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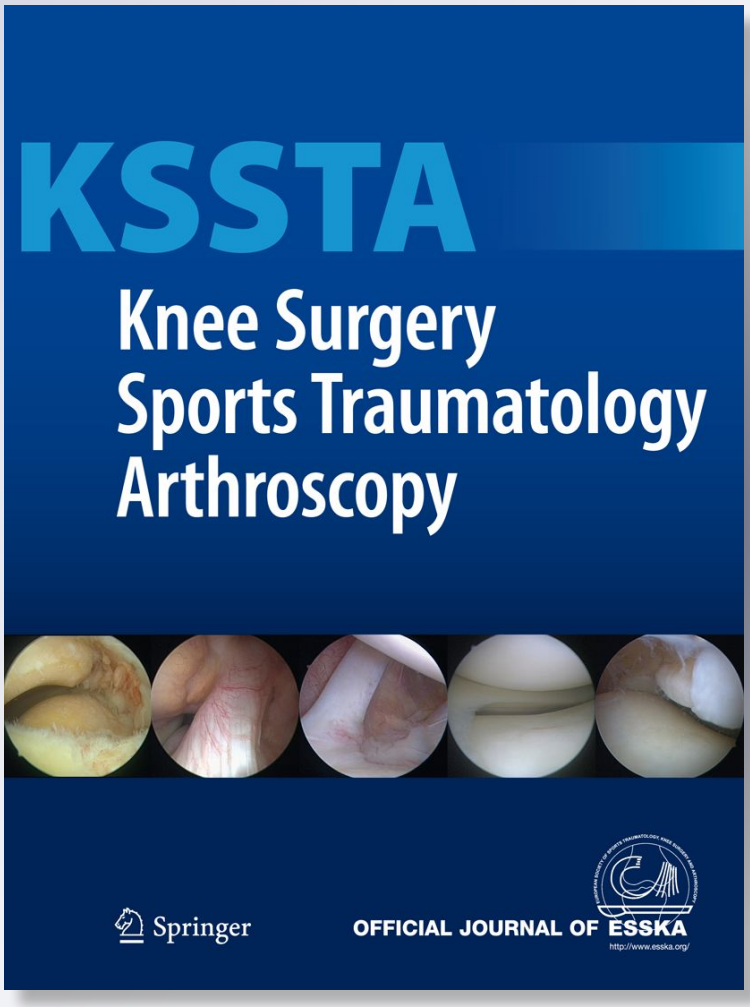
Knee Surgery, Sports Traumatology, Arthroscopy

ISSN 0942-2056

Knee Surg Sports Traumatol Arthrosc
DOI 10.1007/s00167-011-1847-8



Springer

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EXPERIMENTAL STUDY

Cell-free repair of small cartilage defects in the Goettinger minipig: which defect size is possible?

K. Gavenis · U. Schneider · U. Maus · T. Mumme · R. Muller-Rath · Bernhard Schmidt-Rohlfing · S. Andereya

Received: 21 September 2011 / Accepted: 15 December 2011

Abstract
Purpose Cartilage repair of full-thickness chondral defects in the knees of Goettinger minipigs was assessed by treatment with cell-free collagen type-I gel plugs of three different sizes.
Methods In 6 adult Goettinger minipigs, three full-thickness chondral defects were created in the trochlear groove of one knee of the hind leg. These defects were treated with a cell-free collagen type-I gel plug of 8, 10, or 12 mm diameter. All animals were allowed unlimited weight bearing. After 1 year, the animals were killed. Immediately after recovery, a non-destructive biomechanical testing was performed. The repair tissue quality was evaluated immunohistologically, collagen type-II protein was quantified, and a semiquantitative score (O'Driscoll score) was calculated.
Results After 1 year, a high number of cells migrated into the initially cell-free collagen gel plugs and a hyaline-like repair tissue had been created. The O'Driscoll scores were: 8 mm, 21.2 (SD, 2.8); 10 mm, 21.5 (SD, 1.6); and 12 mm, 22.3 (SD, 1.0). The determination of the e-modulus, creep and relaxation revealed that mechanical properties of the two smaller defects were closer to unaffected hyaline cartilage.
Conclusions As cell-free collagen type-I gel plugs of all three different sizes created hyaline-like repair tissue, this system seems suitable for the treatment of even larger defects.

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

Introduction

The regeneration of articular cartilage is still an unsolved issue. Modern techniques of tissue engineering have led to the formation of hyaline-like cartilage, but this repair tissue is still limited in terms of its biochemical and biomechanical properties.

Today, most cartilage repair techniques rely on the implantation of autologous chondrocytes cultivated on 3D scaffolds [1]. Despite of promising results, these approaches display some inherent disadvantages, as being a time-consuming and expensive two-step procedure. In order to obtain autologous chondrocytes, healthy cartilage has to be harvested, thus limiting cell number and displaying the potential risk of donor-site morbidity.

To avoid the mentioned disadvantages, interest is growing on the development of cell-free approaches in the treatment of cartilage defects. The implantation of cell-free scaffolds offers the opportunity of a one-step procedure, minimising surgical time and patients' detraction. However, inhabiting cells are still needed for matrix remodelling. The immigration of chondrocytes from the surrounding healthy cartilage has recently been reported [19]. Regarding potential scaffolds, a cell-free collagen gel demonstrated the ability to trigger chondrocyte in-growth, proliferation and matrix formation in vitro and in a nude-mouse-based contained defect model [7]. As collagen gels can fit into every defect shape, they are increasingly used as a 3D scaffold for cartilage repair techniques [11].

In a previous study, a cell-free collagen gel proved the ability to create a repair tissue similar to a cell-seeded

Published online: 28 December 2011

treatment group [29]. However, the critical defect size for cell-free cartilage defect repair still had to be evaluated, which was the aim of the present study. Therefore, cartilage defects of different size were created in Goettinger minipigs, a large animal model widely applied in cartilage repair studies [8], and treated with a cell-free collagen gel. Subsequently, matrix remodelling was investigated with regard to cellular in-growth and the production of matrix constituents like collagen type-II and proteoglycans. Additionally, the O'Driscoll score and mechanical properties of the repair tissue were determined.

Methods

Defect creation and treatment

A total of 6 male adult Goettinger minipigs were included in this study. The mean age was 2.6 years (2–4 years), the mean weight at study start was 37 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).

A standardized surgical procedure was used to create three full-thickness cartilage defects of different size in the trochlear groove of the left hind leg of all animals. Defect size was 8, 10 or 12 mm, respectively. Therefore, in each group, 6 samples were investigated. Anaesthesia 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 into the joint was set by transligament access through the ligamentum patellae. The defects were set in the area of the trochlear groove as cylinders with a hollow cutter and a sharp angulated raspatorium. The articluar cartilage was debrided meticulously down to the subchondral bone avoiding bleeding.

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™, Arthro Kinetics, Esslingen). The plugs were supplied in PBS solution and stored at 4°C until implantation. The plugs were trimmed to a diameter of 8, 10 or 12 mm, respectively, with a hollow cutter, and the height was 3 mm.

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 transplants.

At 52 weeks after surgery, the animals were killed by injection of an overdose of barbiturate under general anaesthesia. 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 cylindrical 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 with a nominal force of 5 kN and an accuracy of 20 mN. 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 of the force in relation to distance was found. Then indentation was stopped in order to not destroy the samples. This was repeated investigating the cartilage surrounding the repair tissue. Test–retest reliability was not assessed regarding the biomechanical testing.

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 $\times$ sample thickness)/(Δheight $\times$ 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-micrometre sections were subject to further processing. Haematoxylin/Eosin and Safranin O staining were performed according to standard protocols. For the immunochemical detection of collagen type-II protein, sections were deparaffinised, 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. A monoclonal anti-collagen type-X antibody was purchased from Sigma (St Louis, MO, USA) and incubated in a dilution of 1:2,000 in 1% normal goat serum in phosphate-buffered saline (PBS) overnight. Bound antibody was detected by incubation with goat-anti-rabbit antibody or goat-anti-mouse antibody diluted 1:200 for 1 h at RT. 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, 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 three independent observers. For the quantitative histological evaluation, the O′Driscoll score was applied.

Quantification of collagen type-II protein

To quantify the amount of collagen type-II, a col-II-stained slide of each specimen was examined. To avoid differences in the staining intensity due to technical variations, all samples were stained in parallel. A cell-free collagen sample was stained in parallel, giving the background staining of the col-II antibody. All areas with a more intense staining were considered as col-II positive. With the help of the Adobe Photoshop software (version 7.0), pixels of col-II positive areas and the total pixel number of a given area of the slides were determined. The results were expressed as a ratio with the total pixel number of the image serving as the denominator.

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.

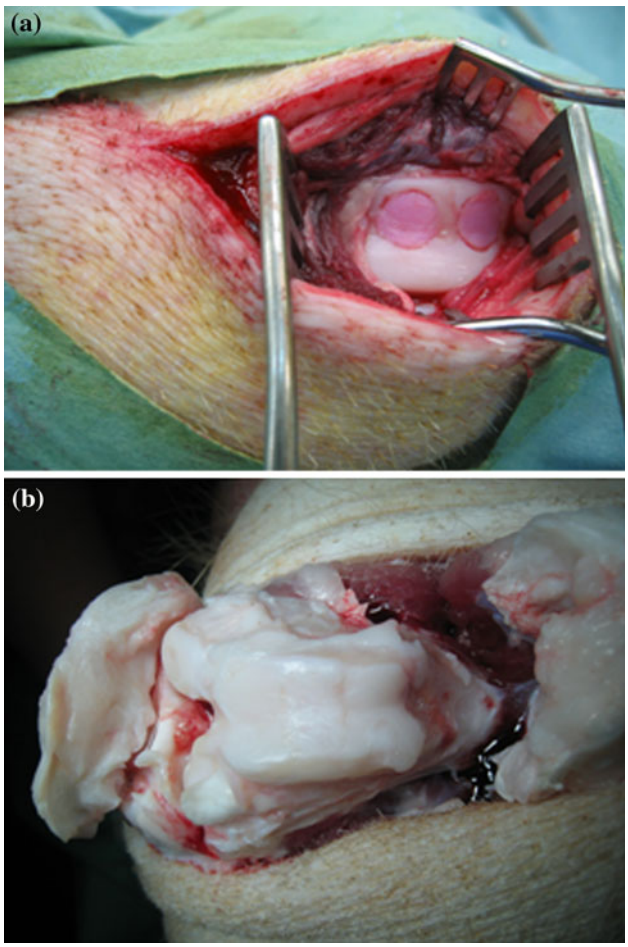

Fig. 1 Macroscopic picture after implantation of the matrix (**a**) and after recovery one year postoperatively (**b**)

Results

After 1 year post-operatively, the macroscopic assessment of the recovered defect areas revealed no signs of inflammation or degeneration. All defects displayed a complete filling of the defect and a smooth surface (Fig.1). The integration of the repair tissue to the underlying bone and adjacent cartilage was excellent (Fig. 2). The initially cell-free collagen gel plugs were homogeneously inhabited by cells, regardless of defect size (Fig. 3). No quantification of cell number has been performed. The immigrated cells showed a roundish, chondrocyte-like morphology and produced collagen type-II and proteoglycans (Fig. 3). The collagen type-II was stored mainly pericellularly, but some areas revealed a more cartilage-like collagen structure with an expanded ECM. While the cellular organization in some areas was low, cells in some areas started to build the characteristic palisade-like structure of hyaline cartilage in the deeper zones (Figs. 3, 4). The collagen type-X staining revealed no signs of hypertrophy regardless of defect size (Fig. 4). Counting the pixels of collagen type-II positive areas, we determined 1.0 (SD, 0.7) for the 8-mm defect, 1.3 (SD, 1.2) for the 10-mm defect and 1.9 (SD, 2.3) for the 12-mm defect (Fig. 5). Regarding the mechanical properties, we determined the e-modulus, creep and relaxation of the repair tissue and the surrounding healthy cartilage (Fig. 6). The mechanical properties of the small- and medium-sized defects were close to each other and closer to the healthy cartilage than mechanical properties of the large defect. The overall assessment of the repair tissue quality by O'Driscoll scoring revealed a higher score with regard to the larger defects: 8 mm, 21.2 (SD, 2.8); 10 mm, 21.5 (SD, 1.6); and 12 mm, 22.3 (SD, 1.0). All statistical data were non-significant.

Discussion

The most important finding of the present study was that a cell-free collagen gel can facilitate the production of hyaline-like cartilage in small cartilage defects of up to 12 mm in diameter.

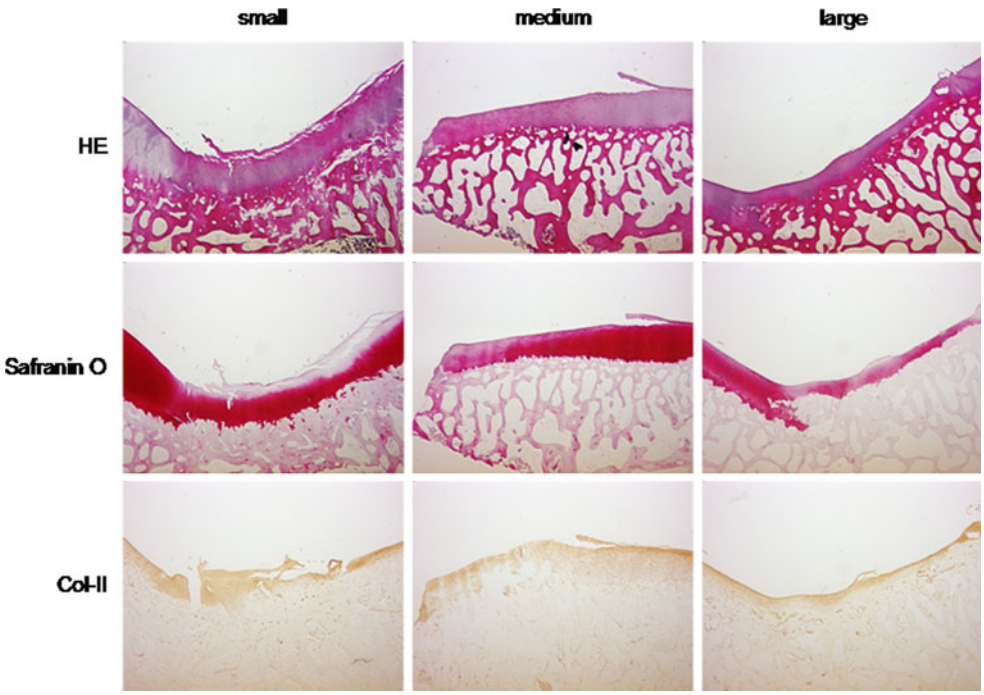

Fig. 2 Hematoxylin–eosin, Safranin O and collagen type-II immunostaining of chondral defects treated with cell-free collagen type-I gel plugs of three different sizes. Original magnification ×16

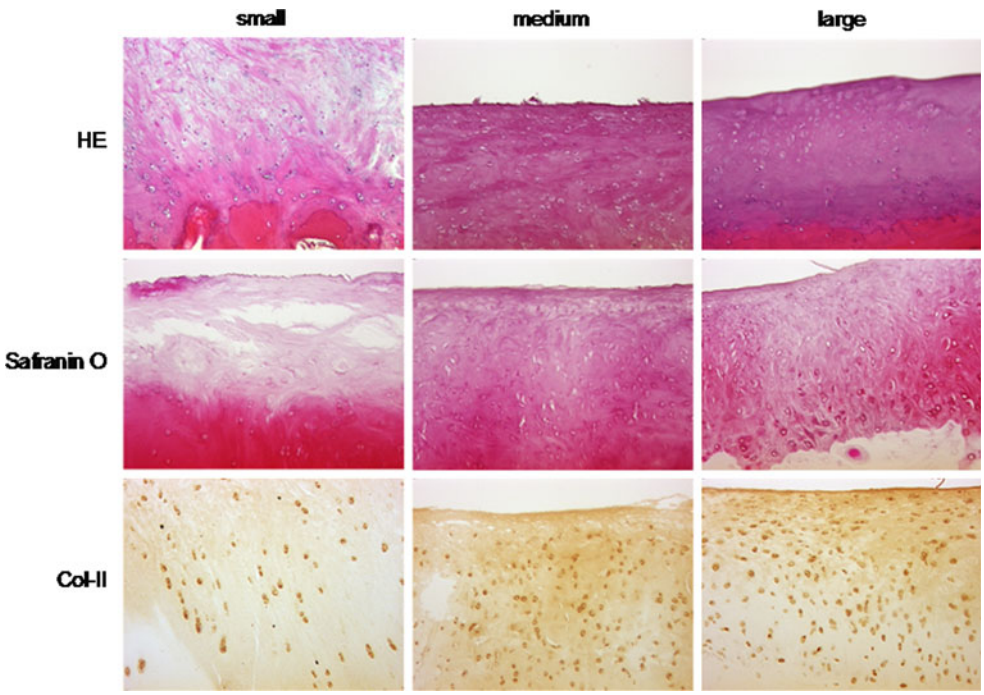

Fig. 3 Hematoxylin–eosin, Safranin O and collagen type-II immunostaining of chondral defects treated with cell-free collagen type-I gel plugs of three different sizes. Original magnification ×200

Cartilage defect size has a major impact on the induction of osteoarthritis. However, defect size has to be determined with care, as arthroscopic assessment tends to overestimate defect size, especially with regard to small cartilage defects [22]. Even small defects of 7 mm in diameter induced local osteoarthritis at the medial compartment in sheep joints after 12 weeks, whereas a 14-mm defect caused significant degenerative changes at the tibial articular cartilage [28]. Guettler et al. [9] demonstrated an altered load distribution for cartilage lesions of 10 mm in diameter and greater in size. They concluded 10 mm being a threshold for biomechanical alteration. Regarding therapy options, small cartilage lesions are commonly treated with conventional techniques like osteochondral autograft transplantation [16], microfracture [18] or left untreated. Defect size treated in this manner started from 0.84 cm^2 [16].

Today, chondral lesions of 2–3 cm^2 and larger are recommended for cell-based matrix implantation techniques

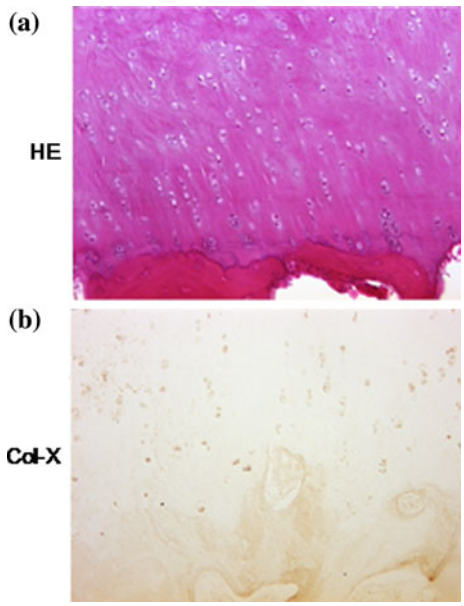

Fig. 4 Hematoxylin–eosin staining (**a**) and collagen type-X immunostaining (**b**) of a chondral defect in the Goettinger minipig treated with a cell-free collagen type-I gel plug after 1 year. Original magnification ×200

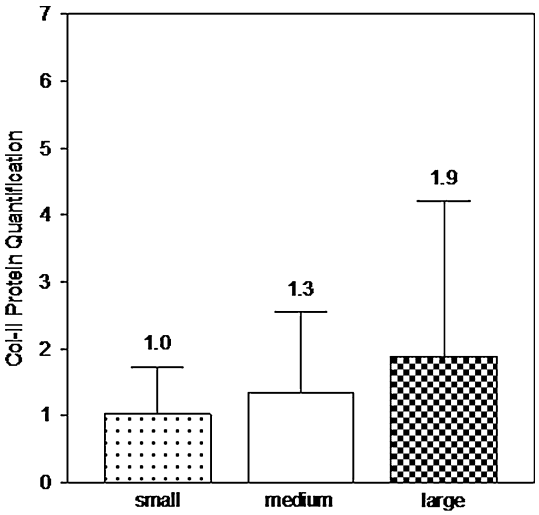

Fig. 5 Quantification of collagen type-II staining intensity of small (8 mm), medium (10 mm) and large (12 mm) cartilage defects treated with a cell-free collagen gel plug after 1 year. Given are mean ± SD. $N = 6$

(ACI) [2], although a direct comparison of ACI versus microfracture revealed a comparative functional outcome after 2 years [30]. Importantly, long-term results and repair tissue quality may differ from short-term outcome.

In recent studies, chondral lesions treated with advanced tissue engineering techniques reached from 2.5 ± 1 cm^2 [5] to larger than 10 cm^2 [23]. In the present study, three chondral defects of 8 mm in diameter to 12 mm in diameter were compared, in line with the mentioned studies on small cartilage defects.

Several large animal models have been used to assess cartilage repair, including sheep [10], horse [31] and goat [15]. Based on its limited capability for endogenous repair of chondral and osteochondral defects, the Goettinger minipig is regarded as a large animal model well suited for the investigation of cartilage repair [8]. Consequently, a wide variety of cartilage repair techniques has been studied in the Goettinger minipig [14, 20, 25, 26].

Although current tissue engineering techniques revealed promising mid- to long-term results, further improvements are needed. Long-term follow-up of advanced techniques of autologous chondrocyte implantation (ACI) revealed only 57% of complete defect filling and 62% of complete integration of the graft after 7 years [5]. IKDC score improved over time and reached a final value after 7 years.

Moreover, cell-based techniques display some inherent drawbacks. They rely on a two-step procedure with one operation required for cell harvesting and a second operation for implantation of the cell–matrix construct. This holds true for the majority of current cell-based techniques [6, 17]. Consequently, a cell-free system avoids one operation and donor-site morbidity. Additionally, a cell-free technique can be considered time-saving and cost-effective.

A different approach to overcome the limitations of the current tissue engineering techniques is the use of cell sources other than autologous chondrocytes. Peterbauer-Scherb et al. isolated bone marrow mesenchymal stem cells (BMSCs) from Goettinger minipigs and characterized their chondrogenic potential. They found BMSCs in principle suitable for future one-step surgical procedures. Importantly, all experiments were performed in vitro with pellet cultures [24]. Jung et al. treated chondral defects in the Goettinger minipig with a combination of adult mesenchymal stem cells and a covering collagen membrane. They observed a more homogeneous cellular distribution and an improvement of overall repair tissue quality compared with defects treated with collagen membrane alone [13].

Only little evidence is gained with cell-free repair of articular cartilage defects. This treatment has been used mostly as a control for a cell-based approach [12] or in combination with microfracture for defect coverage [4]. Moreover, the implanted scaffold is of crucial importance.

Zantop et al. introduced a cell-free approach, combining microfracturing with a resorbable polymer felt and a sodium hyaluronan matrix delivered in a one-step procedure. They observed a complete defect filling with cartilaginous repair tissue [32]. Recently, a composite scaffold of chitosan micro- and nanofibres and collagen-I failed in building a good-quality repair tissue in the Goettinger minipig when compared with cell-seeded scaffold [21]. The O'Driscoll score of this cell-free scaffold was 5.3 ± 2.88. In comparison, the O'Driscoll scores of the

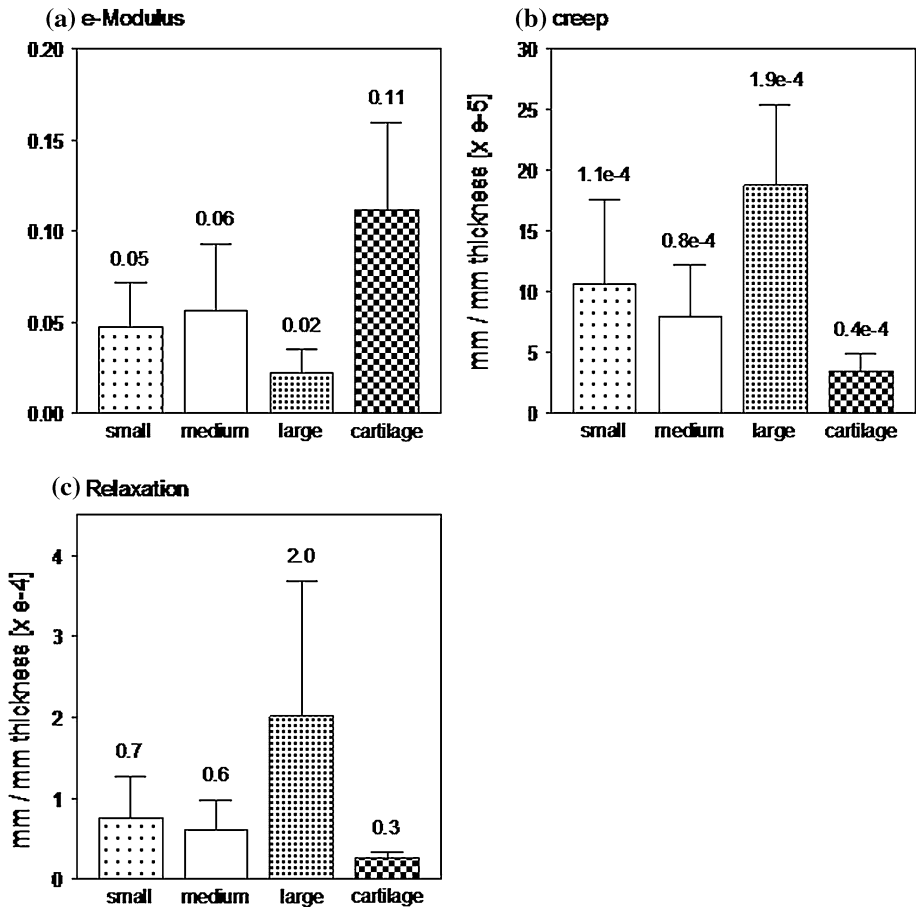

Fig. 6 Mechanical determination of the e-modulus (**a**), creep (**b**), and relaxation (**c**) of chondral defects treated with cell-free collagen type-I gel plugs of three different sizes, compared with the surrounding healthy cartilage. Given are mean ± SD. $N = 6$

cell-free collagen gel used in the present study were 21.2 (8 mm; SD, 2.8), 21.5 (10 mm; SD, 1.6) and 22.3 (12 mm; SD, 1.0) after 1 year follow-up. Interestingly, quantification of col-II protein revealed higher amounts in the larger defects. Unfortunately, the data were not significant due to a high inter-individual variability. Moreover, the computer-aided quantification of histological slides has some inherent drawbacks and has to be interpreted with care.

The ability for cartilage repair of the cell-free collagen gel investigated in this study recently was demonstrated in a similar study [29]. The O'Driscoll score of this cell-free gel was comparable to collagen gel seeded with autologous chondrocytes and twice as high as empty defect included as a control.

Regarding the mechanical properties of the repair tissue, small- and medium-sized defects were closer to the surrounding healthy cartilage. As only very basic biomechanical testing has been performed in the presented set-up, which do not fully describe the biomechanical characteristics of the repair tissue, this has to be verified by more sophisticated methods.

A different study demonstrated the ability of a cell-free alginate–gelatin hydrogel to facilitate chondral regeneration in the sheep model, although a control group with autologous chondrocytes revealed slightly better results [27]. Eggelet et al. [3] treated full-thickness chondral defects in sheep with a combination of a cell-free PGA scaffold, autologous serum and hyaluronan following microfracture. After 3 months, a cartilaginous repair tissue was formed. Regarding the results of the present study, no microfracture was needed for cellular recruitment and repair tissue formation.

In contrast, a cell-free collagen gel demonstrated the ability to trigger chondrocyte in-growth, proliferation and matrix formation in vitro and in a nude-mouse-based contained defect model [7]. As collagen gels can fit into every defect shape, they are increasingly used as a 3D scaffold for cartilage repair techniques [11] and seem to be well suited for cell-free cartilage repair.

Regarding the presented data, no quantification of cell number after recovery has been performed. Additionally, cellular origin remains a matter of speculation, as they may consist of immigrated cells or cells produced by proliferation. Future studies should address repair tissue formation in larger defects, as 12 mm does not seem to be the defect limit for the investigated cell-free collagen gel. To avoid non-significant data, more elaborated investigational methods, namely in the field of biomechanical testing, have to be applied.

Tissue engineering techniques are of growing clinical relevance in the field of cartilage defect treatment. The presented cell-free cartilage repair method is a one-step technique and therefore can be considered time-saving and cost-effective. The use of cell-free matrix systems, namely collagen gels, may lead to the improvement of existing tissue engineering techniques, eventually by arthroscopic treatment of small cartilage defects.

Conclusion

A cell-free collagen gel triggered cellular in-growth and matrix remodelling in small cartilage defects in the Goettinger minipig. As the assessment of the repair tissue revealed a good quality according to the O'Driscoll score even in the largest defect of 12 mm in diameter, there are indications that this cell-free system seems suitable for the treatment of even larger defects. This has to be investigated in future studies.

Conflict of interest The authors declare that they have no conflict of interest.

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