INFLUENCE OF *IN VITRO* MATURATION OF ENGINEERED CARTILAGE ON THE OUTCOME OF OSTEOCHONDRAL REPAIR IN A GOAT MODEL

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Abstract

This study was designed to determine if the maturation stage of engineered cartilage implanted in a goat model of cartilage injury influences the repair outcome. Goat engineered cartilage was generated from autologous chondrocytes cultured in hyaluronic acid scaffolds using 2 d, 2 weeks or 6 weeks of pre-culture and implanted above hydroxyapatite/hyaluronic acid sponges into osteochondral defects. Control defects were left untreated or treated with cell-free scaffolds. The quality of repair tissues was assessed 8 weeks or 8 months post implantation by histological staining, modified O'Driscoll scoring and biochemical analyses. Increasing pre-culture time resulted in progressive maturation of the grafts *in vitro*. After 8 weeks *in vivo*, the quality of the repair was not improved by any treatment. After 8 months, O'Driscoll histology scores indicated poor cartilage architecture for untreated (29.7 ± 1.6) and cell-free treated groups (24.3 ± 5.8). The histology score was improved when cellular grafts were implanted, with best scores observed for grafts pre-cultured for 2 weeks (16.3 ± 5.8). As compared to shorter pre-culture times, grafts cultured for 6 weeks (histology score: 22.3 ± 6.4) displayed highest type II/I collagen ratios but also inferior architecture of the surface and within the defect, as well as lower integration with native cartilage. Thus, pre-culture of engineered cartilage for 2 weeks achieved a suitable compromise between tissue maturity and structural/integrative properties of the repair tissue. The data demonstrate that the stage of development of engineered cartilage is an important parameter to be considered in designing cartilage repair strategies.

Key words: Tissue engineering, osteochondral composite, functional graft, scaffold, autologous cells, animal model.

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Introduction

Injuries of the articular cartilage have a poor healing capacity and when left untreated may progress to symptomatic joint degeneration (O'Driscoll, 1998). Large osteochondral defects are associated with joint mechanical instability and are accepted indications for surgical intervention to prevent development of degenerative cartilage disease (Buckwalter and Mankin, 1998). However, the repair tissue resulting from conventional treatment techniques often shows limitations in quality and duration as compared to native tissues (Temenoff and Mikos, 2000). Among the innovative, so called 'cell-based' therapies, a promising approach involves engineering cartilage grafts using scaffolds seeded with cells, possibly pre-cultured *in vitro* before implantation. A number of studies performed in rabbit (Ball *et al*., 2004; Grigolo *et al*., 2001; Schaefer *et al*., 2002), sheep (Kandel *et al*., 2006), or goat (Niederauer *et al*., 2000) aimed at investigating the outcome of cartilage or osteochondral repair using engineered cartilage. Cells were seeded in the scaffolds either directly after isolation or after an expansion phase and further pre-cultured *in vitro* for 48 h (Niederauer *et al*., 2000), 7 d (Ball *et al*., 2004), 4 to 6 weeks (Schaefer *et al*., 2002), or 8 weeks (Kandel *et al*., 2006) before implantation in an orthotopic site. With only one exception (Niederauer *et al*., 2000), an improved healing was observed when cells were added to the scaffolds as compared to implantation of cell-free materials. To our knowledge, however, the influence of engineered cartilage maturation stage, achieved by pre-culture for different time points, on the healing of chondral or osteochondral defects has not yet been systematically investigated.

The stage of biochemical and biomechanical development of an engineered cartilaginous tissue could play a crucial role to determine the load-bearing capacity of the tissue and its integration with surrounding native cartilage upon implantation. Ball *et al.* (2004) suggested that implanting more developed engineered tissues could support enhanced repair. Using a rabbit model, this study reports that preincubating cells in a polylactic acid scaffold resulted in greater donor cell retention in the repair tissue of osteochondral defects. On the other hand, Obradovic *et al.* (2001) pointed out that less developed engineered cartilage could integrate more efficiently with native cartilage due to higher accessibility of cells at the interface. Currently, engineered constructs derived after 2 weeks of culture of autologous human articular

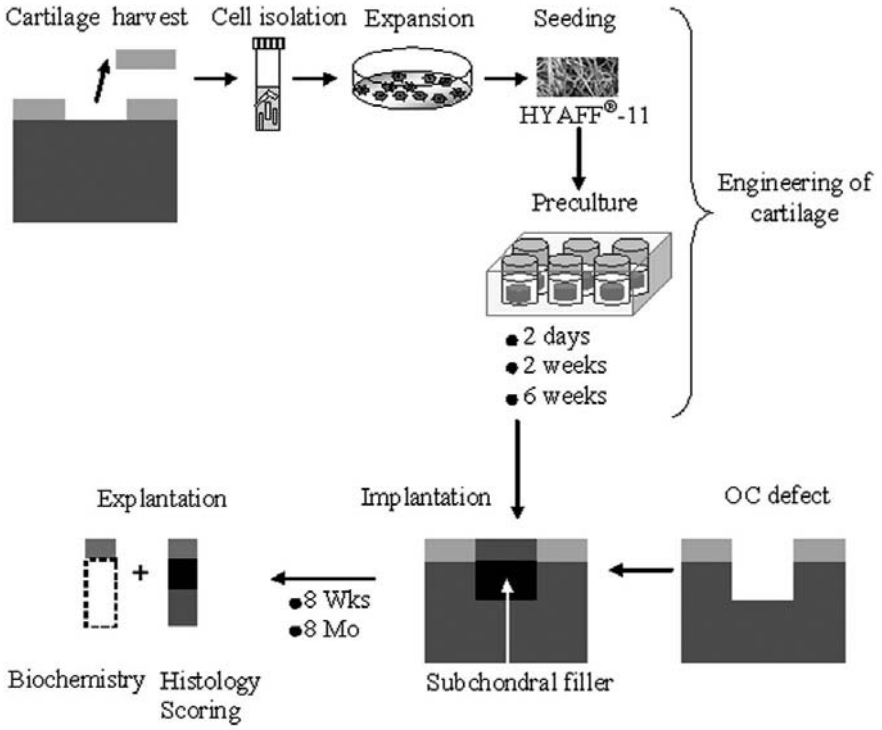

Fig. 1. Schematic representation of the procedure starting from cartilage harvesting from goat knee, engineering of cartilage grafts, creation of osteochondral defects (OC defect) and implantation of the engineered cartilage on top of the subchondral filler. After 8 weeks (8 Wks) or 8 months (8 Mo), explants were analysed biochemically (cartilage repair tissue) and histologically (osteochondral section).

chondrocytes into non-woven meshes of esterified hyaluronic acid (HYAFF®-11) are used as grafts for the repair of cartilage defects in humans (Marcacci *et al*., 2005; Nehrer *et al*., 2006). Indeed, this choice is consistent with a recent study demonstrating that engineered cartilage pre-cultured for 2 weeks has a superior capacity to further develop upon ectopic implantation than constructs grafted without pre-culture (Moretti *et al*., 2005). However, it is still controversial whether implantation of pre-cultured cartilaginous implants as compared to freshly seeded implants can enhance the outcome of orthotopic cartilage repair, and what is the optimal stage of biochemical and biomechanical development which should be reached by an engineered graft to support efficient cartilage repair.

The present study was thus designed to address if the repair outcome of critically sized osteochondral defects (6 mm diameter, 5 mm deep) is influenced by different maturation stages of cartilage grafts in a goat model (Watanabe *et al*., 2009). The goat species, frequently used for cartilage repair studies (Barry, 2003; Dell'Accio *et al*., 2003; Hunziker, 2003; Jackson *et al*., 2001; Murphy *et al*.,2003), was selected due to a combination of relevant cartilage thickness, relatively large stifle size and ease of use, cost and availability as compared to other large size animal models. Moreover, we previously established specific conditions for goat articular chondrocytes isolation, expansion and culture in 3D scaffolds, and demonstrated the feasibility to engineer goat cartilaginous tissues at different stages of development by varying culture time (Miot *et al*., 2006). Goat engineered cartilage was generated *in vitro* from autologous articular chondrocytes pre-cultured in HYAFF®-11 scaffolds for either 2 d, 2 weeks or 6 weeks in a chondrogenic medium and subsequently implanted on top of a subchondral support made of hydroxyapatite and HYAFF®-11 into surgically created osteochondral trochlear defects, which were previously shown not to heal spontaneously in adult Spanish goats (Jackson *et al*., 2001). Experimental settings included osteochondral defects that were left untreated and defects treated with cell-free HYAFF®-11 scaffolds on top of subchondral fillers. The repair of osteochondral defects was assessed 8 weeks or 8 months post-implantation in young adult goats both macroscopically and by means of histological and biochemical analysis.

Materials and Methods

Scaffolds

Cartilaginous constructs were engineered using HYAFF®-11, a non-woven esterified form of hyaluronic acid (Aigner *et al*., 1998). The subchondral support consisted of sponges made of 65 % hydroxyapatite and HYAFF®-11, a composite material with a biological performance so far only assessed in an *in vitro* model (Giordano *et al*., 2006). Disk shaped HYAFF®-11 scaffolds (diameter 6 mm by 1 mm thick) were sterilised by γ-irradiation and provided by FAB S.r.l. (FIDIA Advance Biopolymers, Abano Terme, Italy).

Operative procedures

Twenty seven adult female Saanen goats aged above 18 months were used following the required authorisations and in agreement with institution ethical guidelines. The entire procedure is schematically described in Fig. 1. All animals were unilaterally operated on the posterior left leg and in the first open surgery, under irrigation with PBS, three circular defects of 6 mm diameter and 400-500 μm deep were created in the cartilage of the trochlea groove (proximomedial, distal and lateral locations) using a specially designed punch for a total of 81 cartilage defects. Excised cartilage was either discarded or used for the isolation and expansion of autologous chondrocytes,

Table 1. Description of experimental groups detailing the number of osteochondral defects in each group for the short term follow up (8 weeks; total of 30 defects made in 10 goats) and long term follow up (8 months; total of 51 defects made in 17 goats).

Number of defects	**Group 1:** Untreated defects	**Group 2:** Cell-free scaffold	**Group 3:** Preculture 2 days	**Group 4:** Preculture 2 weeks	**Group 5:** Preculture 6 weeks
8 weeks *in vivo*	6	6	6	6	6
8 months *in vivo*	6	9	12	12	12

as described below. Surgical procedures were performed under general anaesthesia and with the use of sterile techniques. The goats received midazolam (0.4 mg/kg of body weight; Dormicum; Roche Pharma, Reinach, Switzerland) intravenously for sedation. Anaesthesia was induced with a combination of ketamine (3 mg/kg of body weight; Narkan 100; Dr. E. Gräub AG, Berne, Switzerland) and propofol (1 mg/kg of body weight; Propofol 1 % Fresenius; Fresenius Kabi, Stans, Switzerland). After endotracheal intubation, anaesthesia was maintained with one minimal alveolar concentration (MAC) (2.3 %) end-tidal sevoflurane (Sevorane, Abbot AG, Baar, Switzerland). Perioperative and postoperative analgesia was achieved by administering flunixin meglumine (Finadyne, Biokema, Crissier, Switzerland). Penicillin was administered for perioperative antibiosis. Eight weeks after creating the chondral lesions, in a second surgical intervention, osteochondral defects (6 mm diameter x 5 mm deep) were created in each of the three biopsy sites prior to performing the autologous grafting, as described below. This size of defect was previously showed not to heal spontaneously in adult Spanish goats (Jackson *et al*., 2001).

Our experimental design included 5 groups (Table 1). In group 1, osteochondral defects were left untreated. In group 2, defects were treated with a cell-free HYAFF®-11 scaffold and subchondral support. In groups 3, 4 and 5, osteochondral defects were treated with engineered cartilage generated from autologous goat chondrocytes pre-cultured respectively for 2 d, 2 weeks or 6 weeks *in vitro* into HYAFF®-11 and implanted on top of the subchondral support. In groups 2, 3, 4 and 5 the sponges made of HYAFF®-11 and hydroxyapatite were placed at the bottom of the osteochondral defect and then the cell-free HYAFF®-11 scaffolds (Group 2) or engineered constructs (Groups 3, 4 and 5) were placed on top without any additional fixation in order that their surface appeared flush with the articular surface. The operated limb was immobilised for 4 weeks using a cast in order to avoid excessive motion of the joint, previously shown to be associated with a high rate of flap loss (Driesang and Hunziker, 2000). After removal of the cast, animals were returned to free activity and allowed full weight bearing. Goats were sacrificed 8 weeks (10 goats) or 8 months (17 goats) post-operatively by injection of an overdose of Phenobarbital.

Cell cultures

Cell isolation

A minimum of 170 mg of cartilage tissue was harvested from the trochlear defects from each goat. The cartilage was cut into pieces and chondrocytes were isolated upon 22 h incubation at 37 °C in 0.15 % type II collagenase (5.0 units collagenase/mg tissue). Cells were re-suspended in Dulbecco's modified Eagle's medium (DMEM) containing 10 % foetal bovine serum, 4.5 mg/mL D-glucose, 0.1 mM non essential amino acids, 1 mM sodium pyruvate, 100 mM HEPES buffer, 100 U/mL penicillin, 100 µg/mL streptomycin and 0.29 mg/mL glutamine (complete medium).

Cell expansion

Chondrocytes were plated on tissue culture dishes at a density of 10^4 cells/cm^2 and expanded in complete medium supplemented with 5 ng/mL fibroblast growth factor-2 (FGF-2, R&D Systems, Minneapolis, MN, USA) previously shown to enhance goat articular chondrocytes proliferation rate (Miot *et al*., 2006). Cells were cultured in a humidified 37 °C/5 % CO_2 incubator. When cells were sub-confluent, they were detached by treatment with 0.3 % type II collagenase, followed by 0.05 % trypsin/0.53 mM EDTA and subsequently frozen. From the creation of the cartilage defect till the implantation of the engineered tissues, a fixed time of 8 weeks was introduced (Fig. 1). Considering the variable time required for construct cultivation (i.e., from 2 d to 6 weeks), cells from each animal were frozen after the first passage and thawed as required. Expanded goat chondrocytes were re-plated at 5 x 10^3 cells/cm^2. Throughout the expansion phase, medium was replaced twice weekly. Prior to seeding into the scaffold, goat articular chondrocytes had undergone an average of 5.8 ± 1.1 population doublings.

Cell cultivation into 3D scaffolds

HYAFF®-11 scaffolds were pre-wet for a few hours in complete medium containing FCS prior to seeding and quickly blot dried at the time of seeding. Once reaching confluence, chondrocytes were detached and statically seeded (3 x 10^6 cells/scaffold) on the HYAFF®-11 scaffolds placed in a dish coated with a thin film of 1 % agarose. A total of 7 constructs were prepared for each animal. Constructs were statically pre-cultured for 2 d, 2 weeks or 6 weeks in complete medium supplemented with 10 µg/mL insulin, 0.1 mM ascorbic acid 2-phosphate and 10 ng/mL TGFβ3 in a 37 °C/5 % CO_2 incubator. Medium was changed twice weekly. After *in vitro* pre-culture, 3 constructs were implanted in the trochlea (see operative procedures) and 4 constructs were harvested for analysis. Constructs were bisected and analysed by histological and biochemical analysis.

Table 2. Description of modified O'Driscoll scoring system and outcome variables for each category. Normal score for osteochondral tissue corresponds to 0 point whereas the maximal score is 31 points.

Characteristics		Score
1. Filling of defect relative to surface of normal adjacent cartilage	111-125%	1
	91-110%	0
	76-90%	1
	51-75%	2
	26-50%	3
	<25%	4
2. Integration of repair tissue with surrounding articular cartilage	Normal continuity and integration	0
	Decreased cellularity	1
	Gap or lack of continuity on one side	2
	Gap or lack of continuity on two sides	3
3. Matrix staining with safranin O-fast green	Normal	0
	Slightly reduced	1
	Moderately reduced	2
	Substantially reduced	3
	None	4
4. Cellular morphology	Normal	0
Mostly round cells with morphology of chondrocytes	>75% of tissue with columns in radial zone	0
	25-75% of tissue with columns in radial zone	1
	<25% of tissue with columns in radial zone (disorganised)	2
50% round cells with the morphology of chondrocytes	>75% of tissue with columns in radial zone	2
	25-75% of tissue with columns in radial zone	3
	<25% of tissue with columns in radial zone (disorganised)	4
	Mostly spindle-shape (fibroblast-like) cells	5
5. Architecture within entire defect (not including margins)	Normal	0
	1-3 small voids	1
	1-3 large voids	2
	>3 large voids	3
	Clefts or fibrillations	4
6. Architecture of surface	Normal	0
	Slight fibrillation or irregularity	1
	Moderate fibrillation or irregularity	2
	Severe fibrillation or disruption	3
7. Percentage of new subchondral bone		
If new bone is below original tidemark	90-100%	0
	75-89%	1
	50-74%	2
	25-49%	3
	<25%	4
If new bone is above original tidemark		
(average percent of original thickness of repair articular cartilage)	90-100%	0
	75-89%	1
	50-74%	2
	25-49%	3
	<25%	4
8. Formation of tidemark	Complete	0
	75-99%	1
	50-74%	2
	25-49%	3
	<25%	4

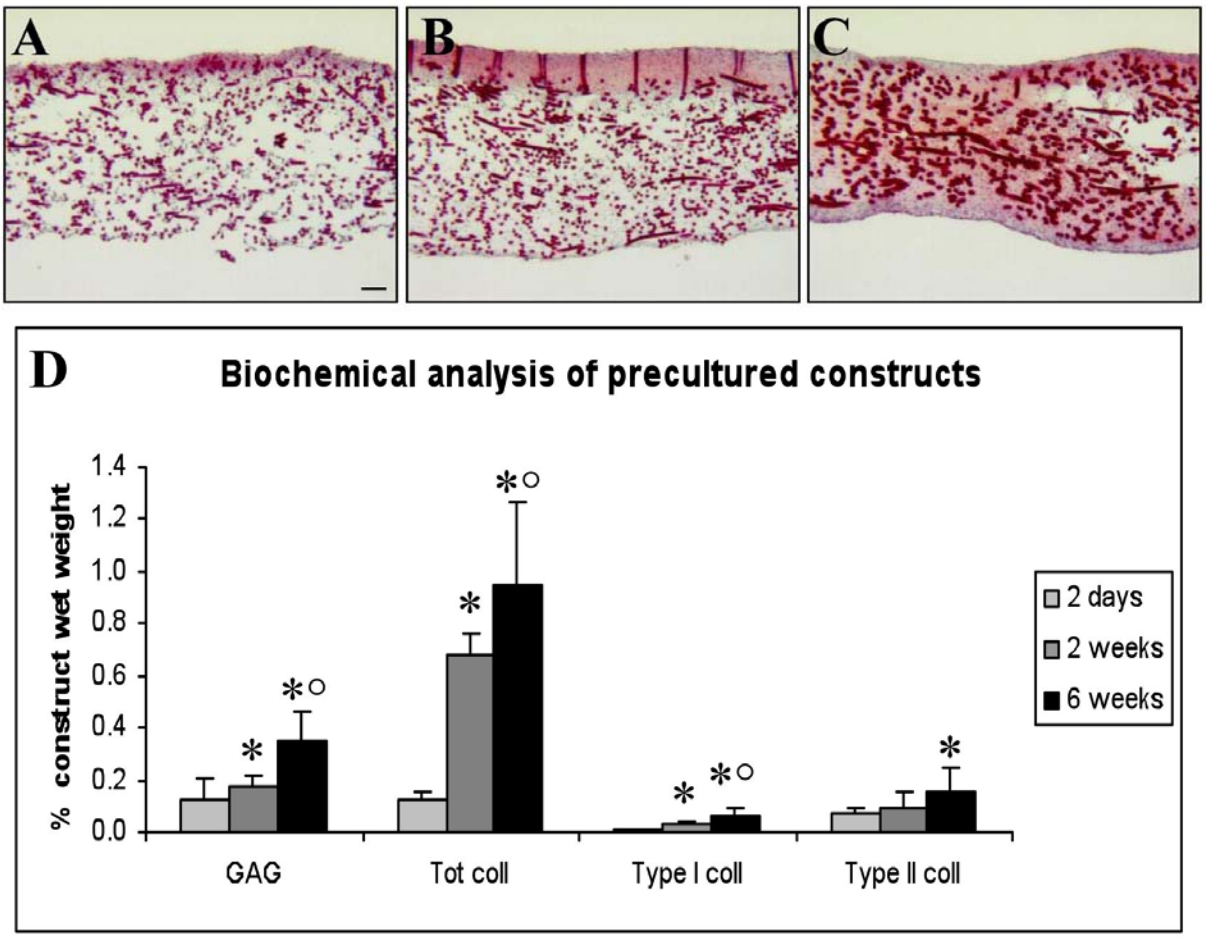

Fig. 2. Histological and biochemical analysis of goat cartilage constructs generated *in vitro*. Safranin O staining of representative constructs generated by goat chondrocytes pre-cultured in HYAFF®-11 for 2 d (**A**), 2 weeks (**B**) or 6 weeks (**C**). Undegraded HYAFF®-11 fibres are strongly stained red by Safranin O. Scale bar: 100 µm. GAG, total collagen, and type I and II collagen contents normalised to the wet weight of tissue in goat cartilaginous constructs pre-cultured for 2 d, 2 weeks or 6 weeks (**D**). * = statistically significant difference from 2 d pre-culture; ° = statistically significant difference from 2 weeks pre-culture.

Histological and biochemical analysis

Cell-scaffold constructs following *in vitro* cultivation were bisected. One half of each sample was fixed in 4 % formalin, embedded in paraffin and cross-sectioned (5 µm thick). Sections were stained for sulphated glycosaminoglycans (GAG) with Safranin O. The second part was used for biochemical analyses, as described below. Tissues following explantation were prepared in small blocks (15 mm W, 15 mm L, 10 mm H). Each block was bisected using a diamond blade saw and the two halves used respectively for histological and biochemical analyses. For histological processing, the explants were decalcified for 8 weeks in EDTA and embedded in paraffin. Using a Leica motorised microtome, sections were cut at different levels of the defect, sequentially stained with Masson trichrome, Safranin O and Alcian blue and scored by one of the authors (PM-V) according to a Modified O'Dricoll classification (Table 2). The following histological variables were separately assessed: filling of the defect, integration of repair tissue, matrix staining with Safranin O, cell morphology, architecture within the entire defect and at the surface, percentage of newly formed subchondral bone, and formation of the tidemark. The grades for each variable were then summed to yield an overall mean O'Driscoll score.

Samples for biochemical analysis were first dissected from the subchondral bony tissue (if derived from explants) and weighed to determine the wet weight. The samples were solubilised by digestion with TPCK-treated bovine pancreatic trypsin in 50 mM Tris-HCl, pH 7.6, using an initial incubation of 15 h at 37 ºC followed by a further 2 h incubation at 65 ºC after the addition of further fresh trypsin (Dickinson *et al*., 2005). Samples were boiled for 15 min to inactivate the enzyme. The amount of GAG present in each trypsin digest was measured using the dimethylmethylene blue colorimetric assay (Handley and Buttle, 1995). Amounts of type II collagen (CII) were assayed by inhibition ELISA using a mouse IgG monoclonal antibody to denatured CII (Hollander *et al*., 1994) and levels of type I collagen (CI) were measured by inhibition ELISA using a rabbit anti-peptide antibody against CI (Dickinson *et al*., 2005). Mature and immature collagen cross-links were measured in the trypsin digests, as previously described (Kafienah and Sims, 2004).

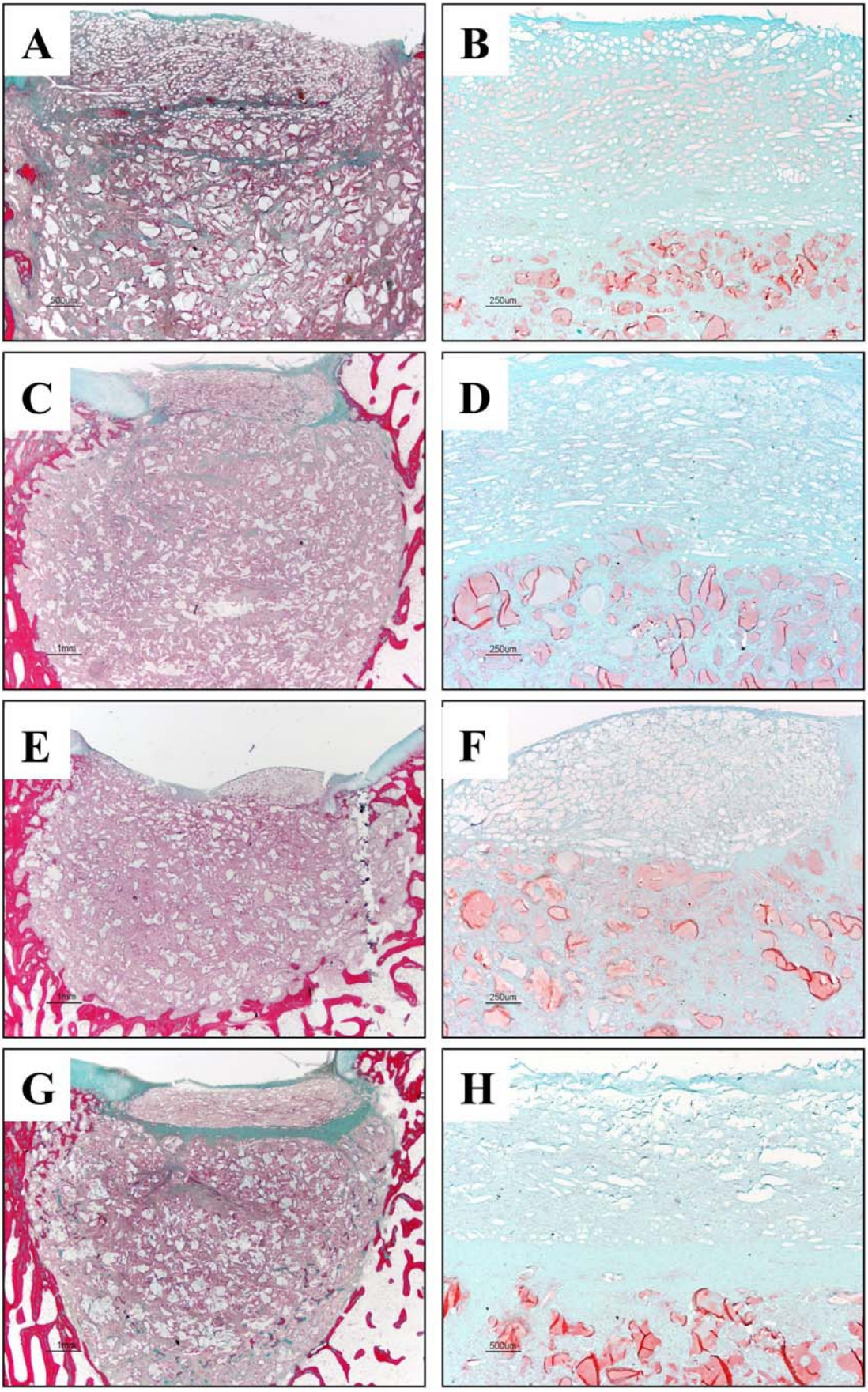

Fig. 3. Histological analysis of osteochondral repair tissues 8 weeks post-implantation (8 goats). (**A**, **C**, **E**, **G**) represent sections of the entire defects stained by Masson trichrome; (**B**, **D**, **F**, **H**) represent sections of the cartilaginous repair tissue from the same defects stained by Safranin O. Defects were either treated with a HYAFF®-11 scaffold without cells (**A**, **B**); or with autologous chondrocytes pre-cultured for 2 d (**C**, **D**), 2 weeks (**E**, **F**), or 6 weeks (**G**, **H**).

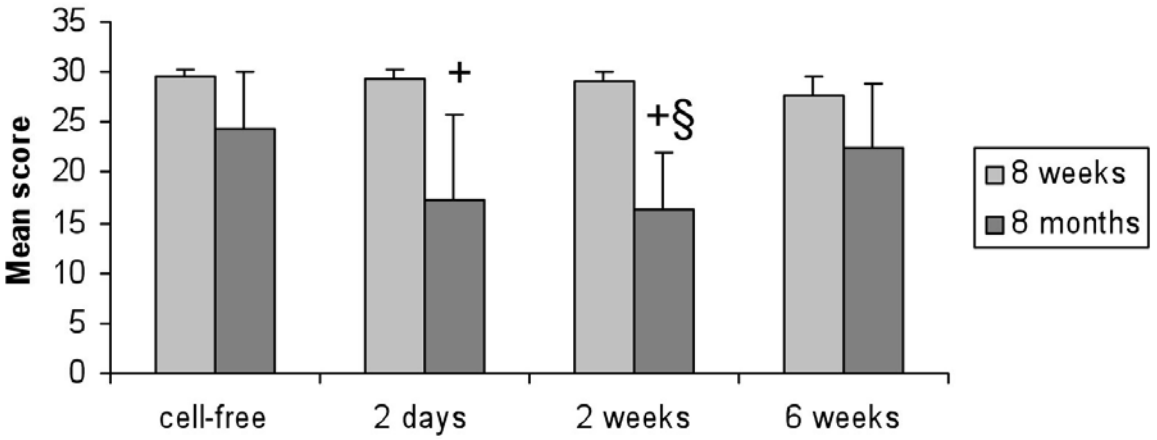

Fig. 4. Modified O'Driscoll scores for osteochondral repair tissues explanted after 8 weeks (8 goats) or 8 months (15 goats) *in vivo*. Defects were either treated with a HYAFF®-11 scaffold without cells (2 goats at 8 weeks; 3 goats at 8 months) or with chondrocytes pre-cultured for 2 d, 2 weeks or 6 weeks (for each cell-treated group: 2 goats at 8 weeks; 4 goats at 8 months). $^{+}$ = statistically significant difference from cell-free for the same implantation time; § = statistically significant difference from 6 weeks for the same implantation time.

Briefly, samples were reduced with sodium borohydride to stabilise the immature cross-links. That was followed by acid hydrolysis in 6 N hydrochloric acid at 110 °C for 24 h. Excess acid was removed by lyophilisation and the dried hydrolysate reconstituted in a mixture of butanol-acetic acid-water (4:1:1). An aliquot was removed for determination of total collagen content by hydroxyproline using a Biochrom20 Plus amino acid analyser equipped with post-column ninhydrin detection. The remaining sample was chromatographed on a CF1 cellulose column (Whatman, Maidstone, UK) to remove the non-cross-linking amino acids. The immature and mature cross-links were then eluted from the CF1 column with water and quantified by amino acid analysis on a Biochrom 20 Plus amino acid analyser optimised for the analysis of collagen cross-links.

Statistical analysis

Unless otherwise mentioned, data are presented as mean ± standard deviation. Means were compared using either Student's *t*-test or Mann Whitney test depending on the normality of the populations, which was tested by Shapiro-Wilk tests. To assess any influence of defect location on the total scores, the results were analysed using one-way ANOVA. Statistical analyses were performed using the Sigma Stat Software (SPSS Inc. version 13.0), with $p < 0.05$ as the criteria for statistical significance.

Results

Extracellular matrix deposition in pre-cultured constructs

For each animal, the quality of *in vitro* engineered cartilage was assessed by histological and biochemical analyses in order to confirm that implants exhibited different degrees of maturation according to the pre-culture time. Representative construct sections stained by Safranin O are shown in Fig. 2A-C. The intensity of staining for sulphated GAG was observed to increase with cultivation time, from 2 d to 6 weeks pre-culture. Extracellular matrix (ECM) was initially mainly present at the periphery of the constructs and then its deposition progressed towards the centre of the engineered tissue. Biochemical analysis of pre-cultured constructs showed that GAG, total collagen, type I collagen and type II collagen, expressed per construct wet weight, all significantly increased from 2 d to 6 weeks pre-culture (2.9-fold, 7.5-fold, 10.0-fold and 2.0-fold, respectively) (Fig. 2D) (p values < 0.001, < 0.001, < 0.001, 0.015, respectively). The amount of type II collagen in engineered cartilaginous tissues was 2.5-fold higher than those of type I collagen after 6 weeks pre-culture.

Histological evaluation of osteochondral repair

Constructs pre-cultured for the different times were implanted into osteochondral defects in the trochlea of goats and were harvested at 8 weeks or 8 months after implantation. Controls included untreated defects and defects treated with cell-free scaffolds. The goats presented no restriction in joint motion, and at macroscopic examination the joint was in very good condition without apparent signs of synovitis, such as redness or swelling of the synovial membrane. The arthrotomy wounds exhibited a very good healing without signs of infection, inflammation or dehiscence.

Assessment at 8 weeks

Masson trichrome, Safranin O and Alcian blue stainings were performed on sections of osteochondral explants. Comparison of the untreated defects with the treated ones clearly showed that the implantation of the biomaterial into the subchondral compartment induced a severe remodelling of the subchondral bone. Biomaterial remnants were detected mainly in the subchondral part and sometimes in the cartilage phase. In all experimental groups, independently of the presence of autologous chondrocytes in HYAFF®-11 scaffolds, the defects were filled with a fibrocartilaginous/fibrous tissue. As evidenced by Safranin O staining, the intensity of GAG deposition was extremely low in all experimental groups after 8 weeks implantation (Fig. 3). Masson trichrome staining

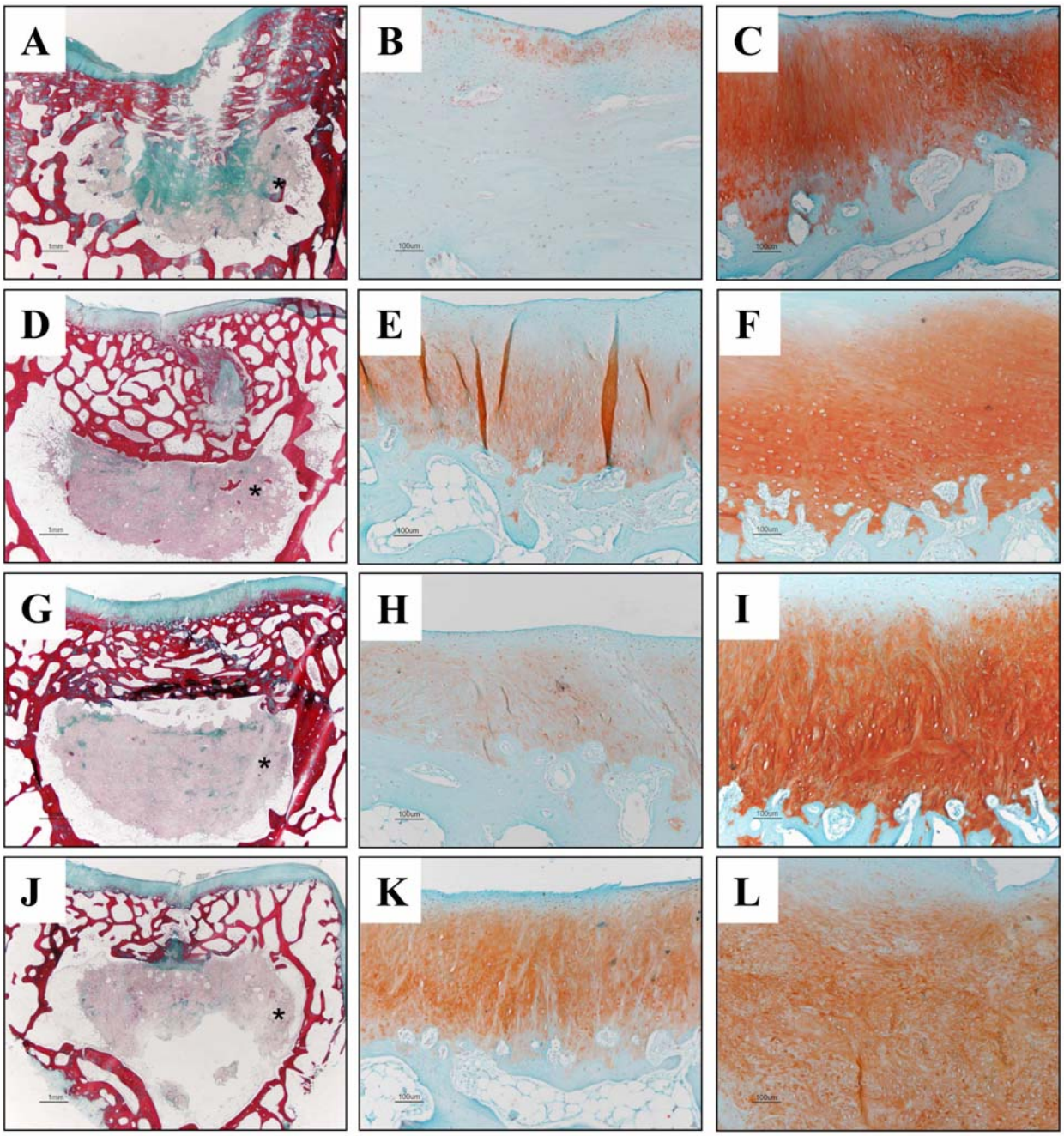

Fig. 5. Histological analysis of osteochondral repair tissues 8 months post-implantation (15 goats). (**A**, **D**, **G**, **J**) represent sections of the entire defects stained by Masson trichrome; (**B-C**, **E-F**, **H-I, K-L**) represent sections of the cartilaginous repair tissue from two defects per experimental group stained by Safranin O. Defects were either treated with a HYAFF®-11 scaffold without cells (**A**, **B, C**); or with autologous chondrocytes pre-cultured for 2 d (**D**, **E, F**), 2 weeks (**G**, **H, I**), or 6 weeks (**J**, **K, L**). *indicates remnants of biomaterial.

displayed a likely ingrowth of donor cells into the areas of the graft, in some cases forming a dense tissue underneath the HYAFF®-11 based layer (Fig. 3G). Within the cartilage compartment, the matrix was surrounded by spindle and round cells reminiscent of the chondrocytic phenotype, as well as abundant macrophages and few giant cells. The observation of macrophages was independent of the presence of autologous chondrocytes in the HYAFF®-11 scaffold, since they were also observed in the cell-free group. The mean O'Driscoll scores are reported in Fig. 4. The quality of repair was not improved when defects were treated with cell-free scaffolds as compared to untreated osteochondral defects (respective scores of 29.7 ± 0.5 versus 28.3 ± 1.6). In the treated groups, the implantation of constructs pre-cultured for 2 d, 2 weeks or 6 weeks did not result in better quality of repair as compared to implantation of cell-free scaffolds, as indicated by mean scores (Fig. 4).

Assessment at 8 months

After 8 months of implantation, the mean O'Driscoll scores of explanted repair tissues showed an improvement as

Table 3. Modified O'Driscoll scores determined 8 months post-implantation for each category and experimental group

Modified O'Driscoll Scores	Untreated (2 goats)	Cell-free (3 goats)	2 days (4 goats)	2 weeks (4 goats)	6 weeks (4 goats)
Category 1	4.0 ± 0.0	2.0 ± 1.10°	1.25 ± 1.22°	0.44 ± 1.01°+	1.45 ± 0.69°*
Category 2	3.0 ± 0.0	2.67 ± 0.5	1.83 ± 1.27°	1.33 ± 1.32°+	2.27 ± 0.90°
Category 3	4.0 ± 0.0	3.44 ± 0.73	2.83 ± 1.11°§	3.00 ± 1.22°	3.64 ± 0.67
Category 4	5.0 ± 0.0	3.78 ± 1.48°	3.08 ± 1.31°	3.56 ± 1.74°	3.82 ± 1.40°
Category 5	4.0 ± 0.0	2.56 ± 0.53°	1.42± 1.38°+*	0.22± 0.67°+§	2.18 ± 1.08°
Category 6	3.0 ± 0.0	2.67 ± 0.50	1.42±1.16°+§	1.22± 1.09°+§	2.36 ± 0.92°
Category 7	3. 33 ± 0.82	3.0 ± 1.5	2.33 ± 1.50	2.78 ± 1.48	3.27 ± 0.90
Category 8	3.33 ± 0.82	4.0 ± 0.0	3.08 ± 1.31+	3.78 ± 0.67	3.36 ± 1.21
Total score	29.66 ± 1.6	24.3 ± 5.8°	17.24 ± 8.4°+	16.33± 5.8°+§	22.35 ± 6.4°

° = statistically significant difference from untreated defect for the same category
+ = statistically significant difference from cell-free for the same category
* = statistically significant difference from 2 weeks for the same category
§ = statistically significant difference from 6 weeks for the same category
(*t*-tests for independent samples)

compared to 8 weeks (e.g. lower mean scores) (Fig. 4), except for the untreated group (score of 29.7 ± 1.6 at 8 months versus 28.3 ± 1.6 at 8 weeks).

A significant improvement was observed in the cell-free scaffold group as compared to the untreated group (p = 0.027), although cartilage repair was still of poor quality. The area of subchondral bone remodelling that was observed at the early stage of the implantation (8 weeks, Fig. 3) was reduced in size and some remnants of biomaterial, surrounded by the shell of neobone formation, were still observed within the medullar cavity (Fig. 5A-C). The subchondral bone was infiltrated by numerous giant cells and macrophages. At the cartilage level, mostly fibroblast-like cells with spindle morphology were observed, along with some macrophages and few giant cells.

In all treatment groups where chondrocytes were pre-cultured in HYAFF®-11 scaffolds, an enhanced repair process was determined by statistically significant lower scores in the 2 d (p = 0.034) and 2 weeks groups (p = 0.010) (Fig. 4) as compared to the cell-free group. The repair tissue consisted mainly of an unorganised fibrocartilaginous tissue, with occasional columnar organisation of chondrocytes (Fig. 5E-F,H-I,K-L). Safranin O-stained sections of two defects per experimental group are displayed (Fig. 5B-C, E-F, H-I. K-L) in order to illustrate the variability of the intensity of staining for GAG in the cartilage compartment within the same group. In the 6 weeks pre-culture group, more macrophages could be observed between the remaining matrix fibres. The overall degree of inflammation was moderate, but still present in all groups. Within the bone compartment, the histological picture was similar to the one observed in the cell-free treated group, with remnants of the biomaterial still present.

The scores attributed by category for all experimental groups after 8 months of implantation are detailed in Table 3. No statistically significant effect of defect location was found by one-way ANOVA using data from all experimental groups (p = 0.197) or only data from the cell-treated groups (i.e., 2 d, 2 weeks and 6 weeks) (p = 0.280). The filling of the defect relative to the surface of adjacent cartilage (Category 1) was significantly improved in all treated groups as compared to the untreated one (cell-free, p = 0.001; 2 d, p < 0.001; 2 weeks, p < 0.001; 6 weeks, p < 0.001). In particular, implantation of cartilage grafts pre-cultured for 2 weeks led to a significantly lower score than implantation of grafts pre-cultured for 6 weeks (p = 0.016) or implantation of cell-free scaffolds (p = 0.002). Lateral integration of repair tissue with surrounding native cartilage (Category 2) was significantly better when HYAFF®-11 scaffolds were implanted with cells as compared to untreated defects (2 d, p = 0.009; 2 weeks, p = 0.005; 6 weeks, p = 0.024). Moreover, the score of grafts pre-cultured for 2 weeks was significantly lower than for the cell-free scaffold (p = 0.018). The intensity of staining for GAG in the repair tissue (Category 3) was significantly higher when cartilaginous tissues were pre-cultured for 2 d as compared to 6 weeks (p = 0.049). Cellular morphology (Category 4) was significantly closer to the typical round shape of chondrocytes in all treated groups as compared to the untreated group (cell-free, p = 0.038; 2 d, p < 0.001; 2 weeks, p = 0.038; 6 weeks, p = 0.019), with no significant influence of pre-culture time prior to implantation of the graft. The architecture within the defect (Category 5), reflecting the number/size of voids within the subchondral tissue, was significantly closer to normal in all groups where defects were treated as compared to the untreated group (cell-free, p < 0.001; 2 d, p < 0.001; 2 weeks, p <

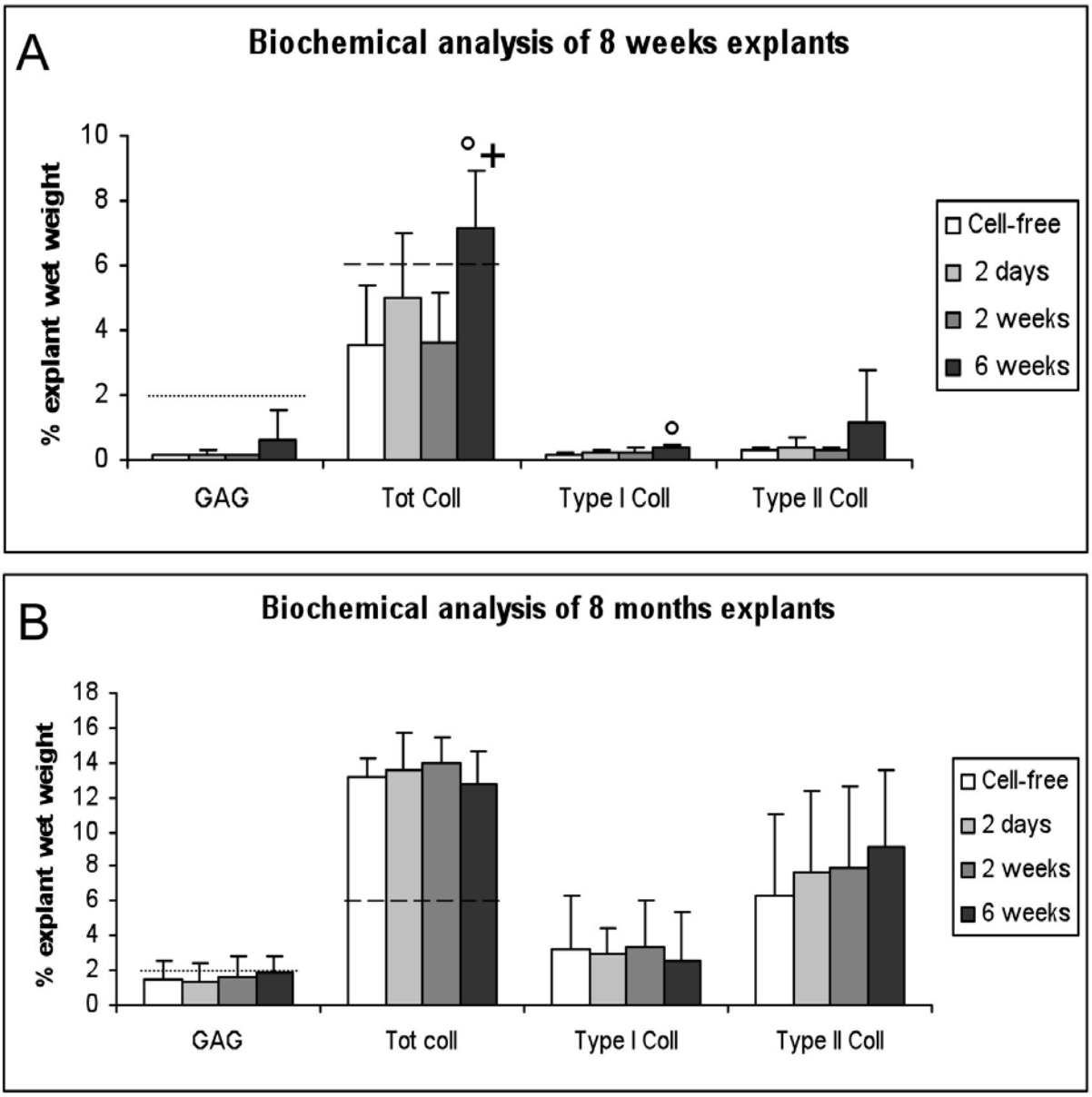

Fig. 6. Biochemical analysis of explants after 8 weeks (8 goats) or 8 months (15 goats) *in vivo*. GAG, total collagen, and type I and II collagen contents normalised to tissue wet weight in cartilaginous repair tissues after 8 weeks (**A**) or 8 months (**B**) *in vivo*. The dotted and dashed lines represent the average contents of GAG and total collagen, respectively, in native goat articular cartilage. ° = statistically significant difference from cell-free scaffolds for the same parameter; $^{+}$ = statistically significant difference from 2 weeks pre-culture for the same parameter.

0.001; 6 weeks, $p < 0.001$), with the significantly lowest score for grafts pre-cultured for 2 weeks as compared to cell-free ($p < 0.001$), 2 d ($p = 0.018$), or 6 weeks ($p < 0.001$) groups. The architecture at the surface of the defect (Category 6) was improved by the presence of cells in the scaffold. Implantation of a graft pre-cultured for 2 d or 2 weeks led to a significantly lower presence of fibrillations and irregularities than pre-culture for 6 weeks (2 d, $p = 0.044$; 2 weeks, $p = 0.021$) or a cell-free scaffold (2 d, $p = 0.004$; 2 weeks, $p = 0.004$). Examining the scores related to the architecture of the repair tissues, i.e. scores for categories 1, 2, 5 and 6 (Table 3), showed that engineered grafts pre-cultured for 6 weeks led to significantly inferior architecture of repair tissue (highest scores) as compared to those pre-cultured for 2 d ($p = 0.012$) or 2 weeks ($p < 0.001$), suggesting a limited remodelling capacity of the most developed constructs. Implantation of engineered grafts pre-cultured for 2 weeks led to a better architecture of repair tissues as compared to cell-free ($p < 0.000$), 2 d ($p = 0.012$) or 2 weeks ($p < 0.001$) pre-cultured groups. The percentage of newly formed subchondral bone (Category 7) was not improved by any treatment. Finally, the formation of the tidemark (Category 8) was significantly improved when engineered cartilaginous tissues pre-cultured for 2 d were implanted as compared to cell-free scaffolds ($p = 0.034$).

Biochemical evaluation of cartilage repair tissues

Assessment at 8 weeks

After 8 weeks *in vivo* (Fig. 6A), limited differences among the experimental groups were identified in the amounts of the assessed ECM components. The amount

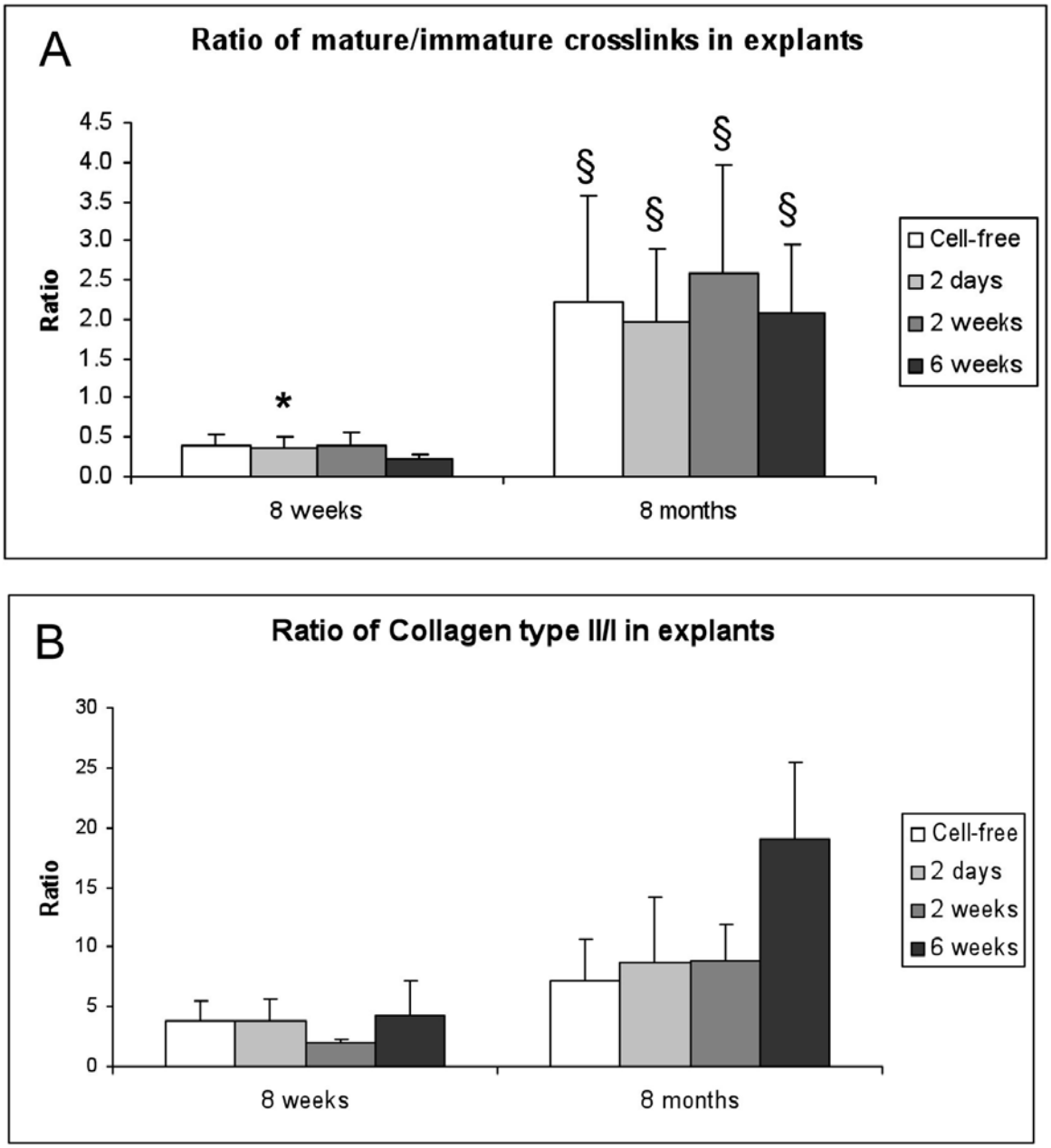

Fig. 7. Ratios of mature/immature cross-links (**A**) or collagen type II /I (**B**) in explants after 8 weeks (8 goats) or 8 months (15 goats) *in vivo*. Prior to implantation, HYAFF®-11 scaffolds were either not seeded (cell-free) or pre-cultured for 2 d, 2 weeks or 6 weeks. Data are presented as mean ± standard error. * = statistically significant difference from 6 weeks for an implantation time of 8 weeks *in vivo*, § = statistically significant difference from 8 weeks *in vivo* for the same culture condition.

of total collagen was significant higher in the repair tissues resulting from engineered grafts pre-cultured for 6 weeks versus 2 weeks ($p = 0.007$) or cell-free implants ($p = 0.011$). The amount of type I collagen was significantly higher in the repair tissues resulting from engineered grafts pre-cultured for 6 weeks than cell-free implants ($p = 0.014$).

The ratio of mature versus immature collagen cross-links in repair tissue, determined as a measure of collagen turnover, was also relatively similar in all experimental groups (Fig. 7A) and was significantly lower in the repair tissues resulting from engineered grafts pre-cultured for 6 weeks as compared to 2 d ($p = 0.031$). Type II/type I collagen ratio was similar in all treated groups after 8 weeks (Fig. 7B).

Assessment at 8 months

The content of GAG, total collagen, and type I and II collagens in the cartilaginous repair tissues generally increased after 8 months as compared to 8 weeks *in vivo*. No significant differences or even trends in the amount of any of these ECM components were detected among experimental groups (Fig. 6B). A high variability in the amounts of these proteins was observed among animals from the same experimental groups and even among the three defects within the same animals. Type II/type I collagen ratio, previously described to monitor the maturation of repair tissue (Hollander *et al.*, 2006), was about double in repair tissues resulting from engineered grafts pre-cultured for 6 weeks as compared to the other

experimental conditions (Fig. 7B), although due to large variability the difference was not statistically significant.

The mature/immature collagen cross-link ratios were not significantly different among the experimental groups, though they were significantly increased as compared to the values measured following 8 weeks *in vivo* (Fig. 7A) (cell-free, $p = 0.003$; 2 d, $p < 0.001$; 2 weeks, $p < 0.001$; 6 weeks, $p = 0.001$).

Discussion

In this study, we demonstrated that varying pre-culture time and consequently the maturation stage reached by an engineered cartilage graft during *in vitro* culture (Miot *et al*., 2006) had an influence on the outcome of osteochondral repair in the described caprine model, although the repair tissues remained fibrocartilaginous in all groups. In particular, the influence was predominantly observed by histological analyses, with the best score obtained for the 2 weeks pre-culture group. Interestingly, a more extensive maturation of engineered tissues for 6 weeks increased type II/I collagen ratio in repair tissues but led to inferior structural properties and overall did not enhance the outcome of the repair.

A subchondral bone remodelling was observed in all treated experimental groups. This phenomenon does not seem to be intrinsic to our caprine model but rather related to the biomaterial, since it did not occur by implantation of other types of scaffold based on synthetic polymers in the same goat model (unpublished data). This bone remodelling was most likely due to the presence of the subchondral filler and in particular to the HYAFF®-11 component, since biomaterial remnants were detected both in the remodelling subchondral bone and in the cartilage phase. The resorption of HYAFF®-11 material took longer than in a previous study by Campoccia *et al.* (1998), where HYAFF®-11 was reported to resorb almost completely in about 4 months following subcutaneous implantation in rats. The presence of inflammatory cells, which is usually associated with the beginning of chemical and mechanical degradation of the material (Campoccia *et al*., 1998), was also observed for an extended period of time (at least 8 months post implantation) in our caprine model. Such an accumulation of giant cells has previously been observed 6 weeks after implantation of a HYAFF®-11/polycaprolactone composite for meniscus regeneration in a sheep model (Chiari *et al*., 2006) and up to 5 months after implantation of HYAFF®-11 scaffold into the dorsolumbar musculature of rats (Campoccia *et al*., 1996).

From 8 weeks to 8 months after implantation, the structural appearance of the defects treated with engineered cartilage improved, in contrast to untreated defects, thus excluding any spontaneous repair in our model. For all treated groups, increased amounts of GAG, total collagen and type I and II collagens from 8 weeks to 8 months were mirrored by an improved repair, as demonstrated histologically through decreased O' Driscoll scores. However, for each implantation time, no significant differences between experimental groups in terms of GAG, total collagen, and type I and II collagen contents could be detected. After 8 months, GAG contents in repair tissues reached values in the range of those measured in native goat articular cartilage (Miot *et al*., 2006), and total collagen contents even exceeded those of native cartilage. The relatively high GAG and collagen contents, which are consistent with those described in a previous study on engineered goat cartilage grafts (Brehm *et al*., 2006), may reflect the phase of active ECM production at still early stages of tissue formation, before an active remodelling and tissue homeostasis are reached. The ratio of mature versus immature collagen cross-links in explants was significantly increased (3.8 fold) from 8 weeks to 8 months, but no significant differences among experimental groups were detected. Analysis of the quantitative collagen cross-link ratio is used as a surrogate marker of matrix turnover (Hollander *et al*., 2006) and has been shown to be a good predictor of tensile strength (Williamson *et al*., 2003). The low collagen cross-link ratios measured here indicate that the repair tissues were turning over quite rapidly and still in a maturation phase. Thus, despite the different degrees of maturation, the repair cartilaginous tissues did not reach the stage of hyaline cartilage in any of the experimental groups. After 8 months *in vivo*, the differences observed in quality of repair amongst experimental groups were mainly related to the overall architecture of the repair tissues, as shown by modified O'Driscoll scores.

The histomorphometric analysis showed that, compared to all other experimental groups, pre-culture of the cartilage graft for 2 weeks resulted in repair tissues which (i) were more congruent with the surface of native adjacent cartilage, (ii) were better integrated with surrounding cartilage, and (iii) displayed an architecture with less voids in the subchondral part and less fibrillations and irregularities at the surface. All together, the features may reflect an increased remodelling capacity. Engineered cartilaginous tissues pre-cultured for 6 weeks consisted of more abundant ECM at the time of implantation than 2 d or 2 week pre-cultured constructs. In a previous study using the same materials and protocols, the improved biochemical composition of the tissues pre-cultured for 6 weeks was mirrored by an increase in the equilibrium and dynamic stiffness from the 2 d pre-cultured constructs (1.3- and 16.0-fold, respectively) (Miot *et al*., 2006). However, grafts pre-cultivated for 6 weeks resulted in overall inferior histological quality of repair tissue as compared to those pre-cultured for 2 d or 2 weeks. While the type II/I collagen ratio seemed to indicate an increase of maturity of the repair tissue, integration with surrounding native cartilage and structure of repair tissue were clearly worse. The presence of ECM is expected to protect cells from mechanical loading (Ball *et al*., 2004), but a too mature and dense ECM might have prevented the integration with native cartilage (Obradovic *et al*., 2001), as well as the migration of precursor cells from the subchondral bone and/or from the adjacent cartilage. Indeed, in a different goat model of osteochondral repair, the participation of cells from adjacent cartilage/bone has been suggested to contribute more significantly to the repair process than implanted cells, masking their contribution (Niederauer *et al*., 2000). To the best of our knowledge, whether integrative properties of engineered cartilage grafts can be modulated by the pre-culture time and thus

by developmental stage has only been demonstrated *in vitro*, where immature constructs integrated better than the more mature ones (Obradovic *et al*., 2001). The use of mature cartilage grafts for repair of osteochondral defects is partly dependent on the need for lateral integration which might be overcome by the use of enzymatic treatment for proteoglycans removal, shown to increase adhesive strength at the integration interface (Obradovic *et al*., 2001), or the use of an immature cell/scaffold layer at the interface (Pabbruwe *et al*., 2009).

The intensity of staining for GAG was significantly improved when grafts were pre-cultured for 2 d as compared to 6 weeks, a difference difficult to visualise through Safranin O staining due to variability between defects within the same experimental group and not revealed by the biochemical analysis. The apparent lack of a direct correspondence between GAG staining and biochemical quantification in explants of repair tissue is consistent with a previous clinical study performed using HYAFF®-11 for chondrocyte delivery, where histology-based classification of the repair tissues did not always correspond to separate ranges of measured GAG amounts (Dickinson *et al*., 2005).

Our assessments did not include mechanical tests of the repair tissue. Due to the relatively thin goat cartilage layer (0.7-1.5 mm (Frisbie *et al*., 2006)), indentation tests of the whole articular surface would have represented the only feasible option. However, the reliability of these tests would be biased by the osteochondral nature of the defects, since the measured stiffness could not have been decoupled from the subchondral tissue properties. Other groups also showed that in cartilage repair studies in goats (Lind *et al*., 2008) mechanical tests could not capture differences between treatment groups which were demonstrated histologically.

One limit of the current study is that it did not assess whether the implanted autologous cells contributed directly to the healing or if they played an indirect role in attracting precursor cells into the defect sites. In order to determine if the repair fibrocartilaginous tissue was derived from the implanted graft or from the host, implanted chondrocytes could have been labelled using fluorescent dye such as PKH26 (Dell'Accio *et al*., 2003) or transduced by a lentivirus allowing long term expression of a green fluorescent protein (Miot *et al*., 2009).

In conclusion, we demonstrate that pre-culture of engineered cartilage for 2 weeks seems to be a good compromise to achieve both intrinsic tissue maturity and suitable structure/integration of repair fibrocartilaginous tissue in a goat model. One can speculate that with longer implantation time, repair tissues could further mature towards a more hyaline phenotype. Implantation of engineered grafts pre-cultured for 2 d was inferior in respect to both structure and composition, while those pre-cultured for 6 weeks resulted in a more mature composition but an inferior remodelling capacity. While these findings should be validated in other pre-clinical models or in a clinical scenario, they highlight that the maturation stage of engineered cartilage is an important parameter to be considered in designing cartilage repair strategies.

Acknowledgments

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Conflict of interest

We certify to have no financial conflict with the subject matter or materials discussed in this manuscript at any of our academic institutions.

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Discussion with Reviewers

Reviewer I: Is there any reference/previous work to the compatibility of Hyaff and the chosen hydroxyapatite scaffold? May this have affected the outcome and quality of repair tissue? Please state any references.

Authors: The subchondral support consisted of sponges made of 65 % hydroxyapatite and HYAFF®-11, a composite material with a biological performance so far only assessed in an *in vitro* model (Giordano *et al*., 2006). Considering the well established general biocompatibility of hydroxyapatite, the raised question could be directly

related to the compatibility of HYAFF®-11. A previous study reported the accumulation of giant cells up to 5 months after implantation of HYAFF®-11 into the dorsolumbar musculature of rats (Campoccia *et al*., 1996), although the material was almost completely resorbed after about 4 months (Campoccia *et al*., 1998). One other study reported on the biocompatibility of HYAFF®-11 in a joint environment, specifically when used in a composite scaffold with polycaprolactone as meniscus substitute in sheep (Chiari *et al.*, 2006). Six weeks after implantation, histological analysis of the implants showed in all specimens accumulation of giant cells in contact with the biomaterial, mixed with fibroblast-like cells. The authors concluded that the impact of the giant cell reaction was difficult to be estimated since it could have been part of the physiological resorption process and neither the synovial biopsies nor the smears revealed acute inflammatory cells. Thus, implantation of HYAFF®-11, even in a joint environment, seems to be associated with the prolonged presence of inflammatory cells, but the overall impact of this phenomenon on the quality of repair tissue remains an open issue.