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Treatment of articular cartilage defects in horses with polymer-based cartilage tissue engineering grafts

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Abstract

The objective of our study was to evaluate the integration of autologous cartilage tissue engineering transplants based on resorbable polyglactin/polydioxanone scaffolds into full-thickness cartilage defects of horses. Cartilage biopsies were taken from the non-load-bearing area of the lateral talus of the left tibiotarsal joint of eight healthy Haflinger horses. Tissue engineering cartilage transplants were generated by three-dimensional arrangement of autologous chondrocytes in biocompatible and resorbable polymer scaffolds. Full-thickness cartilage defects of 8 mm in diameter were created in the tubular bone condyle of the fetlock joint and cartilage grafts were fixed using an anchor system, while defects without grafting served as controls. After 6 and 12 months the repair tissue was evaluated histologically and showed formation of a cartilaginous tissue and good integration into the surrounding host tissue with firm bonding of the graft to the adjacent cartilage and the underlying subchondral bone. Biochemical analysis demonstrated that the content of glycosaminoglycans and hydroxyproline is comparable in repair tissue derived from treated and control defects. The use of three-dimensional autologous cartilage transplants based on resorbable polymer scaffolds ensures secure fixation, good integration of the graft into cartilage lesions, and is therefore suggested as a promising therapeutic option for the treatment of cartilage defects.

Keywords: Tissue engineering; Autologous cartilage repair; Cartilage regeneration; Polyglactin/polydioxanone scaffold; Horse model

1. Introduction

Traumatic joint defects and degenerative joint diseases such as osteoarthritis lead to severe cartilage lesions that may be accompanied by pain, immobility, stiffness and progressive joint destruction. Since articular cartilage has a low capacity to renew, surgeons aim to cover cartilage lesions and to restore articular surfaces. In recent years, cartilage tissue engineering evolved promising approaches for the regeneration of articular defects based on the application of autologous chondrocytes [1–4]. The most common tissue engineering approach for the restoration of cartilage surfaces and treatment of full-thickness cartilage defects of the knee joint is the autologous chondrocyte transplantation (ACT). Since the report by Brittberg and colleagues, who introduced the clinical application of autologous chondrocytes for the treatment of cartilage defects, several thousands cases have been documented and reported in various publications, showing good results for femoral condyle, osteochondrosis dissecans, patellar and trochlear lesions in at least 75% of the cases [5–7]. The technique was developed in 1994 and is characterized by the injection of an in vitro expanded chondrocyte suspension under a periosteal flap, sewed on the defect [8]. Despite the clinical success of ACT, the technique is limited to distinct and circumscribed defects requiring healthy

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cartilage surrounding the defect. A periosteal flap or adequate biomaterials like collagen sheets are sewed onto the healthy joint cartilage or sealed with fibrin glue. This "pocket" serves as a reservoir for the injection of the chondrocyte suspension.

Second generation chondrocyte transplantation approaches focus on the use of appropriate biomaterials combined with autologous chondrocytes. Promising matrix-induced autologous chondrocyte implantation (MACI) approaches include the use of chondrocytes embedded in a collagen-based matrix. The combination of cells and a collagen sponge leads to vital grafts that can be fixed by sewing and additional sealing with fibrin glue. Because of the adhesive properties of the collagen sponge an additional fixation is not necessary in small defects [9,10]. In contrast to gel-like biomaterials or a cell suspension, fixation of matrix-based cartilage grafts is feasible, for instance, with minipins also in critical load-bearing sites [11]. Furthermore, hyaluronan-based scaffolds in combination with autologous chondrocytes showed promising results in the treatment of articular cartilage defects. Based on a cohort of 67 patients, the implantation of culture-expanded chondrocytes in a three-dimensional hyaluronan-based scaffold resulted in improvement of knee conditions, patients' quality of life and knee function. Histological assessment of the grafted site demonstrated formation of hyaline-like repair tissue in the majority of specimens [12].

A more advanced matrix-based ACT technique for the regeneration of cartilage lesions combines the mechanical properties of bioresorbable polymer scaffolds and the tissue development promoting properties of gel-like matrices with autologous chondrocytes [13]. The advantage of those polymer-based grafts is the homogenous three-dimensional cell distribution within the scaffold, the initial mechanical stability and the use of no cover materials like periosteum, resorbable sheets or foils [14].

Mechanical and chondrogenesis promoting properties of polymer-based cartilage grafts were assessed in different animal model systems and showed good formation of a hyaline-like cartilaginous repair tissue with mechanical characteristics that were comparable to native cartilage [15–17]. Thus, the three-dimensional rearrangement of autologous chondrogenic cells in a temporary gel-like extracellular matrix and resorbable polymer scaffolds generates biologically functional cartilage grafts for the repair of human articular cartilage lesions. Taking surgical issues into account, Erggelet and colleagues demonstrated that the use of such a polymer-based cartilage tissue engineering approach allows the generation of customized grafts properly fitting the defect that can be introduced arthroscopically and securely fixed by transosseous anchoring of the grafts with resorbable vicryl sutures [18].

Although initial studies in the field of cartilage regeneration are performed generally in small animal models, large animal models such as dogs, goats and horses more closely resemble the human situation and may therefore be necessary to confirm clinical relevance of a given tissue engineering approach [19]. Aiming for a comprehensive preclinical evaluation of our technique to restore articular surfaces and to confirm existing preclinical findings, we performed the present study in a large animal model system using Haflinger horses. Autologous equine articular chondrocytes were expanded under conventional cell culture conditions, rearranged three-dimensionally using bioresorbable polymer scaffolds, and were used to fill articular defects in the fetlock joint. After 1 year, cartilage grafts were evaluated histologically and showed a good integration of the cartilaginous repair tissue into the surrounding native cartilage and the underlying subchondral bone tissue. Therefore, the use of autologous three-dimensional tissue engineering cartilage grafts is considered to be a promising and clinical relevant approach to regenerate articular lesions also in humans.

2. Materials and methods

2.1. Animals and cartilage harvest

Eight healthy horses (Haflinger horses; gelding (5), mare (3); age 2; weight: approximately 400 kg) were used in this study. The horses were housed in box stalls (3.65 m × 3.65 m) after surgery for the duration of the experiment. The study was approved by the department of animal protection of the regional authorities of Thuringia, Germany in compliance with legislation on animal welfare. After physical and radiographic confirmation that preceding pathologic features did not exist in the fetlock of the left anterior limb, cartilage biopsies were taken from the talocalcanean joint under general anesthesia (isoflurane inhalation). The cartilage harvest was performed arthroscopically from the non-load-bearing area of the lateral talus of the left tibiotarsal joint. To prevent bacterial infections and inflammatory processes after surgery, antibiotics (procain-benzylpenicillin, 10000 I.E./kg body mass, i.m.) and flunixin meglumin (Finadyne, 1.1 mg/kg, body mass, i.v.) were administered for 3 days. At the same time, 100 ml blood was taken from the vena jugularis for serum preparation. Cartilage was harvested aseptically and transported to the laboratory under sterile conditions in RPMI medium.

2.2. Cell isolation and transplant fabrication

Cartilage samples were minced and enzymatically digested as described earlier [13]. Resulting chondrocytes were resuspended in RPMI medium (Biochrom, Germany) supplemented with 1% horse serum, 5% fetal bovine serum (Biochrom, Germany), penicillin (100 U/ml) and streptomycin (100 μg/ml) and seeded into tissue culture flasks at a density of 2×10^5 cells/cm^2. The medium was replaced every other day. After reaching 90% confluence, the cells were detached using a solution of 0.05% trypsin and 0.02% EDTA (Biochrom, Germany) and replated.

For transplant fabrication, cells (passage 3–4) were detached and mixed with low melting point-agarose (Sigma, Germany). The cell-agarose-suspension was reassembled three-dimensionally in polyglactin/polydioxanone scaffolds (Ethicon, Germany) as described [20]. The transplants were cultured in Ham's culture medium supplemented with 5% autologous horse serum, penicillin (100 U/ml), streptomycin (100 μg/ml), and 50 μg/ml ascorbic acid (Sigma, Germany) for 3 weeks before transplantation. The medium was replaced every other day.

2.3. Transplantation

Arthrotomy was performed in the tubular bone condyle of the fetlock of an anterior limp 1 cm lateral of the verticillus. A 3 cm incision was made

through the skin, and after dissection of the subcutaneous tissues, the joint was opened. A full-thickness circular articular cartilage defect was created in the tubular bone condyle of the fetlock of an anterior limb with an 8 mm diameter cutter as described previously [21]. Chondrocyte-loaded scaffolds were fixed using a 1.5 mm (diameter) wide titanium thread anchor system (Mitek® Miniquick-Anchor-Plus) with resorbable polyglactin sutures. Anchors were fixed in the subchondral bone via four drilling holes. Full-thickness articular cartilage defects without treatment with cartilage tissue engineering grafts served as controls. The wound was closed and protected by a wound dressing.

To prevent bacterial infections and inflammatory processes after surgery, procain-benzylpenicillin (10000 I.E./kg body mass, i.m.) and flunixin meglumin (Finadyne, 1.1 mg/kg, body mass, i.v.) were administered for 5 days. The wound dressing was replaced on the 2nd and 7th day after surgery. Postoperatively, the animals were allowed to move after resting in boxes for 20 days.

2.4. Clinical examination

A veterinarian examined the horses both clinically and orthopedically. Lameness was evaluated in a standing position, when walking and trotting, the latter one included flexion tests. 6 and 12 months after transplantation, horses were sedated by 0.08 mg/kg body mass Romifidinhydrochloride (Sedivet® Boehringer Ingelheim Vetmedica GmbH, Germany) and were euthanized by means of intravenous injection of 20 mg/kg body mass Embutramide, 5 mg/kg body mass Mebezoniumjodide and 0.5 mg Tetracainhydrochloride (T61 ad us. vet.® Intervet, Germany). After Euthanasia, the entire joint region was dissected, examined macroscopically, radiographically and by Magnetic Resonance Imaging (MRI). Radiographs of the joints were taken using a Hofmann SelectorMD (Gierth HF 300, Germany) and Kodak T-MAT PLUS DG radiographic films (Kodak, Germany). Two different projections were obtained from each horse in a standing position: one dorsopalmar (0°) and one lateromedial (90°). MRI scans were performed in the sagittal, transversal and coronal plane sections with a 1.5 T magnet (Gyroscan Intera Master, Philips Germany), first using T2-weighted sequences, and then a three-dimensional gradient echo sequence. The slice thickness was 2.5–3 mm.

2.5. Histological examination and scoring

To evaluate the formation of a cartilaginous repair tissue, histopathological and biochemical analysis of the samples were performed after 6 and 12 months. Samples were fixed in 4% formalin, embedded in methylmetacrylate (Merck, Germany) and sectioned. Sections were stained with Giemsa and von Kossa/Paragon. To evaluate the outcome of the newly formed cartilaginous tissue in comparison to the natural healing of the untreated control defects, we used the Wakitani Score [22] with slight modifications (Table 1). Two scientists performed independently the scoring of histological specimen. The student's t test was used for the statistical analysis of histological results. Differences were considered statistically significant when p was less than 0.05. Data values are given as the average ± standard deviation (SD).

2.6. Biochemical examination

The content of hydroxyproline was measured by high-performance liquid chromatography (HPLC) and fluorescence detection after acidic hydrolysis of the cartilage samples with 6 M HCl, at 110 °C for 24 h as described [23]. The hydrolyzate was neutralized with NaOH, diluted in distilled water and derivated with borate buffer (pH 9.5). The quantification of glycosaminoglycans was performed after digestion of samples for 4 h, at 60 °C with papain followed by a reaction with 1.9-dimethylmethylen blue and photometric analysis (650 nm). Data values are given as the average ± SD.

Table 1
Modified score according to Wakitani

Characteristics of repair tissue	Score
Thickness of cartilage	
>2/3 of native cartilage	0
1/3–2/3 of native cartilage	1
<1.3 of native cartilage	2
Surface regularity	
Smooth (>3/4)	0
Moderate (>1/2–3/4)	1
Irregular (>1/4–1/2)	2
Highly irregular (<1/4)	3
Integration of grafts into host tissue	
Full integration	0
> 1/2 integrated	1
<1/2 integration	2
Parallelism of cell columns	
And isogenic groups	
Parallelism and isogenic groups present	0
Little divergence, few isogenic groups	1
Strong divergence, minor isogenic groups	2
No parallelism, no isogenic groups	3
Texture of transition line	
Smooth	0
Corrugated	1
Jagged	2
None	3
Characteristic of tangential zone	
Strong	0
Moderate	1
None	2
Maximum score	15

3. Results

3.1. Clinical assessment

All horses showed a marginal temperature increase over 2 days after surgery. No lameness was observed, except for one horse of the 12-months-transplant group, which showed a distinct lameness for 8 days. After resting in box stalls for 20 days, the horses were taken out to little paddocks during the day. The horses could graze the whole day. Independent of control or transplant group three horses were sound at walk and trot after week 12 of the study and five horses were sound at walk and showed a marginal lameness at trot. There were no differences in motion and flexion of treated and control limbs in all horses. Radiological findings of all horses were normal at all points in time, no signs of osteoarthritis, osteophyte formation, exostosis, nor joint flattening were observed. There were no dislocations of any of the implanted anchors (Fig. 1A). After 12 months, MRI analysis showed that cartilage lesions were covered with repair tissue without damage of the subchondral bone. No abnormalities and covering of the cartilage lesions were detected by MRI

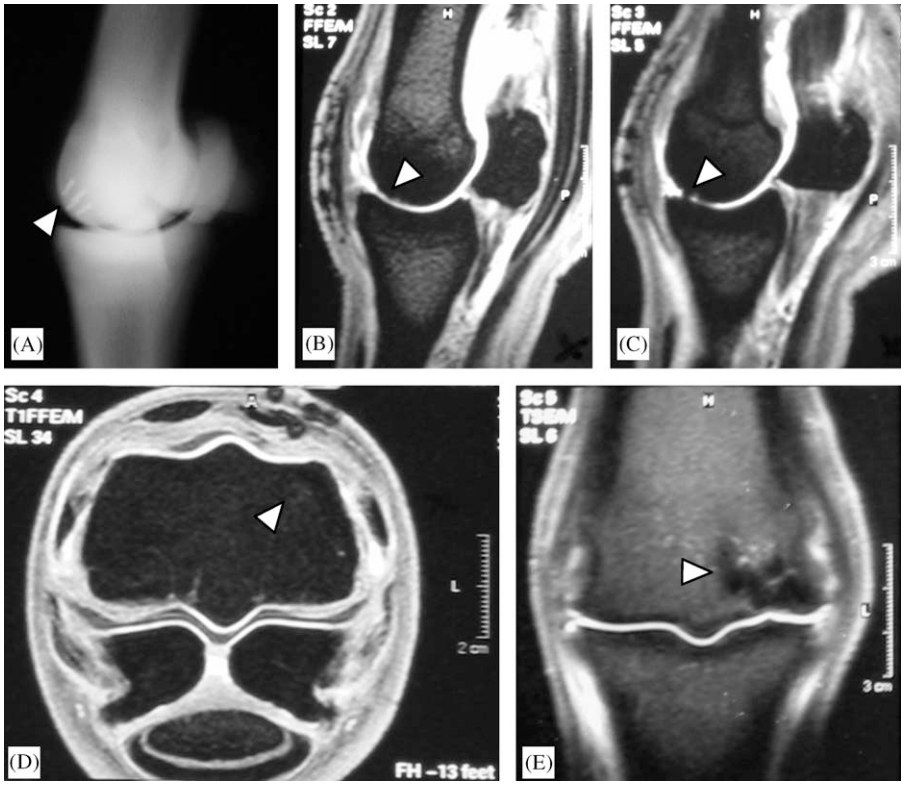

Fig. 1. Radiological and MRI analysis of full-thickness cartilage lesions treated with autologous chondrocytes embedded in polyglactin/polydioxanone scaffolds after a 12-month time period. Horses treated with cartilage tissue engineering grafts showed no radiological abnormalities. Dislocation of implanted titanium anchors (white triangle) was not evident (A). MRI analysis showed no abnormalities and covering of the cartilage lesion (B, white triangle). At the graft site of one horse, small lesions (white triangle) were evident 12 months after transplantation (C, D). On the level of subchondral fixed titanium anchors, the bone marrow gave an altered MRI signal (white triangle) due to remodeling of the subchondral bone (E).

analysis (Fig. 1B). At the graft site of one horse, small lesions were evident that were characterized by an altered MRI signal compared to normal cartilage (Fig. 1C and 1D). The transplants showed a good integration into the surrounding native articular cartilage as well as into the underlying subchondral bone structure. No signs of articular constriction or transplant loosening nor debonding could be detected, no subchondral edema was observed. On the level of subchondral fixed titanium anchors, the bone marrow gave an altered MRI signal (Fig. 1E). Scoring of cartilage lesions according to Outerbridge [24] showed grade 2–3 lesions in the 1-year transplant group, while controls graded 3–4.

3.2. Histological analysis

Macroscopically, the repair tissue was well integrated into the adjacent cartilage in controls and treated defects. In all samples treated with three-dimensional cartilage grafts, we observed two diagonal cracks through the graft site. Transplant integration into the defect site was analyzed histologically by Giemsa staining with emphasis on the transition zone between native cartilage and newly formed repair tissue as well as on bonding of the repair tissue to the subchondral bone (Fig. 2). After 6 months, the defect was filled in full thickness with cartilaginous repair tissue and showed a good integration to the native cartilage. The subchondral bone plate was damaged according to the creation of cartilage lesions, but the repair tissue showed a good bonding to the underlying bone. The repair tissue showed evenly distributed cells and the presence of stained extracellular cartilaginous matrix components (Fig. 2A). Control defects were filled with a thin, cell-rich fibrous tissue layer and showed no signs of extracellular cartilaginous matrix (Fig. 2B). 12 months after implantation of the tissue engineering cartilage grafts, histological staining showed a good defect filling with firm bonding of the newly formed repair tissue to native cartilage and to the subchondral bone plate. The repair tissue was rich in evenly distributed cells and is characterized by the formation of cartilaginous extracellular matrix (Fig. 2C). The thickness of the repair tissue achieved the level of the adjacent cartilage with a regular surface displaying a smooth transition from the native to the repair tissue (Fig. 2E). The control defects filled irregularly with an inhomogeneous fibrous tissue and showed less staining of extracellular matrix, mainly in the depth of the defect near the subchondral bone plate (Fig. 2D). The fibrous repair tissue showed bonding to the native cartilage and an irregularity of the surface at the transition from

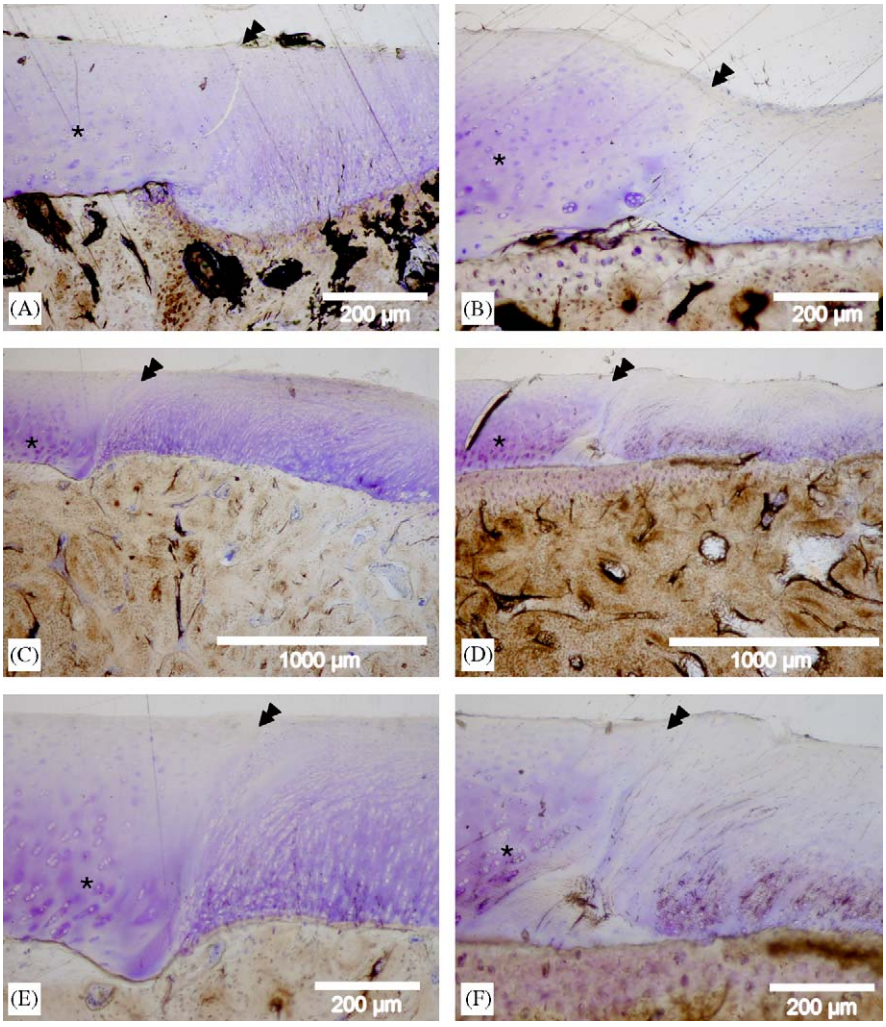

Fig. 2. Histological analysis of autologous cartilage transplants based on polymer scaffolds. Sections of defects treated with autologous cartilage grafts and untreated controls were stained with Giemsa. Histological analysis was performed with emphasis on the transition zone (arrows) of native cartilage (asterisks) and newly formed repair tissue. After 6 months, defects treated with cartilage transplants showed formation of repair tissue with a cartilaginous extracellular matrix. The cartilage tissue engineering graft showed good integration into the adjacent native cartilage (A). Control defects (B) were filled with a cell-rich fibrous tissue with poor cartilaginous matrix formation. 12 months after implantation of the cartilage grafts (C), defects were filled with a cell-rich repair tissue that is characterized by a regular surface, the formation of a cartilaginous extracellular matrix, and by a smooth transition from the adjacent cartilage to the repair tissue (E). Control defects were filled with an inhomogeneous fibrous tissue with less staining of extracellular matrix components (D). The fibrous repair tissue showed bonding to the adjacent cartilage with surface irregularity and a zone almost free of cells bridging the repair tissue and the adjacent cartilage (F).

native to the defect site. In addition, a zone almost free of cells characterized the transition of adjacent cartilage to the fibrous repair tissue in controls (Fig. 2F).

The Wakitani Score was applied for evaluating the formation of a cartilaginous repair tissue and the integration of the newly formed tissue into the host tissue (Fig. 3). A low score (min. 0; max. 15) describes a native-like cartilage tissue and a very good integration of the repair tissue into the surrounding host tissue. Controls were rated after 6 months with a score of 9.4 and showed no improvement after 12 months achieving a score of 9.6. The group that received tissue engineering cartilage grafts reached a score of 7.8 after 6 months and improved after 12 months resulting in a score of 6.2. The transplant group and control group were considered significantly different at either point in time (6 months: $p = 0.03$; 12 months: $p = 0.01$).

3.3. Biochemical examination

To assess the amount of newly formed extracellular matrix at defect sites, the content of glucosaminoglycan and hydroxyproline was quantified biochemically (data not shown). Glucosaminoglycan is an important component of proteoglycan and the amino acid hydroxyproline is incorporated in collagen. Both molecules are typical components of a cartilaginous extracellular matrix. The amount of glucosaminoglycan was 2 µg/ml tissue as

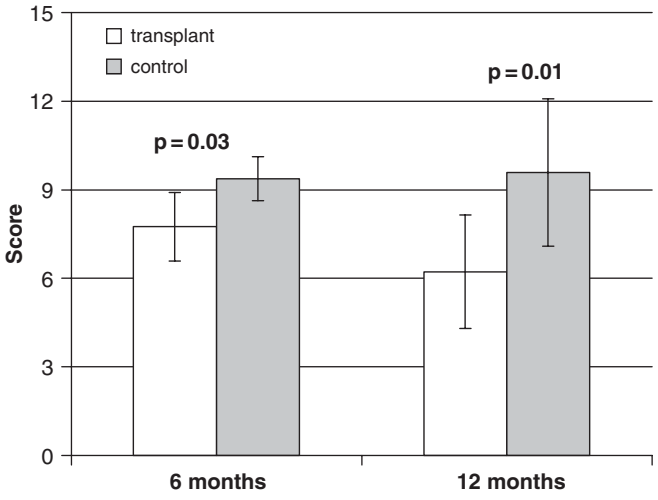

Fig. 3. Histological scoring of newly formed cartilage repair tissue after 6 and 12 months according to Wakitani. Scoring of the newly formed repair tissue according to Wakitani showed that defects that were treated with autologous tissue engineering transplants developed a significantly ($p = 0.03$) better cartilage-related repair tissue than control defects after a time period of 6 months. At 12 months after transplantation, controls showed no improvement with regard to cartilage tissue development, while defects treated with cartilage transplants improved and scored significantly ($p = 0.01$) lower than controls. Error bars represent standard deviation.

determined in biopsies obtained from control defects and defects treated with tissue engineering cartilage grafts. The content of hydroxyproline was similar in biopsies from control and grafted defects with 0.26 μg in controls and 0.3 μg in the tissue engineering repair tissue.

4. Discussion

In the present study, we re-arranged autologous, culture-expanded equine articular chondrocytes three-dimensionally in resorbable polyglactin/polydioxanone scaffolds and used those tissue engineering cartilage grafts to cover full thickness articular cartilage defects. Histologically, cartilage grafts developed after 6–12 months a cartilaginous repair tissue that was firmly bonded to the adjacent native cartilage tissue and to the underlying subchondral bone plate. In contrast, control defects formed a fibrous repair tissue with an irregular surface and less extracellular matrix.

Since cartilage has a low capacity to renew, a variety of surgical techniques evolved to achieve cartilage regeneration. Such techniques include therapeutic interventions like lavage, shaving, debridement, bone marrow stimulating techniques (pridie-drilling, microfracture) and biological replacement strategies. Biological approaches focus on the transplantation of whole tissue, cells as a suspension or combined with different supporting material or scaffolds [25,26]. Meanwhile, although first generation tissue engineering approaches like ACT show good improvement as defined by typical clinical outcome criteria [27,28], techniques using matrix-induced autologous chondrocyte transplantation (MACI) are suggested to be technically more attractive than ACT while clinical, arthroscopic and histological outcomes are comparable for ACT and MACI [2]. In ACT, chondrocyte suspensions are "fixed" in the cartilage defect by closing the defect with a periosteal flap that should prevent the loss of chondrocytes by leakage. In spite of securely suturing or gluing periosteal flaps onto the surrounding cartilage, leakage of chondrocytes into the joint cavity may a major technical drawback of ACT. Using a goat model, Driesang and Hunziker [29] showed that applying ACT and closure of the defects with a periosteal flap led to a delamination of the flaps when free joint movement with or without immobilization was allowed during the recovery period. The immobilization of chondrocytes, as known from MACI, in appropriate biomaterials like fibrin, collagen, and as shown here in resorbable polyglactin/polydioxanone scaffolds prevents loss of chondrocytes into the joint cavity. Moreover, the use of textile polymer structures avoids covering with periosteal flaps and enables secure fixation of the transplant by anchoring of the graft onto the subchondral bone without further damage of the surrounding healthy cartilage. Recently, Erggelet et al. [18] proposed an attractive arthroscopic approach of transplant fixation in human knee joints without introducing non-resorbable titanium anchors into the subchondral bone tissue. The arthroscopic fixation technique uses a transosseous 4-point fixation for the resorbable polymer fleece loaded with autologous chondrocytes and also eliminates a substantial amount of the side effects known to occur after arthrotomic autologous chondrocyte implantation procedures. In addition, resorbable polydioxanone pins may be suited for the fixation of matrix-based cartilage grafts, thus avoiding loosening of the transplant. In a horse model, separated cartilage flaps resulting from osteochondritis dissecans cartilage lesions were reattached by using polydioxanone pins and showed normal radiographic subchondral contours after up to 8 years of follow up [30].

Along with secure fixation of cartilage grafts important issues for the reconstruction of articular surfaces are the firm integration of the grafts into the surrounding bone and cartilage tissue and the formation of a mechanically stable hyaline-like cartilaginous repair tissue.

In recent years, a variety of studies demonstrated that three-dimensional rearrangement of culture expanded chondrocytes in clinically applicable, resorbable biomaterials like collagen [31,32], hyaluronan [33,34], and polyglactin/polydioxanone scaffolds [16,17,35] induces chondrocytic cells to re-differentiate and supports the formation of a hyaline-like cartilage matrix in vitro and in vivo. However, to date, few publications have described the reconstruction of articular cartilage in large animal models, most notably in horses, that underline the clinical relevance of the respective cartilage tissue engineering approach. Using a conventional ACT approach, Litzke and colleagues showed that ACT contributed to the repair of equine full-thickness articular cartilage lesions. After a period of

2 years, clinical and pathological findings were normal in untreated and ACT-treated joints, but ACT resulted in an improved defect filling with a well-integrated repair tissue compared to untreated controls [36]. As a matrix-based cartilage tissue engineering graft, chondrocyte-laden collagen scaffolds were evaluated in full-thickness articular defects in femoropatellar joints of horses. Arthroscopic defect debridement and chondrocyte implantation resulted in increased chondrocyte numbers and cartilage staining in deeper layers of the grafted lesions, whereas ungrafted controls were almost filled with fibrous tissue over an up to 8-month period. Although the collagen-based scaffolds augmented with allogenic chondrocytes resulted in improved cartilage healing, the authors suggested poor long-term durability [37] and that the inadequate matrix content of the repair tissue limits the utility of collagen-based scaffolds for grafting of large articular defects [38]. In contrast, as reported previously by our group, the use of autologous chondrocytes in resorbable polyglactin/polydioxanone scaffolds for the repair of full-thickness articular defects resulted in a similar morphology of the repair tissue compared with the surrounding native cartilage and in the formation of a hyaline-like cartilage tissue rich in typical cartilage matrix molecules [21].

5. Conclusions

In the present study we underlined previous findings by covering full thickness cartilage defects with autologous tissue engineering cartilage grafts in a large animal horse model and demonstrated that the transplantation of autologous chondrocytes in polymer-based scaffolds is a promising approach to cover and reconstruct articular cartilage defects. The use of polyglactin/polydioxanone scaffolds allows the secure fixation of cartilage transplants, which in turn integrate well into the surrounding tissue with firm bonding of the repair tissue to the native cartilage and to the underlying subchondral bone. Therefore, the transplantation of autologous chondrocytes embedded in three-dimensional polymer scaffolds into cartilage defects is suggested to be a clinical applicable and convenient approach to regenerate articular cartilage lesions.

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