DNA content of commercially available ECM scaffold materials and compared their DNA content with scaffolds prepared in the laboratory using different acellularisation protocols. Most of the examined scaffolds still contained some amounts of DNA (4 out of 6 from the commercially available scaffolds and 2 out of 3 from the produced ECM scaffolds). Interestingly, scaffolds

which retained higher amounts of DNA did not show worse results than the scaffolds with less DNA (Gilbert et al., 2008). In a work by Brown et al., it could be shown in an animal model that the presence of cellular components within the extracellular matrix scaffold modulates the phenotype of the macrophages participating in the host response following implantation. Scaffolds containing cellular components (even autologous cellular components), elicited a predominantly M1 type macrophage response, resulting in a more dense connective tissue and/or scarring. Acellular scaffolds, however, were observed to elicit a predominantly M2 type macrophage response, which results in a more constructive type of remodelling response (Brown et al., 2009).

In the same animal model (abdominal wall defect in rats covered with several ECM scaffolds, i.e. porcine small intestinal submucosa (SIS), noncrosslinked SIS, and autologous body wall) the effect of macrophages that derive from peripheral blood in the degradation of ECM scaffolds was examined and Valentine et al. could show, that peripheral blood monocytes are required for the early and rapid degradation of both SIS scaffolds and autologous body wall, and that carbodiimide crosslinked SIS's are resistant to macrophage-mediated degradation (Valentin et al., 2009). Apart from the macrophages the T lymphocytes play a crucial role in organ rejection, especially in cell-mediated immune responses to xenografts (Strom et al., 1996). Th1 lymphocytes produce cytokines such as interleukin (IL)-2, interferon (IFN)- γ , and tumor necrosis factor (TNF)- α , leading to macrophage activation, stimulation of complement and differentiation of CD8⁺ cells to a cytotoxic phenotype, which leads to transplant rejection (Chen et al., 1996). In contrast, Th2 lymphocytes produce IL-4, IL-5, IL-6, and IL-10, cytokines that do not activate macrophages. Resulting from this, the activation of the Th2 pathway is associated with transplant acceptance (Chen et al., 1995). The only ECM scaffold material for which the Th1/Th2 response has been characterized so far is small intestine ECM (Allman et al., 2001). Allman implanted SIS-ECM subcutaneously in mice and could show that the expression of IL-4 (Th2) was increased, while the expression of IFN- γ (Th1) was markedly decreased by 100-fold compared to the response elicited by the xenogeneic muscle group. Although there was an immune response to the SIS-ECM after implantation, this response was dominated by the Th2 pathway mediators, lacking an acute rejection.

Since similar studies have not been conducted for other forms of ECM scaffold materials, it is not possible to determine whether they would elicit the same type of host response. Thinking of the diversity of different tissues and scaffolds, it seems likely that the host response to biologic scaffolds will vary to a large degree following implantation. Further research is needed to understand the immunogenic reactions to the implanted scaffolds and the effects of acellularisation on the repopulation of these scaffolds. One step in this direction are two recent publications by Beattie et al. and Reing et al. who could show that the acellularisation process and the produced degradation products are important modulators for the recruitment and proliferation of appropriate cell types during the remodelling process (Beattie et al., 2009; Reing et al., 2009).

The first study using acellularized tissue in orthopaedic research was performed by Cartmell et al. (Cartmell et al., 2000). Therein, they investigated the effectiveness of treatment of rat tail tendons with SDS, Tri (n-butyl) phosphate and Triton-X and found that both the treatment with SDS (1% for 24h) or TnBP (1% for 48h) resulted in an acellular matrix with retention of almost normal histological structure. The biomechanical properties were similar to native tendons. In contrast, Triton-X (1% for 24h) disrupted the collagen fibers and

was not as effective in acellularization as the two other methods, causing a disruption of the normal D-period of reconstituted rat tail tendon collagen fibers and an approximate 50% decrease in wet tensile strength and stiffness values. Following this, in 2004 Cartmell and Dunn could show that bone-patella-tendon-bone ligaments from rabbits could be successfully acellularized and reseeded with fibroblasts. Fibroblast proliferation rate was thereby slightly retarded on SDS- treated patellar tendons, whereas the repopulation in the TnBP- treated group was successful. Ingram et al. examined the additional effects of ultrasound on the repopulation process of natural tendon scaffolds acellularized with 0.1% SDS and found that repopulation with tenocytes was improved (Ingram et al., 2007). The work of Woods et al. compared the effectiveness of three extraction protocols in the development of an acellularized porcine bone-anterior cruciate ligament- bone graft (Woods et al. 2005). Therefore, they combined Triton-X (1% for 24 hours) either with SDS (1% for 24 hours) or TnBP (1% for 24 hours). The combination of Triton-X with SDS was most effective at removing cell nuclei and intracellular protein (vimentin) from the ACL but affected both the collagen and glycosaminoglycan (GAG) components of the extracellular matrix while increasing the tensile stiffness of the ligament. In contrast, the combination of Triton-X with TnBP was least effective of the three treatments in terms of cellular extraction, anyway its use did not alter the mechanical (stiffness and failure load) and biochemical (collagen and GAG content) properties of the ACL compared to the control group significantly. Triton-TnBP matched the level of decellularization achieved by Triton-SDS in terms of visible cell nuclei. It had lower effects on the extraction of the intracellular vimentin. Though Triton-TnBP did change the collagen content of the ACL (significant reduction of collagen content), the mechanical properties (tensile stiffness, failure load) were left unaffected. Overall, all three decellularization treatments maintained adequate mechanical and biochemical properties of B-ACL-B grafts. In a study by Vavken et al. 2009 the effectiveness of the acellularization of porcine ACL with Triton-X (0.25% for 24 hours), SDS (0.1% for 24 hours) and trypsin (0.1% for 24 hours) was examined. All acellularization protocols reduced DNA content, with triton-X treatment having the greatest effect. Concurrently, acellularization did not affect tissue collagen or total protein content, but did decrease glycosaminoglycan content. In this work Triton-X had the lowest effect on glycosaminoglycan depletion, so that the author favoured the acellularization with Triton-X. These different results show that future research is needed to find the most effective and most gentle way of acellularization to prepare the scaffold for repopulation.

For the acellularization of meniscal samples there is even more research needed. We could show (Sandmann et al., 2009) that the treatment of human meniscal samples with SDS 2% for 2 weeks is highly effective in acellularization (see chapter 5). In another work published by Stapleton et al. 2008 a different protocol was used for the acellularization of porcine meniscal samples. The menisci were acellularized by exposing the tissue to repetitive freeze-thaw cycles, incubation in hypotonic tris buffer, 0.1% (w/v) sodium dodecyl sulfate in hypotonic buffer plus protease inhibitors, nucleases and hypertonic buffer followed by disinfection using 0.1% (v/v) peracetic acid and final washing in phosphate-buffered saline. Histological, immunohistochemical, and biochemical analyses of the acellularized tissue confirmed the retention of the major structural proteins. However, a 59.4% loss of glycosaminoglycan content was noticed but with no significant alteration of the biomechanical characteristics. Furthermore, acellularized tissue and extracts were not cytotoxic to cells.

Up to now, there are many unanswered questions regarding acellularized scaffolds. The method of acellularization seems to be very promising of creating a collagen scaffold for tissue engineering of ligaments and meniscus, as the extracellular matrix and thereby the biomechanical properties are mainly preserved. However, *in vivo* animal studies are still missing. In the future acellularized tendons or meniscus samples might play a crucial role in orthopaedic surgery as do several extracellular matrix materials, which have been commercialized so far and are applied in a variety of indications reaching from the reconstruction of soft tissue defects (Ueno et al., 2004) to grafts in vascular surgery (Hiles et al., 1993) or neurosurgery (LeVisage et al., 2005).

3. Repopulation of acellularized scaffolds

The repopulation of acellularized tissue using detergents/solvents might improve and fasten incorporation and remodelling processes and thereby improve the long-term results. Cell proliferation on acellularized tissue is dependent on the used chemical treatment, with SDS showing the best acellularization rate but slightly diminished cell proliferation compared to Triton-X or TnBP (Gratzer et al., 2006, also see previous section).

In a study published by Cartmell in 2004 the seeding of acellularized tendons was most effective in TnBP treated tendons. They were seeded with fibroblasts and viable tissue-engineered grafts could be created. These modified allografts could be developed into mechanically functional delivery vehicles for cells, gene therapy vectors, or other biological agents. Whereas multiple *in vitro* studies have been performed to repopulate acellular tissue (Cartmell et al., 2004; Gratzer et al., 2006), *in vivo* animal studies are sparse. In a study by Vavken et al. 2009 it could be shown that acellularized tendons treated with Triton-X could best be repopulated as the extracellular matrix was mainly unaffected. SDS treated tissue seems more difficult to repopulate, whereas it shows the best acellularization properties. Harrison et al., 2005 compared the effect of different culture condition variables (i.e. (1) the number of cells used for seeding, (2) the addition of epidermal growth factor (EGF), and (3) culture duration) on the repopulation of porcine ACL with fibroblasts. They found that Triton-X and TnBP acellularized ACL's were suitable for repopulation. Cellular ingrowth in the SDS group was successful, but less effective. At first it was supposed that this might be due to cytotoxic effects of SDS, but Gratzer et al., 2006 showed that not the cytotoxicity but probably matrix alterations were responsible for that.

Nevertheless, little is known about the optimal cell source for tissue engineering of ligaments. Van Eijk et al. 2004 searched for the optimal cell source for tissue engineering of the anterior cruciate ligament. They compared the use of bone marrow stromal cells (BMSCs), ACL fibroblasts and skin fibroblasts and seeded them onto a resorbable suture material [poly(L-lactide/glycolide) multifilaments] at five different seeding densities, and cultured them for up to 12 days. All cell types tested attached to the suture material, proliferated, and synthesized extracellular matrix rich in collagen type I. On day 12 the scaffolds seeded with BMSCs showed the highest DNA content ($p < 0.01$) and the highest collagen production ($p < 0.05$ for the two highest seeding densities). Scaffolds seeded with ACL fibroblasts showed the lowest DNA content and collagen production. Another interesting study examined the proliferation and survival of different cell types (ACL fibroblasts vs. skin fibroblasts) on ligament analogues in the harsh environment of the rabbit knee joint (Belincampini 1998). Liu et al 2008 concluded in their study, that BMSC are a

better cell source than ACL fibroblasts when using silk scaffolds for ACL tissue engineering. The same conclusion was reached in the study by Ge et al. in 2005.

4. Tissue engineering of the ACL - own work

The aim of our study (Tischer et al. 2007) was to investigate whether the treatment of rabbit semitendinosus tendons with SDS leads to cell free constructs and whether these could serve as a scaffold for tissue engineering of the ACL. Therefore allograft semitendinosus tendons were treated with SDS using a special protocol (see table 1).

Day 1	Deionized water	24h
Day 2	1% SDS	24h
Day 3	Deionized water	24h
Day 4	70% ethanol	24h
Day 5	Phosphate buffered saline (PBS)	Several times

Table 1. Acellularization protocol for rabbit semitendinosus tendons

Following the acellularization process, the tendons were seeded with autologous dermal fibroblasts. Therefore dermal fibroblasts were taken from a small skin biopsy, extracted and cultured for approximately 10 days until the cells were confluent. The cells were split and after confluence solutions were made with a cell number of 1.0×10^6 cells/ 0.5ml medium. This suspension was injected into the acellular tendons in line with the collagen fibres. Additionally, cell suspension was applied for 4 days to the tendon surface for outer settlement of the cells.

Following, the tendons were tested biomechanically and histologically. For biomechanical testing three groups were examined, being nine native tendons, nine acellular tendons and nine tendons seeded with autologous dermal fibroblasts for 4 days of culture. The load to failure test was performed on a universal material test machine (Modell Zwicki 1120, Fa. Zwick, Germany) with a preload of 2N. The load- to failure test was performed by increasing the tensile loading continuously with a speed of 10mm/min. Apart from the load to failure the biomechanical properties elongation of tendon until rupture and the stiffness were recorded.

For histological examination six tendons were used in each group. As standard all longitudinal sections were stained with hematoxylin-eosin. For analysis of changes in the extracellular matrix, tendons from each group were stained with a panel of monoclonal antibodies directed against collagen 1, 3, 4, 6, Pro-collagen 1, versican and vimentin.

Histological staining with a standard Hematoxylin/Eosin staining revealed that all cells could be removed from the tendons and the constructs were cell-free (see figure 1). No more cell nuclei could be seen. The acellularization process lead to more interfascicular spaces in the SDS group. The group with the seeded tendon constructs lead to increased irregularities in the tendons. The cells were viable, but most of them did not show the typical spindle shape appearance. Survival of the cells was proven by immunohistochemical staining for pro-collagen I, the precursor molecule of collagen I, which was absent after acellularization but was found around the seeded cells after seeding.

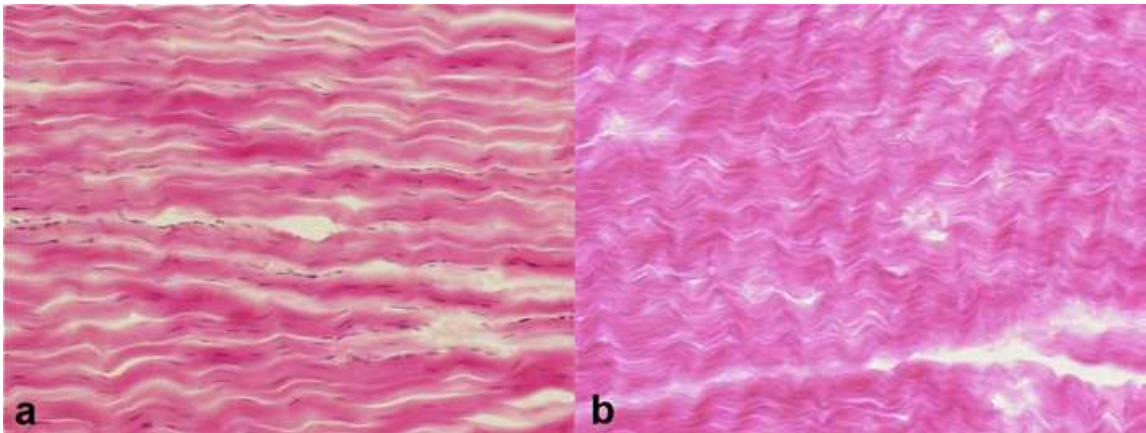


Fig. 1. Histological appearance of native (a) and acellular (b) rabbit semitendinosus tendon. No more cell nuclei can be found after acellularization (b). (HE staining, 100x magnification).

The treatment of the tendon constructs did not show any staining irregularities in the composition of the extracellular matrix. Staining for the collagens I, III, IV and VI did not show significant differences between the groups. Furthermore the distribution of macromolecules like versican was equally distributed in all three tested groups. Vimentin, an intermediary filament especially characteristic for fibroblasts was decreased, but clearly detectable.

Biomechanical results

For biomechanical testing nine native tendons, nine acellular tendons and nine cells seeded for 4 days were tested. The tests were done using a universal material test machine and with load- to failure testing. The maximum load to failure, the elongation of the tendon until rupture and the stiffness could be recorded (see table 2). The load to failure between the three groups was statistically not significant as is shown in the following table.

	Load to failure	Stiffness	Elongation
Native tendons	134.5 ± 12.9N	58.9 ± 6.57N/mm	2.47 ± 0.33%
Acellular tendons	118.5 ± 7.3N	48.5 ± 3.05N/mm	2.46 ± 0.06%
Seeded tendons	132.3 ± 5.6N	18.5 ± 1.73N/mm	7.50 ± 0.5%

Table 2. Results of load to failure testing of rabbit semitendinosus tendons

The elongation of the seeded tendons as well as the stiffness were significantly elevated compared to the acellular or the native control group. The most evident explanation might be that the seeded tendons underwent a swelling process during cell culture, which was not compensated for by our only short pretensioning before testing. However, for comparability between the other groups, pretensioning was not changed.

Animal model

While the *in vitro* results of repopulated acellular scaffold were very promising, *in vivo* results are missing. Therefore the above described scaffold was tested in an *in vivo* rabbit model of ACL replacement (see figure 2). Therein we could show that SDS acellularized, allogenic constructs colonized with autologous fibroblasts (Tischer et al. 2009) had

significantly weaker biomechanical properties than autologous tendons. In histological examination inflammatory reactions and acellular regions could be noticed, which might be due to the used acellularization method. However, since this is the first study in the field, more research as to be performed.

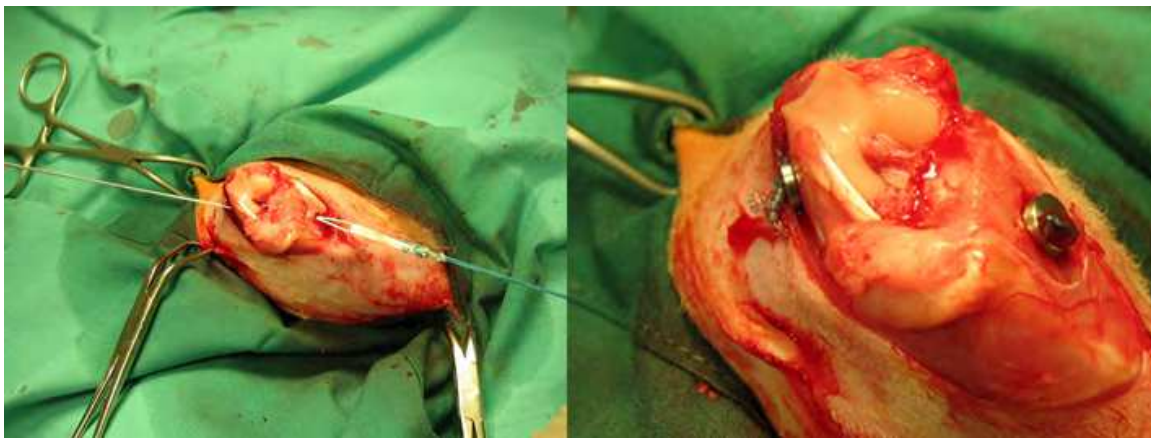


Fig. 2. Rabbit ACL replacement model using double stranded semitendinosus tendon at anatomical insertion site. Fixation was performed using custom made endobuttons.

A lot of ACL research has been done in the rabbit model and though it is a good animal model, future research in the field of double bundle research should take place in bigger animals, as their anatomy and the forces come closer to the forces in human knee joints. Questions, like the best fixation technique or the advantages of the double bundle reconstruction should be best addressed in bigger animals (Tischer et al., 2009).

Discussion

Solvents, like SDS or Triton- X have been shown to be effective agents in acellularizing tissues. In our work we focussed on SDS and our results show that with the solvent SDS it is possible to acellularize tendon tissue. The advantage compared to other methods is that by the acellularization with SDS the constructs are made cell- free and all the cell- detritus is washed out as well. This makes them less immunogenic as the cell-based reactions do not take place in the same way. The acellularization with SDS has been shown to be most effective.

Secondly, SDS acellularized constructs can be reseeded again. This might be a little more difficult than when using other methods due to an alteration of extracellular matrix hindering repopulation (Gratzer et al., 2006), but it has been shown to work (Ingram et al., 2007). Acellular and repopulated scaffolds might be very useful in the future as the integration of transplants can thereby be improved. A small dermal biopsy followed by cell culture might be enough to create a “custom-fit” transplant. Future research has to be done in this field to evaluate the possibilities.

5. Tissue engineering of the Meniscus - own work

Tissue engineering of the meniscus is even more complex than that of ligaments or tendons, as the meniscus has an even more complex collagen network and most of the methods described before have lead to a failure in acellularization or a deterioration of the

biomechanical properties. Inspired by the work mentioned above, we tried to apply the same acellularization protocol and initially failed. After gradually improving the SDS concentration and time we finally succeeded in acellularization using SDS for meniscus samples (see table 3). Since the diffusion of chemicals is time dependent, larger tissue specimens are expected to take longer before acellularization is achieved. A higher concentration of SDS (2% compared to 1%) and a longer duration were needed to get a cell-free meniscus construct.

Day 1	Deionized water	24h
Day 2- 14	2% SDS	
Day 15	Deionized water	24h
Day 16	70% ethanol	24h
Day 17	Phosphate buffered saline (PBS)	Several times

Table 3. Extraction protocol for human meniscus constructs

Histological and immunohistochemical results

After treatment with SDS 2% for 2 weeks all meniscus samples (both medial and lateral) were identified as acellular by hematoxylin/eosin staining. Phase-contrast examinations revealed regular collagen bundle arrangement in the acellular specimens as seen in intact menisci. Immunohistochemically, no differences in the labelling patterns for collagen 1, 2 and 6 were observed when compared with intact menisci. Whereas for collagen I there was strong labelling in the whole meniscus, collagen 2 was labelled only in the fibrocartilaginous section of both groups. Collagen VI staining was evenly distributed throughout the meniscus tissue, both in acellularized and intact menisci.

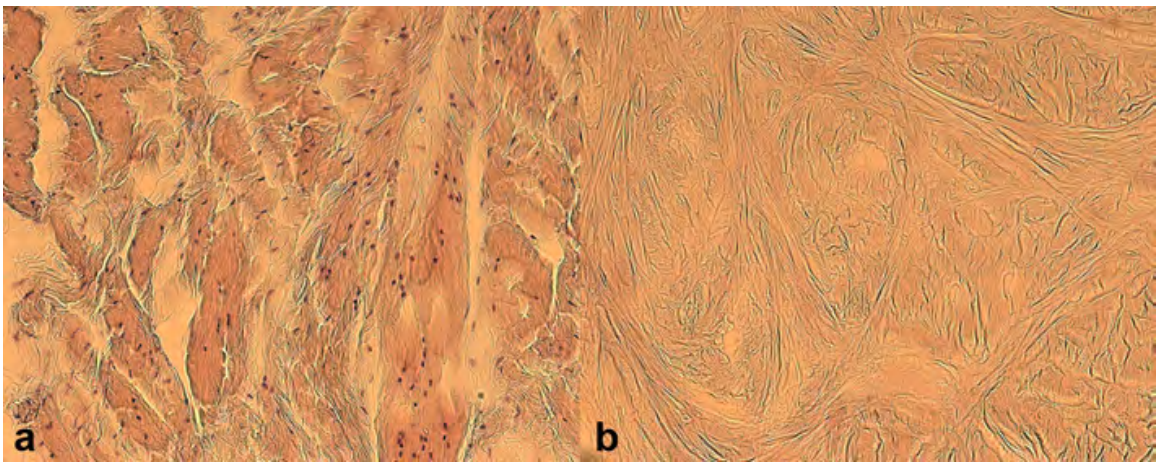


Fig. 3. Native (a) and acellular (b) meniscus samples at 200x magnification.

Biomechanical results

After the acellularization process a total of 5 medial and 5 lateral human menisci were examined using a ball indentation test. 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. The sample 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 testing (see figure 4). The test was performed as a minimally constraint compression-relaxation test. During testing, the meniscus samples were kept moist using physiologic saline solution. 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. Load, indenter position, and time were displayed by the test software TestXpert and the following three biomechanical values could be calculated: (1) *Relative sample compression* calculated by the 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 the tested tissue to evade the indenter) (2) *Stiffness* determined from the linear elastic slope of the loading curve between 2 and 5 N. High stiffness values mean high elasticity and vice versa. (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 show more elastic than viscous properties.

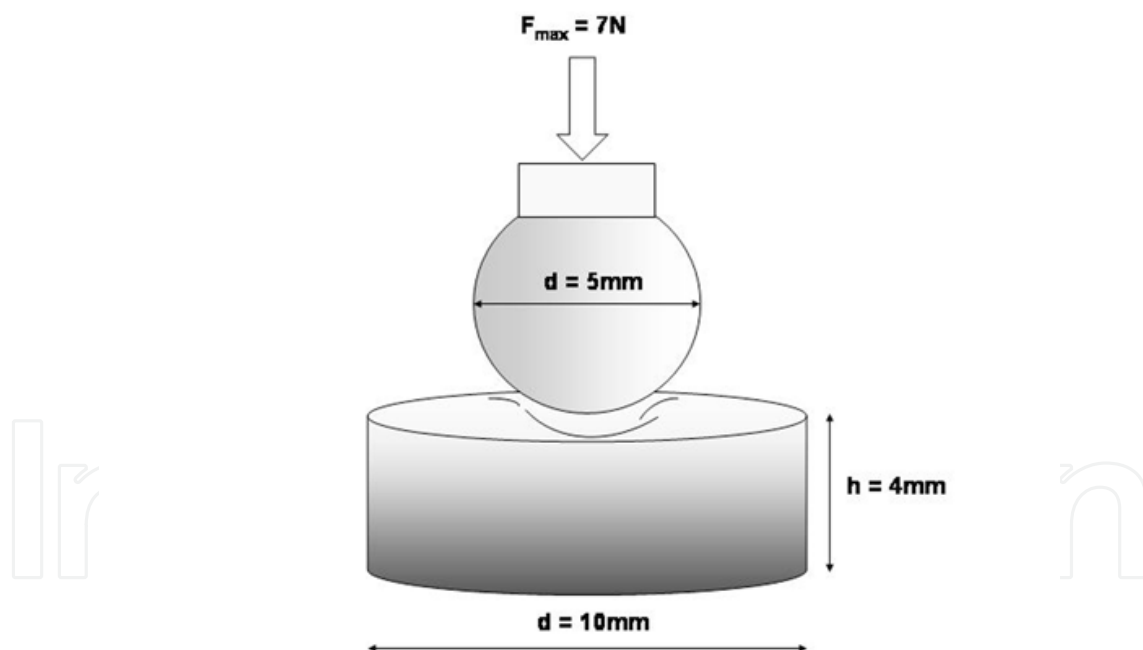


Fig. 4. Scheme of the ball indentation trial (Sandmann et al., 2009).

The testing showed that the acellularization did not affect the biomechanical properties of the tested tissue. The test was performed with cyclic loading in 5 cycles with a maximum load of 7N. Between the cycles 60seconds of relaxation were used. Stiffness increased significantly throughout testing by 162% (native meniscus) and 143% (acellular meniscus). During each testing cycle the differences between the two groups—intact meniscus and

acellular scaffold –were not statistically significant. Mean compression of sample height was 32% ($\pm 7.2\%$) for intact meniscus samples after the fifth cycle compared to 35% ($\pm 8.3\%$) in the scaffolds. Scaffold compression exceeded compression of intact meniscus by 9%, being not statistically significant ($p > 0.05$). The residual force of the two groups increased after each cycle, but no statistically significant difference could be noticed. Biomechanical results were not significantly different comparing processed medial or processed lateral meniscus samples with the control group.

	Stiffness (cycle 1)	Stiffness (cycle 5)	Mean compression height after 5 cycles	Residual force (cycle 1)	Residual force (cycle 5)
Native meniscus	11.6 $\pm$ 3.2 N/mm	31.1 $\pm$ 3.3 N/mm	32% ($\pm 7.2\%$)	1.0 $\pm$ 0.41 N	3.0 $\pm$ 0.36 N
Acellular meniscus	12.3 $\pm$ 2.9 N/mm	30 $\pm$ 3.2 N/mm	35% ($\pm 8.3\%$)	3.1 $\pm$ 0.4 N	3.2 $\pm$ 0.41 N

Table 4. Results of biomechanical testing

Discussion

Comparable to the tissue engineering of tendons the treatment of human meniscus samples with the potent solvent SDS generated cell-free meniscus scaffolds. Theses scaffolds have been shown to be biomechanically stable with characteristics similar to native meniscus samples. Special caution was given to the different biomechanical characteristics of meniscus tissue compared to tendon tissue. Seeding of the acellularized menisci has not been tested so far but future research might have to answer the question, whether repopulation of meniscus samples is possible and whether the repopulation rates can be improved by different stimulation methods like mechanical stimuli generated in bioreactors.

6. Outlook

The ACL and the meniscus are the both structures in the knee joint which are frequently injured and so far in most cases no restoration of the original anatomy is possible. Especially the development of osteoarthritis in the long term is still an unsolved problem. Fortunately, many advances have been made in the surgical reconstruction of the ACL and the disastrous total meniscectomy has been abandoned long ago. The role of new surgical techniques like double bundle ACL surgery still has to be found. Apart from the acellularization of tissues, the use of growth factors, additionally or alone, seems to be a very promising option in improving the long- term results. Especially in meniscus transplants those factors that stimulate matrix synthesis and inhibit degradation of the extracellular matrix could be very useful. Future research has to point on the repopulation process and its improvement. Angiogenic growth factors might contribute to even further improve the results. Maybe the use of bioreactors producing adequate mechanical stimuli, growth factors or synthetic scaffolds (e.g. silk) will help to solve the remaining questions.

7. Conclusion

Tissue engineering provides a lot of possibilities for improving tendon or meniscus scaffolds. Especially, the acellularization process with following cell seeding might be a very effective way of improving incorporation and remodelling processes as well as avoiding the cell-based immune reactions, which may be partially responsible for the deterioration of the allografts over the years. We have shown that the treatment of tendons or meniscus samples with the solvent SDS produces cell-free constructs. Despite acellularization, the extracellular matrix remains widely intact and the biomechanical properties remain unaltered. Whether the acellularization and the seeding will be successful in the long term has still to be shown in further animal models. Only then will human application be possible and improve operative treatment for our patients.

Big efforts have been made during the last years *in vitro* and *in vivo* and we believe that the tissue engineering of tendons and menisci samples might play a crucial role in the future of orthopaedic surgery.

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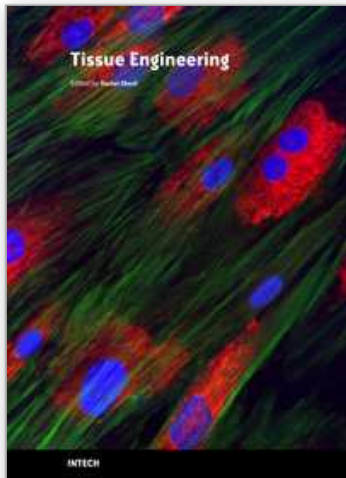
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Tissue Engineering

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The Tissue Engineering approach has major advantages over traditional organ transplantation and circumvents the problem of organ shortage. Tissues that closely match the patient's needs can be reconstructed from readily available biopsies and subsequently be implanted with minimal or no immunogenicity. This eventually conquers several limitations encountered in tissue transplantation approaches. This book serves as a good starting point for anyone interested in the application of Tissue Engineering. It offers a colorful mix of topics, which explain the obstacles and possible solutions for TE applications.

How to reference

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InTech Europe

University Campus STeP Ri
Slavka Krautzeka 83/A
51000 Rijeka, Croatia
Phone: +385 (51) 770 447
Fax: +385 (51) 686 166
www.intechopen.com

InTech China

Unit 405, Office Block, Hotel Equatorial Shanghai
No.65, Yan An Road (West), Shanghai, 200040, China
中国上海市延安西路65号上海国际贵都大饭店办公楼405单元
Phone: +86-21-62489820
Fax: +86-21-62489821

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