Arch Orthop Trauma Surg (2006) 126: 228–234
DOI 10.1007/s00402-005-0025-1

ORIGINAL ARTICLE

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Second generation of meniscus transplantation: in-vivo study with tissue engineered meniscus replacement

Received: 9 July 2003 / Published online: 8 October 2005

Abstract *Introduction*: The options available after meniscus loss offer only limited chances for a long-term success. In the following experimental study, we investigated the effect of meniscus tissue engineering on properties of the collagen meniscus implant (CMI). *Methods*: Autologous fibrochondrocytes, obtained per biopsy from adult Merino sheep ($n=25$), were released from the matrix, cultured in-vitro and seeded into CMI scaffolds ($n=10$, group 1). Following a 3-week in-vitro culture, the tissue engineered menisci were used for autologous transplantation. Macroscopical and histological evaluation were performed in comparison with non-seeded CMI controls ($n=10$, group 2) and with meniscus-resected controls ($n=5$, group 3) after 3 weeks (each 1 animal group 1 and 2) and 3 months. *Results*: The lameness score did not show any difference between the groups. Meniscus tissue was found in seven knee joints (group 1), in five knee joints (group 2) and in two knee joints (group 3). The size of the transplants reduced from 25.9 ± 4.5 to 20.1 ± 10.8 mm (group 1) and from 25.9 ± 1.5 to 14.4 ± 12.5 mm (group 2). Histologically, enhanced vascularisation, accelerated scaffold re-modelling, higher content of extra-cellular matrix and lower cell number were noted in the pre-seeded menisci in comparison with non-seeded controls. Dense high-cellular fibrous scar tissue was found in two of five cases in the resection control group. *Conclusion:* Tissue engineering of meniscus with autologous fibrochondrocytes demonstrates a macroscopic and histological improvement of the transplants. However, further development of the methods, especially of the scaffold and of the cell-seeding procedure must prove the feasibility of this procedure for human applications.

Keywords Meniscus · Tissue engineering · Fibrochondrocyte · Meniscus transplantation · Meniscal reconstruction

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Introduction

Meniscus replacement still represents an unsolved problem in orthopaedics and traumatology [8, 18, 25]. Following meniscus resection only two applicable methods are available for substitution: allograft transplantation or implantation of a collagen meniscus scaffold [8, 35]. However, both therapeutic methods showed only suboptimal clinical results which caused the search for alternative methods [19, 43].

Since the first successful allogenic meniscus transplantation 1984 in Munich, Germany, this operation is used in patients worldwide [28]. However, meniscus allograft transplantation can be considered only as a temporary limited salvage procedure in younger patients [24]. Transplanted allografts never reach the tissue quality of normal menisci [13]. Slow immuno-rejection processes during the first 2 years following meniscus implantation lead to shrinkage of the allografts. Frequent tears and tissue destruction take place as consequence of impaired tissue mechanical strength [5, 18, 27, 29, 33, 42, 43].

Several biologic and synthetic materials such as autologous tendons, perichondrium, fibrin clot, fat,

submucosa, xenografts, collagen matrices or carbon-fibre prostheses were developed for meniscal replacement in-vivo [2, 3, 4, 15, 16, 17, 37, 38, 44]. The transplantation of the Collagen Meniscus Implant (CMI) represents the only treatment method that was introduced for clinical usage [35, 38, 39]. CMI is a resorbable type I collagen-based matrix chemically engineered from bovine Achilles tendons, which was developed as a template for meniscal cartilage regeneration [38]. This meniscus prosthesis can be implanted arthroscopically with sutures to the remaining rim of the meniscus where it provides a matrix for in-growth of cells and generation of a new meniscus.

Clinical data presented in a small group of patients validated the ability of CMI to support the regeneration of a new tissue and to improve the symptoms in patients [35]. In a newly finished multicentre study performed in 288 patients, pain and self evaluation was improved in the majority of the patients after 2 years [34]. In histological findings, however, CMI remnants with cellular in-growth and some dense meniscus-like tissue were found in about 50% of the cases [34]. A similar experience was made in our own patient collective following arthroscopic implantation of CMI: a clinical improvement was associated with inferior histological results [22]. The invading reparatory cells were able to produce matrix proteins in some areas. However, newly created tissue presented histologically as a scar tissue with a marked difference to the meniscus fibrocartilage.

Tissue engineering represents an important option for the generation of tissues or organs of the musculoskeletal system with a low potential for healing [1]. While great efforts have been made to engineer bone or cartilage, there is a dearth in the amount of work done to create meniscus [12, 20, 30]. The purpose of this pilot study was to establish a tissue engineering concept for meniscus replacement. We investigated the feasibility and the biological effect of in-vitro pre-seeding of a collagen matrix (CMI) with autologous fibrochondrocytes in a large animal model.

Materials and methods

Twenty five adult female merino sheep with an average weight of 80.0 ± 10.6 kg (mean ± SEM) were used for the experiments. Three groups were formed: group 1 ($n = 9$) meniscus resection and transplantation with tissue engineered CMI scaffold seeded with autologous fibrochondrocytes, group 2 ($n = 9$) meniscus resection and transplantation with CMI scaffold only and group 3 ($n = 5$) meniscus resection without transplantation. All animals in each of the three groups were sacrificed 3 months following this operation and studied as described below. Two additional animals (one CMI seeded, one CMI non-seeded) were used preliminarily 3 weeks after transplantation to examine the early effect of the cell seeding.

Meniscus biopsy

For the meniscus biopsy, a 3 cm long skin incision and an anteromedial exposure of the right knee joint under aseptic conditions were used. The biopsy (3 mm^2) was taken from the anterior horn of the medial meniscus leaving the entire meniscal base intact. Cells were isolated from the meniscus tissue with the method described by Green [9]. The tissue was cut into small pieces and digested with 0.2% trypsin for 30 min and 0.2% collagenase for 3 h at 37°C, cells were seeded into tissue-culture flasks.

Tissue engineering in-vitro

Collagen meniscus implant (CMI, ReGen Biologics, USA) was used as the matrix for the tissue engineering of the meniscus. This meniscus prosthesis is a bioresorbable device developed and already available for replacement after subtotal meniscus loss in humans [34]. CMI is manufactured from type-I collagen fibers purified from bovine Achilles tendons with chemical treatments including cross-linkage with aldehydes [39]. For the reason of primary toxicity of the CMI-aldehydes we have observed in our preliminary in-vitro experiments, an extensive washing of the scaffold was performed with sterile Dulbecco's Modified Eagle Medium (DMEM, Gibco) over a period of 3 weeks before seeding with autologous fibrochondrocytes.

Autologous fibrochondrocytes were obtained per biopsy from ovine menisci. The cells were isolated from the meniscus tissue and cultured in-vitro under standard conditions in DMEM supplemented with 10% fetal bovine serum and 1% penicillin and streptomycin for 3 weeks [47]. The cultured fibrochondrocytes (10×10^6 cells/3.25 cm CMI scaffold) were dissolved in 10 µl DMEM and distributed into the washed CMI collagen matrices with a 27 Gauge needle. Before transplantation, the CMI-seeded constructs were kept in tissue culture for 3 weeks under sterile standard conditions (Fig. 1).

Meniscus transplantation

Meniscus transplantation with the tissue engineered menisci was performed 6 weeks following the biopsy procedure. During this time, the animals were allowed to move with full range of motion and full weight bearing. A prolonged anteromedial exposure of the right knee joint was used for the transplantation. To reach the posterior parts, the medial collateral ligament was cut above the base of the medial meniscus. The medial meniscus was removed subtotally cutting sharp along the base and leaving 2 mm of a peripheral meniscal ridge intact. The tissue engineered CMI was shaped into the defect using the removed meniscus as template (2.5 ± 0.3 cm) and sutured to the remaining meniscus

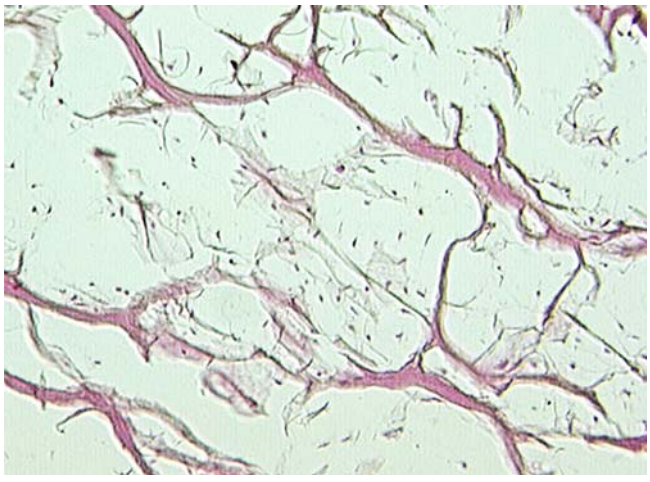

Fig. 1 Histology of in-vitro seeded CMI scaffold. Fibrochondrocytes are positioned in the free spaces of the collagen scaffold

base with four non-resorbable Ethibond 3–0 sutures (Fig. 2). Finally, an intra-ligamentous suture of the medial collateral ligament and a closure in layers were performed. To reach non-weight bearing status of the operated legs for 4–6 weeks, 3 cm of the ipsilateral Achilles tendons were removed using a 2 cm longitudinal lateral skin incision 5 cm proximally to the tendon origin [3].

Evaluation

The animals were regularly monitored concerning wound healing, their general behaviour and agility during the entire study period. A standardised examination for joint range of motion, stability and gait was performed weekly.

Following the final clinical evaluation, the animals were sacrificed and right knee joints were disarticulated. Detailed observations of the menisci, tibial and femoral cartilage were made, including the presence of effusion, signs of infection, and cartilage defects. The macroscopic damage to the cartilage of the medial tibial and femoral condyles was graded as described by Szomor [41]. The site of meniscus transplantation was photographed, the size of the meniscus regenerate measured and the meniscus tissue judged for integrity, form, colour, surface, elasticity and vascularisation. The menisci with their attachment at the capsula were carefully dissected together and placed into 6% formalin. The specimens were embedded in paraffin, cut transversely at 5 µfm sections and stained with hematoxylin/eosin, van Giesson and Azan blue. The meniscus regenerates were evaluated on light microscopy in terms of cellularity, nature of cells, inflammatory signs, presence or absence of transplanted and new collagen as well as of presence of newly produced glycosaminoglycans.

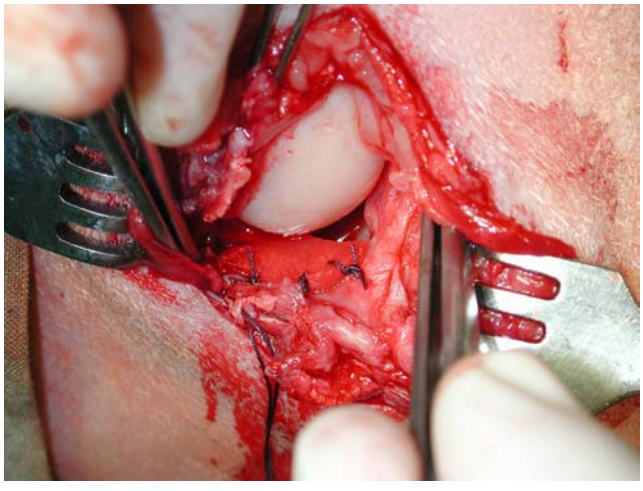

Fig. 2 Operation situs of the CMI implantation with sutures to the remnant meniscal rim

Statistical analysis

As this publication represents the preliminary results of a pilot study with a limited number of animals, statistical analysis was pointless and therefore not performed.

Results

All animals recovered from both operations. There were no intra- or post-operative complications. One infection of the knee joint occurred after 4 weeks (group 1, seeded CMI), one animal (group 2, non-seeded CMI) suffered heart insufficiency 6 weeks following the second operation. Both were excluded from the study evaluation so that each of eight animals from group 1 and 2 and 5 animals from group 3 could be included into the final results.

Clinical findings

Due to the Achilles tendon resection, all animals did not weight bear the treated legs for at least 4 weeks. After this period of time, partial weight-bearing was observed for 2–4 weeks; however, all animals were able to ambulate with full weight-bearing at 8 weeks after the second operation. At the final evaluation prior to sacrification, all animals had completely healed scars and showed normal agility and gait. All knee joints were stable to anterior, posterior, varus and valgus testing and had a normal range of motion. No differences could be detected between the animals of different groups.

Macroscopic findings

After the animals were killed, a discrete effusion in form of a clear synovial fluid was detected in seven of eight knee joints in group 1 (seeded CMI), in four of eight

knee joint in group 2 (non-seeded CMI) and in one of five knee joint in group 3 (resection control). Gross observation of the medial articular compartment revealed one cartilage defect (grade 2) on the femoral and no cartilage defect on the tibial articular surface in group 1, while there were two cartilage defects on the femoral site (one grade 1, one grade 2) and one cartilage defect on the tibial side (grade 3) in group 2, respectively two cartilage defects on the femoral site (one grade 1, one grade 4) and one cartilage defect on the tibial side (grade 2) in group 3 (Table 1).

Medial meniscus regenerate tissue was found in seven of eight knee joints in group 1, in five of eight knee joints in group 2 and in two of five knee joints in group 3 (Table 2). There was no difference between seeded and non-seeded meniscal transplants in group 1 and 2 regarding colour, surface and elasticity of the meniscus transplants or their integration to the capsula. In cases of transplant destruction in group 1 and 2, no correlation was found to the co-incidence of articular cartilage damage. The mean length of the transplanted meniscus reduced from time point of implantation to time point of explantation from 25.9 ± 4.5 to 20.1 ± 10.8 mm in group 1 and from 25.9 ± 1.5 to 14.4 ± 12.5 mm in group 2. The meniscus regenerate tissue was 10 mm in one and 5 mm in the second cases in group 3. A prominent vascularisation at the surface of meniscal base near insertion at the capsula was seen in seven of seven explants in group 1 and in two of five explants in group 2. No vascularisation was detected in both regenerates in group 3 (Table 2).

Histology

Histologically, a difference between the group 1 (seeded CMI) and group 2 (non-seeded CMI) was detected already in the 3 weeks sections (Fig. 3). While the implanted CMI scaffold was filled with a high number of fibrocytes embedded in a small volume of matrix (Fig. 3a), the free spaces of the seeded CMI scaffold were occupied by a small number of fibrochondrocytes and filled with a relatively large volume of inter-cellular matrix (Fig. 3b).

Table 1 Cartilage defects on the femur and tibia observed 3 months after implantation of seeded CMIR, non-seeded CMIR and resection respectively

	Seeded CMIR $n=8$	Non-seeded CMI $n=8$	Resection controls $n=5$
Cartilage defect femur	1/8	2/8	2/5
Cartilage defect tibia	0/8	1/8	1/5
Total number of defects	1/8	3/8	3/5

Group 1 (seeded CMI) demonstrated the fewest chondral defects probably due to the protecting effect of the implant. In each group cartilage defects on the femoral sites were more frequent than on the tibial sites

Table 2 Meniscus regenerate tissue and vascularisation of the implants found in the different groups 3 months after implantation of seeded CMI, non-seeded CMI and resection respectively

	Seeded CMI $n=8$	Non-seeded CMI $n=8$	Resection controls $n=5$
Meniscus regenerate	7/8	5/8	2/5
Vascularisation	7/7	2/5	0/2

Group 1 (seeded CMI) showed the most meniscus regenerates and the best vascularisation at the meniscal bases while the regenerates in group 3 did not show any vascularisation

The 3 months specimens showed different stages of scaffold re-modelling in the group of seeded CMI (Figs. 4b and 5b). In three specimens, no signs of resorption were found, while a partial resorption was detected in three (Fig. 5b) and a complete resorption in other two implanted seeded collagen matrices (Fig. 4b). The cell density in the group of seeded transplants was low in four, moderate in two and high in one specimens. In this group, the scaffolds were filled with a large amount of matrix proteins in four, with moderate amount in one and with low amount in two explants.

In comparison, no evident re-modelling of the implanted collagen scaffold was seen in the five available tissue probes in the group of non-seeded CMI (Figs. 4a and 5a). The cell density in the group of unseeded transplants was high in three and low in two specimens. In this group, all implanted scaffolds were filled only with a low amount of matrix proteins.

The tissue found in two of five cases in the resection control group demonstrate the histological appearance of a dense, less-organised, high-cellular fibrous tissue similar to a scar formation.

Discussion

Clinical and histological findings after CMI transplantation demonstrate several problems [22, 34]. Following CMI implantation, the scaffold undergoes a slow process of resorption and the new originated tissue is identical with scar tissue [34]. The cells ingrowing from the capsula into the meniscus scaffold are responsible for creation of a scar tissue and not for creation of a fibrocartilage tissue [25]. The biomechanical properties of this scar tissue are far inferior to the properties of the normal viable meniscal tissue. This is the reason, the implanted CMI scaffolds are damaged in the posterior parts—at the sites of the highest mechanical stress [22].

One of the potential solutions for meniscus replacement is offered by tissue engineering [1, 12, 40]. Up to date, only few experimental tissue engineering studies focussed on the subject of the meniscus [12, 45]. Walsh et al. used type I collagen sponges loaded with autologous, bone marrow derived, cultured mesenchymal stem cells for meniscus replacement in rabbits [45]. Although,

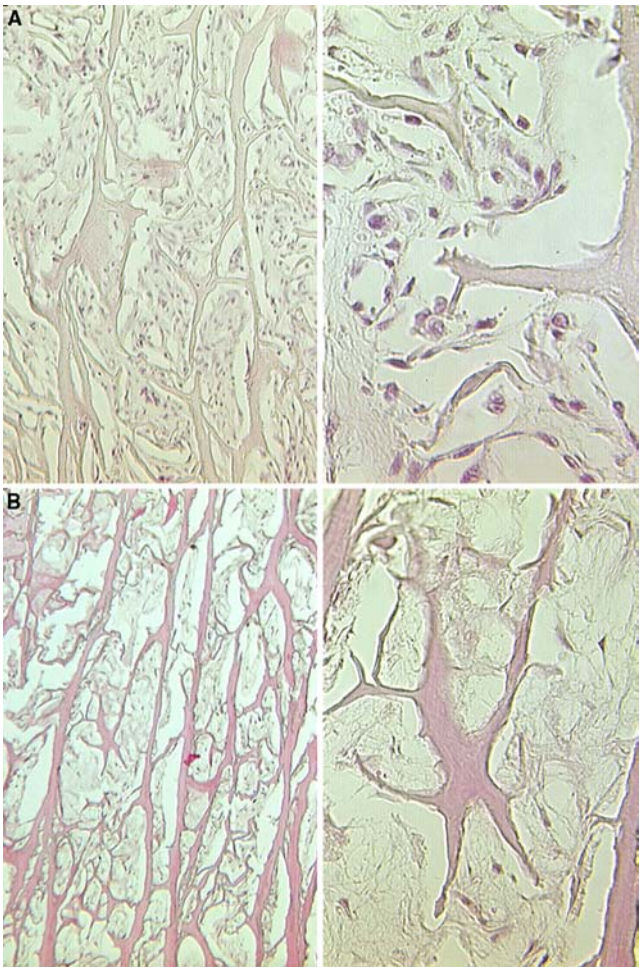

Fig. 3 Histology of meniscus transplant 3 weeks following implantation. In the non-seeded specimen (**a**), the spaces between the collagen fibres are filled with a high number of fibrocytes with less intercellular matrix. In the seeded specimen (**b**), the spaces between the collagen fibres are filled with a low number of fibrochondrocytes and more intercellular matrix

the cultured mesenchymal stem cells augmented the repair process in some specimens, degenerative changes were not prevented in this experiment. Ibarra et al. [12] seeded PGA and PGLA scaffolds with meniscal cells and implanted the constructs subcutaneously for 4 weeks before placing them in a meniscus defect for 6 weeks using an ovine model. The results were promising and showed tissue rich with proteoglycans and organized collagen fibre matrix, however, the need for further developments were stated [12].

The idea of the own tissue engineering experiment was the delivery of fibrochondrocytes into the meniscal scaffold prior to the implantation in the knee joint. As demonstrated by histologies obtained 3 weeks following the in-vivo implantation (Fig. 3), the pre-seeding of the meniscal matrix with autologous fibrochondrocytes prevented the invasion of destructive inflammatory and reparatory cells. As a result, an improvement of the macroscopic and histological findings was evident in the treated knee joints up to 3 months following implantation of the seeded scaffolds. As positive effect of meniscus engineering, larger and better vascularised menisci were found in comparison to the specimen following implantation of non-seeded CMI. The resorption of the implanted porcine collagen matrix, lasting more than 12 months as known from human application [39], was accelerated and the histological analysis demonstrated a lower cell number associated with higher portions of extracellular matrix in the engineered menisci. Due to the small number of cases, less cartilage lesions in the medial joint compartments were seen in joints with tissue engineered menisci than in joints with unseeded CMI scaffolds. The creation of a meniscus regenerate following total meniscectomy, as seen in some of the meniscetomised knee joints, is well known from former experiments in rabbits and dogs [45]. After removal of the meniscus, bleeding from the perimeniscal vessels results in an organized clot within the joint space which serves as scaffold for migration of cells from the synovium and capsule [4, 45]. This aspect of tissue regeneration was confirmed in our study. However, the results also show the insufficiency of this regenerate and the low quality in comparison to seeded or non-seeded CMI.

Our study is the first to demonstrate a successful in-situ tissue engineering of the meniscus in a large animal model. However, the results appoint also the critical issue of the experiment. Despite the improved histological appearance, the tissue engineered meniscus is biomechanically unstable and undergoes a process of mechanical destruction. Despite the prevention of weight-bearing for 4–8 weeks through the separation of the ipsilateral Achilles tendon, the size of the implants reduces during the period of observation, and in one case even a complete elimination of the engineered meniscus occurs. The short-time tissue engineering under standardised laboratory conditions could mechanically stable meniscus fibrocartilage [8, 25, 40].

The improvement of biomechanical properties of the engineered meniscus will require major developments of the scaffold and changes in the environmental conditions during the tissue engineering procedure. The used collagen CMI scaffold is a 3-d mesh-like construct of collagen fibres that are, differently to the native meniscus, randomly organized in the matrix. The majority of collagen fibers within the substance of the meniscus have a predominantly parallel circumferential orientation allowing the meniscus to resist tensile forces and function as a transmitter of load across the knee joint [31]. Additionally, few small, radially placed fibres provide structural rigidity and help to resist compression forces. The challenge of future projects will be the construction of biomechanically stable meniscal substitutes with a microstructure similar to the native meniscus [26]. Although, several other matrices such as biodegradable polymers [11] or small intestine submucosa [4, 7] have been proposed and investigated for the meniscus replacement, collagen-based-matrices seem to be the best

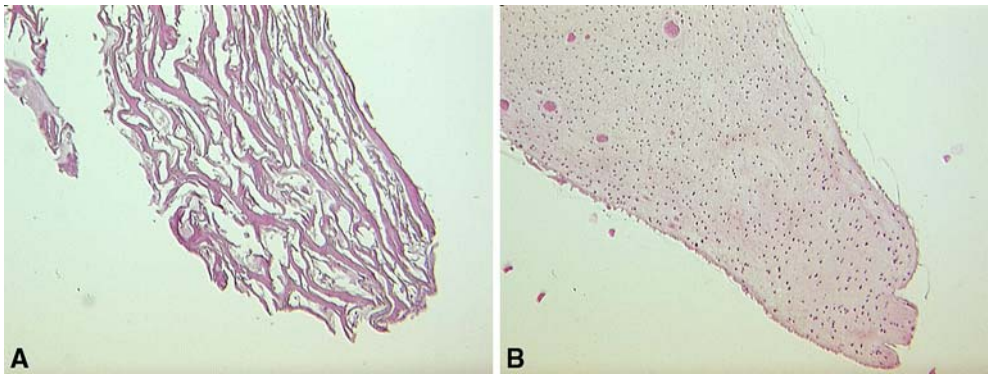

Fig. 4 Histology of the tip of meniscus transplant 3 months following implantation. Poorly re-populated scaffold without signs of resorption in the non-seeded specimen (**a**). In the seeded specimen (**b**) complete resorption of the collagen scaffold which was replaced by a meniscus-like tissue

alternative today to accomplish appropriate construction of a stable meniscus prosthesis [1].

An important factor in the meniscus engineering could also be a proper biophysical environment [6, 36]. Similar to other tissues such as cartilage, meniscus cells need a physical stimulus for keeping their phenotype and their capability for a sufficient matrix production [10]. Meanwhile, several studies have demonstrated the positive effect of an intermittent physical stimulus on the behaviour of fibrochondrocytes [36]. In order to optimise the meniscus engineering process, the usage of bioreactors with precise determined conditions will be obligatory in future tissue engineering projects [1, 40]. It is also expectable that the application of growth factors such as TGF-ß, bFGF or IGF-1 that proved to influence meniscus biology will be necessary to develop a new meniscus [14, 21, 46]. Due to safety obstacles, it cannot be answered today, if gene transfer will be suitable for the delivery of growth factors into meniscal cells or scaffolds in the near future [20, 23].

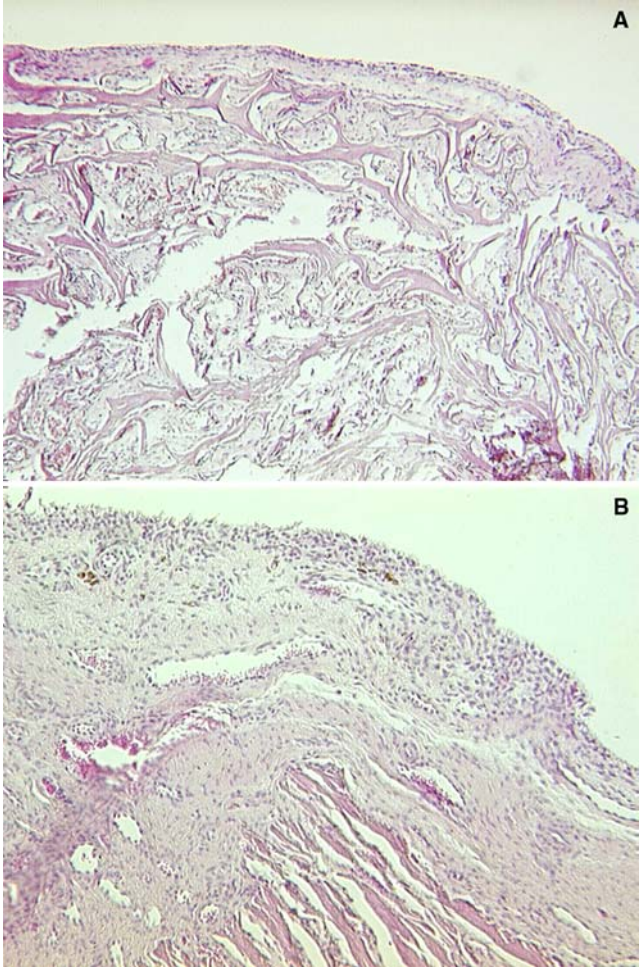

Fig. 5 Histology of the base of meniscus transplant 3 months following implantation. In the non-seeded specimen (**a**), the scaffold without signs of resorption is filled with a cell-rich scar tissue. In the seeded specimen (**b**), the partially resorbed collagen scaffold is well integrated and filled with meniscus-like tissue

Conclusions

Tissue engineering of the meniscus represents today the most promisive alternative for future meniscus replacement [1, 26, 40]. This experimental study shows the first step on the way toward the creation of a perfect meniscus substitute. Various biologic considerations such as meniscus scaffold, cell type, cytokines or design of bioreactor with specific environmental conditions have to be addressed to reach functional capabilities of a normal meniscus in the future.

Acknowledgements Funds from German speaking Association for Arthroscopy (AGA), Arthrosehilfe e.V. and Commission for Clinical Research (KKF) of the Technical University Munich, Germany provided partial support for the research presented in this article.

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