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EXPERIMENTAL STUDY

A comparative study of 3 different cartilage repair techniques

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Abstract
Purpose The value of cell-free techniques in the treatment of cartilage defects remains under debate. In this study, cartilage repair of full-thickness chondral defects in the knees of Goettinger minipigs was assessed by treatment with a cell-free collagen type-I gel or a collagen type-I gel seeded with autologous chondrocytes. As a control, abrasion arthroplasty was included.
Methods In 18 adult Goettinger minipigs, three full-thickness chondral defects were created in one knee of the hind leg. They were either treated with a cell-free collagen gel, a collagen gel seeded with 2×10^5/ml chondrocytes, or left untreated. All animals were allowed unlimited weight bearing. At 6, 12, and 52 weeks, 6 animals were sacrificed. Immediately after recovery, a non-destructive biomechanical testing was performed. The repair tissue quality was evaluated histologically, and the O'Driscoll score was calculated.
Results After 6 weeks, a high number of cells migrated into the initially cell-free collagen gel. After 1 year, a hyaline-like repair tissue in both groups has been created. As assessed by O'Driscoll scoring and col-II staining, repair tissue quality of the initially cell-free gel was equal to defects treated by cell-seeded collagen gel implantation after 1 year. All untreated control defects displayed a fibrous repair tissue. The mechanical properties represented by the e-modulus were inconsistent in the course of the study.
Conclusions The implantation of a cell-free collagen type-I gel can lead to a high-quality repair tissue in the Goettinger minipig that equals a cell-based procedure after 1 year postoperatively. This study demonstrates the high chondrogenic potential of the applied collagen gel, which might help to overcome the disadvantages inherent in conventional cartilage tissue engineering methods.

Keywords Collagen type-I gel · Goettinger minipig · Cartilage tissue engineering · Cell-free implant

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Introduction

Tissue engineering has become an important part within the treatment options available for articular cartilage defects of human knee joints. As the capacity of ACT in rebuilding hyaline-like cartilage is limited, various three-dimensional matrix systems have been developed [7, 8, 13, 17, 22, 27, 30]. Among the growing range of carrier systems for matrix-based chondrocyte transplantation, collagen gels have gained special attraction. They have proven to promote cellular proliferation while preserving the chondrocyte phenotype [18]. Moreover, it is possible to achieve a homogeneous cellular distribution.

The classical matrix-based transplantation of autologous chondrocytes is a two-step procedure. In the first surgery, a cartilage biopsy is taken in order to obtain a sufficient number of autologous cells. Chondrocytes are either directly seeded on the carrier (CaReS^{TM} and others) [9] or pre-amplified in monolayer culture until seeding of the carrier ([MACT/MACI^{TM}] and others) [2, 3, 5]. In a second step, the cell suspension or chondrocyte-seeded carrier is

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transplanted. These operative techniques are expensive and time consuming. Moreover, the taken biopsy causes the potential risk of donor-site morbidity, although this phenomenon is more likely to occur when osteochondral plugs are harvested [31]. Therefore, interest is growing in the development of cell-free repair techniques, avoiding the mentioned disadvantages.

The purpose of this study was to evaluate the potential value of a cell-free collagen type-I gel for cartilage repair in a standardized, well understood, and accepted large animal model, the Goettinger Minipig [14, 24, 32]. Therefore, cellular immigration, defect filling and bonding, maturation of the repair tissue over time, and mechanical behavior were investigated. As a reference system, a collagen gel seeded with autologous chondrocytes in low cell density was included, corresponding to the CaReS^{TM}-method. Although most cartilage repair techniques require a much higher chondrocyte number, the CaReS^{TM} system has proven to promote cartilage regeneration in the clinical setting with a chondrocyte concentration of 2×10^5/ml [1].

Materials and methods

Cartilage sample preparation

A total of 18 male adult Goettinger minipigs were included in this study. The median age was 3 years (2–4 years); the median weight at study start was 36 kg (30–45 kg). Approval of the local ethical board and the competent authority was obtained prior to operation (AZ: 50.203.2-AC 41 55/06).

Cartilage biopsies were obtained from the left hind legs of all animals. This surgery was a standardized procedure to create the defects and to gain a cartilage biopsy for isolating autologous chondrocytes. Anesthesia was established with incubation narcosis. For exposing the joint, a 4-cm skin incision was made at the ventral side of the median plane. The arthrotomy was set by transligament access through the ligamentum patellae. Three defects were set in the area of the trochlear groove as cylinders with a diameter of 6.3 mm with a cutter and a sharp angulated raspatory. The articular cartilage was debrided meticulously down to the subchondral bone avoiding bleeding. To obtain autologous chondrocytes for transplantation, the biopsies were transferred into sterile transport containers filled with culture media and processed immediately.

Cell preparation and cultivation

Samples were collected in DMEM medium containing 10% fetal calf serum (FCS), 100 U/ml penicilline, 100 µg/ml streptomycine. The cartilage was cut into 1–2 mm^3 pieces and digested with 1 mg/ml Liberase 3 (Roche Diagnostics, Indianapolis, MN, USA) overnight. The released chondrocytes were washed subsequently for 3 times, and cell number was determined on a CASY1 cell counter (Schärfe System, Reutlingen, Germany). Viability was assessed by Trypan blue staining.

Preparation of cell-seeded collagen samples

Rat tail collagen type-I gel was provided by Arthro Kinetics (Esslingen, Germany). The collagen type-I was supplied as an aqueous solution of 0.6 mg/ml in 0.1% acetic acid. It remained liquid when stored at 4°C and gelled when transferred to 37°C.

2×10^5 chondrocytes/ml gel was resuspended in 1 vol collagen type-I gel mixed with 1 vol 2× DMEM/2 M HEPES (0.93:0.07). Cell-seeded collagen gel was given into a well of a 12-well plate to reach a final height of the specimens of 3 mm. After gelling, samples were overlaid with medium until implantation.

The cell-free collagen type-I plug

Collagen type-I plugs were obtained from Arthro Kinetics (Esslingen, Germany). They consisted of 4.8 mg/ml rat tail collagen type-I gel, a gel already in clinical application (CaReS^{TM}, Arthro Kinetics, Esslingen). The diameter of the samples was 9 mm with a height of 3 mm. The plugs were supplied in PBS solution and stored at 4°C until implanted.

Cartilage defect treatment techniques

All 18 animals were operated 2 weeks following first surgery. Access to the joint was performed in same way as during the first surgery. Regenerated tissue which eventually filled the defect zone of the first surgery was excised by hollow cutter (6.3 mm) down to the subchondral bone (Fig. 1). In few cases, a punctual bleeding of the subchondral bone occurred. The joint was washed, and defects were either filled with the prepared collagen gels or left untreated. In this set of experiments, exclusively chondral defects were treated.

With regard to the defects to be filled with collagen gel, a 7.3-mm cylinder was cut from the cell-free and corresponding cell-seeded transplants. The transplants were fixed with fibrin glue (Tissucol, Baxter, Deerfield, IL, USA) and pushed into the defect with 11-mm stamp. The joint was flexed to check fixation of the transplant.

At 6, 12, and 52 weeks after surgery, the animals were sacrificed by injection of an overdose of barbiturate under general anesthesia. The complete hind limb was removed, and the involved knee joint was exposed. The repair tissue

was macroscopically assessed with regard to pathologic changes. The macroscopic appearance of the defect was documented, and the specimens were recovered and subject to further processing.

Mechanical testing

Immediately after recovery, a biomechanical testing was performed on all specimens. The samples were placed on an open cylindric vessel allowing free lateral deformation. During the examinations, the samples were kept moist using DMEM medium. Mechanical indentation tests were performed on a material testing machine (Zwick 1455, Zwick, Ulm, Germany) with a calibrated load cell (accuracy 0.02 N). The diameter of the indentor was 4 mm.

Repair tissue of all specimens was compressed with a constant speed of the indentor (1 mm/min) until a rapid increase in the force in relation to distance was found. This point was considered as the failure load (or max. force). This was repeated investigating the cartilage surrounding the repair tissue.

The exact repair tissue thickness of all examined specimens was determined on HE stained slides following histological preparation. E-modulus was calculated according to the following formula: (Δforce * sample thickness)/(Δheight * surface indentor). It was expressed as a ratio with respect to the surrounding healthy cartilage.

The biomechanical testing was non-destructive. The elastic deformations of the tissue recovered completely.

Histological and immunochemical evaluation

After recovery, samples were decalcified, fixed with 4% paraformaldehyde, and embedded in paraffin. Five-micrometer sections were subject to further processing. Haematoxylin/Eosin staining was performed according to standard protocols. For the immunochemical detection of collagen type-II protein, sections were deparaffinized, blocked for 1 h with 1% NGS, and incubated with a polyclonal antibody to human collagen type-II (Biotrend, Cologne, Germany) diluted 1/50 overnight. Bound antibody was detected by incubation with goat-anti-rabbit antibody diluted 1:200 for 1 h. Staining was visualized using the streptavidin/biotin technique (Vectastain ABC Kit, Vector Laboratories, Burlingame, VT, USA) with Diaminobenzidine (DAB Peroxidase Substrate Kit, Vector Laboratories, Burlingame, VT, USA) as the developing substrate. All images were captured by a Leica microscope (Leica, Wetzlar, Germany) and prepared using the Discus software by the same manufacturer. The histological specimens were blinded, and repair tissue quality was rated by an independent observer. For the quantitative histological evaluation, the O'Driscoll score was applied.

Statistical analysis

Statistical analysis was performed with the help of the SAS system (SAS 9.1 software package, SAS Institute, Heidelberg, Germany). Due to dependency within the subjects as an adequate analysis procedure, we used repeated measure ANOVA, with the subject number as repeated factor and an "unstructured" covariance structure. *P* values were adjusted using the method of Tukey–Kramer. All results are given with an accuracy of one decimal.

Results

The macroscopic assessment of the recovered defect areas revealed no signs of inflammation or degeneration. All defects treated with collagen gel, regardless of cell-seeded or not, displayed a complete filling of the defect and a smooth surface at all time points. The integration of the repair tissue to the underlying bone and adjacent cartilage was excellent (Figs. 2, 3). After 6 weeks, histological staining revealed a high content of homogeneously distributed cells inhabiting the initially cell-free collagen gel (Fig. 2). Cellular morphology was chondrocyte-like in both treatment groups. After 1 year, both treatment groups showed a mature hyaline-like repair tissue, while the untreated control defects were filled with a fibrous repair

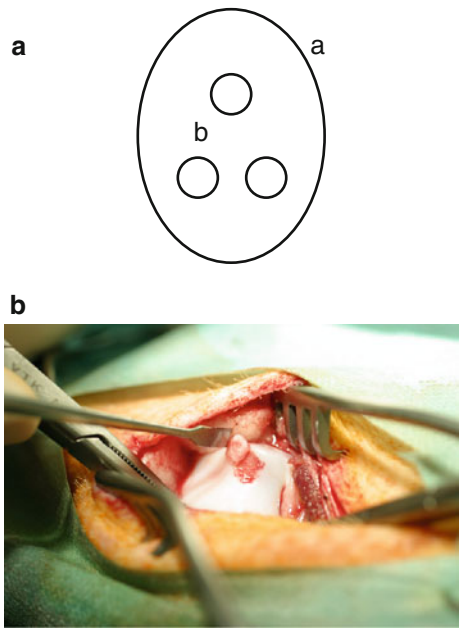

Fig. 1 **a** Schematic drawing of chondral defect positioning on the condylus (*a*) condylus; (*b*) chondral defects. **b** Intra-operative picture of defect creation

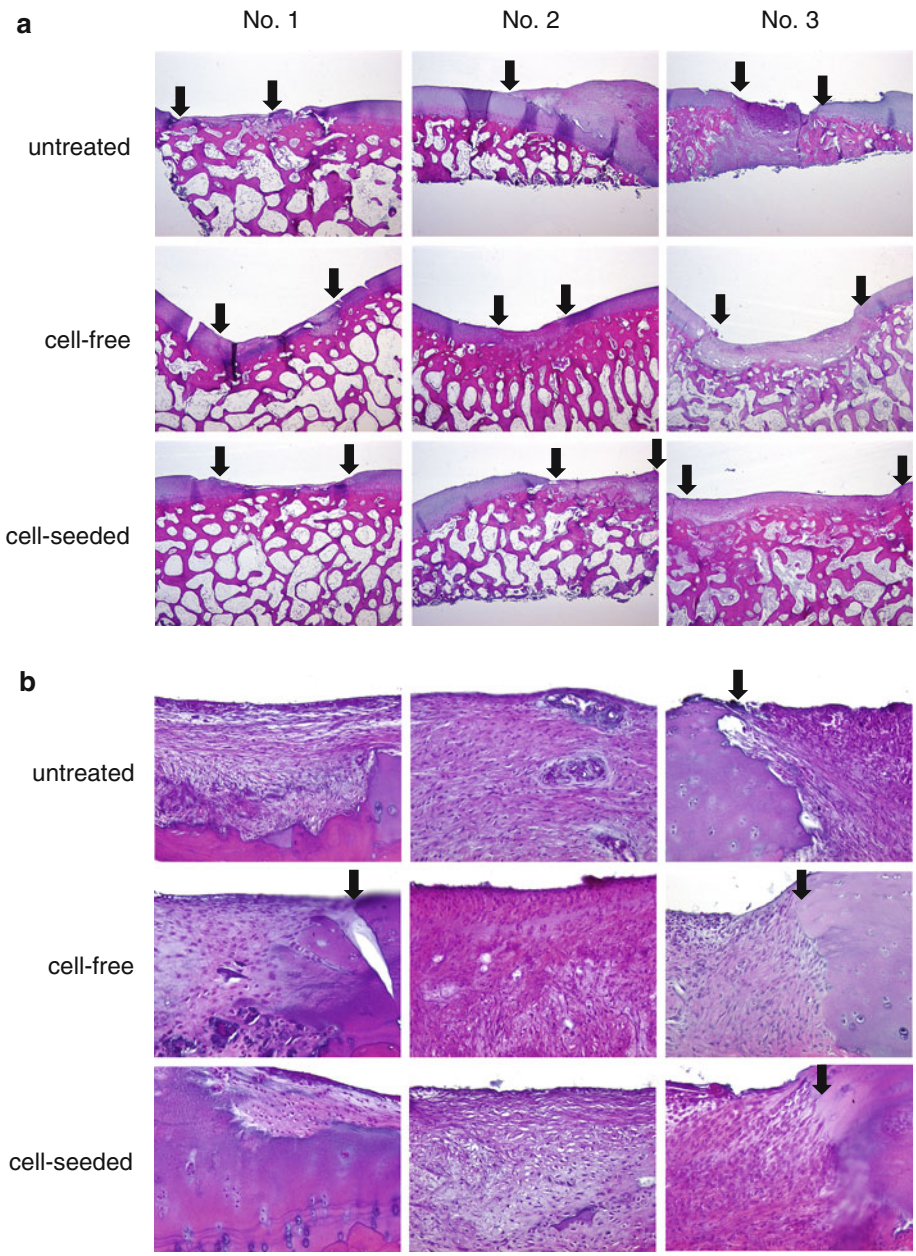

Fig. 2 HE staining of cartilage defects treated with cell-seeded collagen gel, cell-free collagen gel or left untreated after 6 weeks in the Goettinger Minipig. Shown are three of six specimens. **a** Original magnification ×16; **b** Original magnification ×200. *Borders* between surrounding cartilage and regenerative tissue are marked with an *arrow*

tissue, which started to shrink after 1 year (Figs. 2, 3). Figure 4 illustrates the maturation of the immigrated cells during maturation.

The collagen type-II staining of defects treated with an initially cell-free collagen gel was weak after 6 weeks and continuously increasing until 1 year (Fig. 4). Cell-seeded collagen gel revealed an equal amount of collagen type-II protein based on the semiquantitative immunostaining, whereas col-II staining of untreated control defects stayed weak (data not shown).

Statistical evaluation of the O'Driscoll score revealed higher scores in the two groups treated with collagen gel with respect to the untreated control group (Fig. 5). The multivariate analysis showed a treatment ($P < 0.0001$) and time ($P = 0.0488$) effect. Using contrasts for pair comparisons of the treatments showed a significant difference between the control (group 1) and active treatments (group 2: cell-free gel, group 3: cell-seeded gel) ($P < 0.0001$ for both contrasts) and no differences between the two active treatments of groups 2 and 3 ($P =$ n.s.).

Looking at the data, for the time variable, a contrast with weights (1, −0.5, −0.5) was chosen yielding a significant difference between 6 weeks on the one hand and 3 month and a year each weighted half on the other hand ($P = 0.0156$).

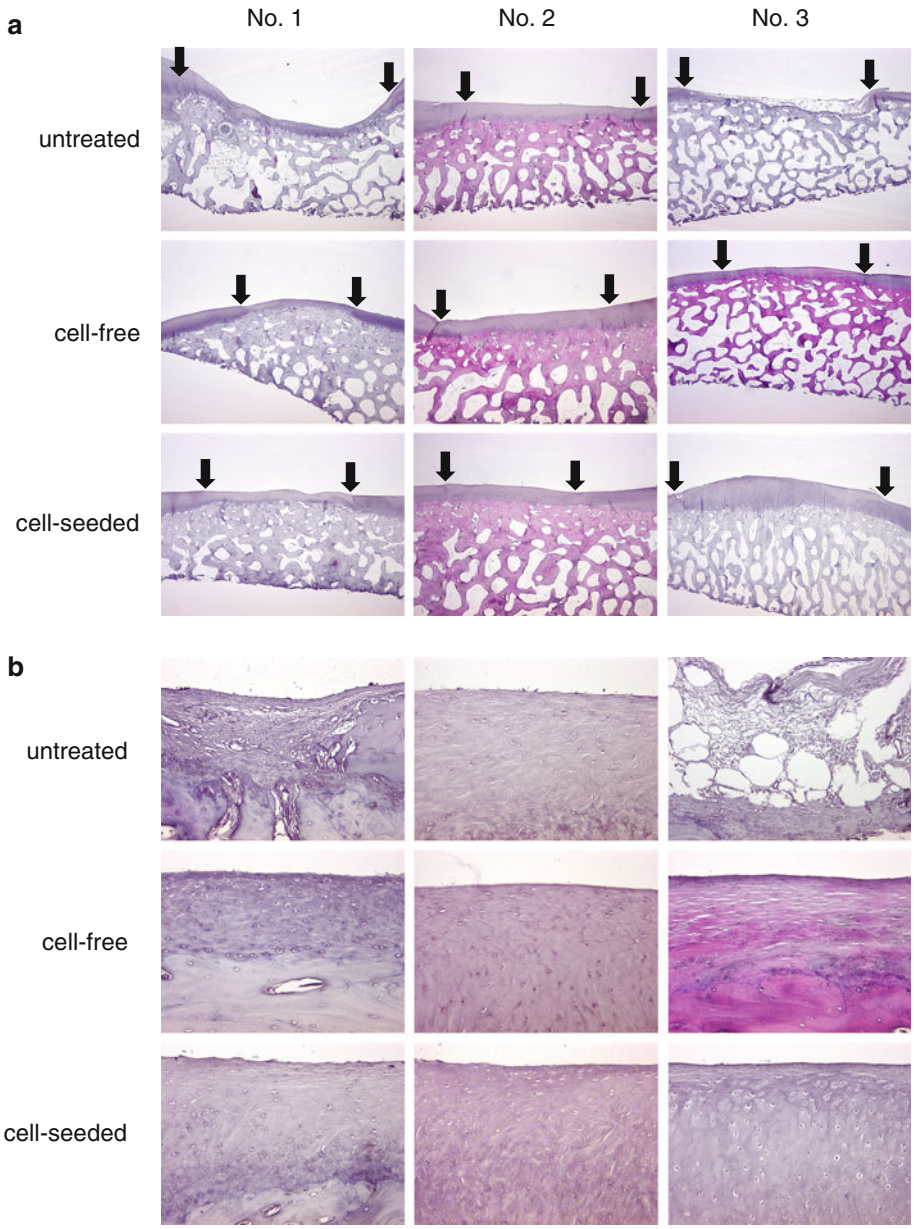

Fig. 3 HE staining of cartilage defects treated with cell-seeded collagen gel, cell-free collagen gel or left untreated after 1 year in the Goettinger Minipig. Shown are three of six specimens. **a** Original magnification ×16; **b** Original magnification ×200. *Borders* between surrounding cartilage and regenerative tissue are marked with an *arrow*

Repair tissue quality with respect to this scoring system was improving in the two collagen gel-treated groups until week 12. After 1 year, no further improvement was observed. The O'Driscoll scores of initially cell-free collagen gel and cell-seeded collagen gel were nearly the same at all three time points. The scores of both groups were significantly elevated with respect to the untreated control at all time points.

The calculated relative E-modules of the untreated control group and the first treatment group with cell-free collagen gel were increasing after 3 months followed by a decrease after 1 year (Fig. 6), whereas the relative E-modules of the second treatment group with cell-seeded collagen gel showed a continuous increase. After 1 year, this group revealed the highest relative E-module.

Discussion

The presented study demonstrated an equal articular cartilage repair tissue quality of cell-free and cell-seeded collagen gel implants in the Goettinger minipig.

Tissue engineering techniques are increasingly used to treat symptomatic knee defects. In the meantime, the implantation of autologous chondrocytes, initially invented

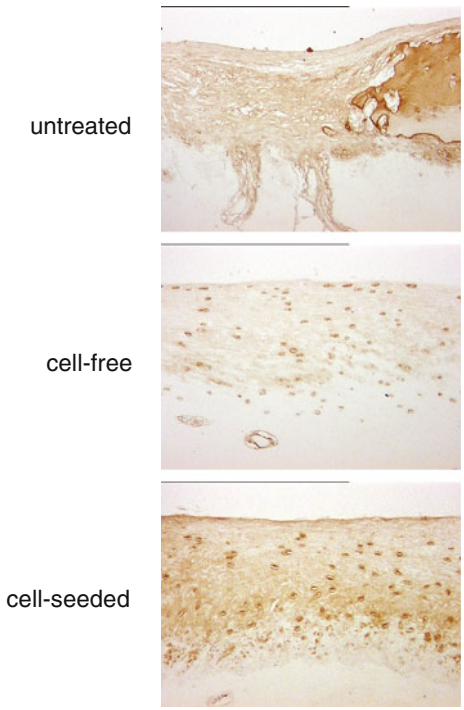

Fig. 4 Collagen type-II immunochemical staining of cartilage defects treated with an initially cell-free collagen gel after 1 year in the Goettinger Minipig. Original magnification ×200

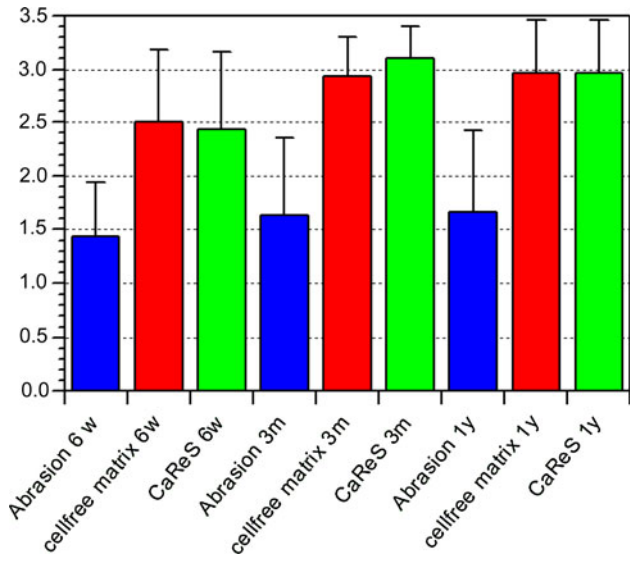

Fig. 5 O'Driscoll scores of cartilage defects left untreated (*left column*), treated with cell-free collagen gel (*middle column*) or cell-seeded collagen gel (*right column*) after 6 weeks, 3 months or 1 year in the Goettinger Minipig. Given are the means ± SD. $N = 6$

by Brittberg et al. in 1994 [5], has been improved by delivering autologous chondrocytes seeded on 3D matrices of different origins. As an alternative cell source, mesenchymal stem cells have been frequently used in the assessment of cartilage repair [23]. During the last years,

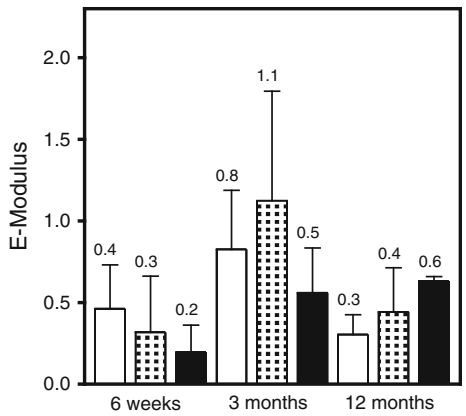

Fig. 6 E-modules of the repair tissue relative to the surrounding healthy cartilage after 6 weeks, 3 months, and 1 year. *Left column* untreated defect, *middle column* cell-free collagen gel, *right column* cell-seeded collagen gel. Given are the means ± SD. $N = 6$

collagen has been widely used as a matrix material and has been applied as sponges or gels [15]. Due to its good biocompatibility, various cell-seeded collagen matrices have been studied in cartilage repair [7, 8, 13, 22, 27, 30]. In particular, collagen gels have gained attraction as a three-dimensional culture system. They have demonstrated their capacity to promote chondrocyte proliferation and proteoglycan synthesis in vitro [34, 35].

In the presented study, a collagen type-I gel made of tails of inbreeding rats has been used, which is in clinical practice for some years (CaReS^{TM}, Arthro Kinetics, Esslingen, Germany) [1, 9, 10]. The main feature of this gel is that chondrocytes are seeded in low density of approximately 2×10^5 chondrocytes/ml, avoiding a prolonged cellular amplification and dedifferentiation. This is in contrast to most other cartilage repair systems, as they require a much higher chondrocyte number.

To investigate cartilage repair, several large animal models have been applied, including goat [26], sheep [16], horse [36], and pig. To study the effects of cell-free and cell-seeded matrix implantation, the current study used the Goettinger minipig, a large animal model that has been widely applied in cartilage and bone repair studies [12, 14, 29]. Jung et al. [21] compared the healing capacity of osteochondral defects created in the patellar groove and the medial femoral condyle and found both localizations suitable for studies on osteochondral healing. The chondral defects of the current study were set in the trochlear groove.

With the help of this model, a wide variety of matrix-based repair techniques has been assessed [4, 32]; even a direct intra-articular injection of mesenchymal stem cells has been performed [24] and an injection of mesenchymal stem cells in chondral defects covered with a collagen I/III membrane [20].

Some studies have addressed the question of a cell-free treatment of cartilage defects, mostly as a control for a cell-based approach. Besides several advantages as being a relatively short and inexpensive procedure, cell-free treatments of cartilage defects lack the risk of potential donor-site morbidity. These degenerative changes are often related to the harvesting site, mostly when osteochondral plugs are taken [30]. Recently, this issue has been reviewed by Matricali et al. [28].

To our knowledge, until now, no study has demonstrated an equal repair tissue quality of cell-seeded and cell-free implants. Li et al. reported the production of a mostly fibrocartilage-like tissue, when full cartilage defects in a porcine model were treated with an acellular biodegradable poly (epsilon-caprolactone) (PCL) nanofibrous scaffold [25]. The same observation was made by Filová et al. [6].

The importance of a matrix system best suited for the specific application is highlighted by another study. Jung et al. evaluated the effect of growth and differentiation factor-5 (rhGDF-5) combined with a cell-free collagen type-I/hyaluronate matrix (c/h) on osteochondral defect repair in a minipig model [19]. After 1 year postoperatively, they observed an insufficient bone and cartilage healing and concluded that other matrices may be better suited in improving osteochondral defect treatment.

In contrast, the presented study revealed an excellent repair tissue quality with both approaches, keeping in mind that all defects were chondral without any stimulation of the bone marrow. Unfortunately, the data evaluation of this prospective study revealed no statistical significance. Matrix remodeling was monitored for up to 1 year postoperatively, which is comparable to several other studies [19, 32]. Regarding the presented results, no inflammatory reaction at all has been observed, demonstrating the good biocompatibility of the collagen gel source.

The cell-free collagen type-I gel used in the presented set of experiments proved to facilitate cellular in-growth in vitro and in a nude mouse-based contained cartilage defect model [11]. Cellular in-growth of host cells into initially cell-free implants of different matrix materials has been observed before [33]. However, with regard to the presented experiments, the source of the immigrating cells remains unclear and may be consisting of cells from the synovial fluid and the surrounding cartilage. Additionally, self-regenerative processes in cartilage healing may have contributed to the observed matrix remodeling. This has been described in previous studies [21]. Therefore, the results presented in this study may differ from the human setting. This has to be evaluated in future experiments. In contrast to other matrix systems, the current study demonstrated a comparable repair tissue quality regardless of the concomitant implantation of autologous chondrocytes. This may be a major advancement in cartilage tissue engineering as surgical techniques based on cell-free implants require only one treatment. Additionally, they may be less time consuming, expensive, and wearisome for the patient.

Conclusion

In the Goettinger minipig, the implantation of a cell-free collagen type-I gel triggered cellular in-growth and created a good quality repair tissue after 1-year follow-up. This repair tissue equals a cell-based approach with respect to cellular morphology, collagen type-II production, and the O'Driscoll scoring system. In the histological evaluation, a slight hypertrophic reaction of the underlying bone was found. Because no autologous cartilage is needed, the donor-site morbidity could eventually be reduced by this method. This study demonstrates the high chondrogenic potential of the applied collagen gel.

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Conflict of interest None.

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