

AUTOLOGOUS CARTILAGE FRAGMENTS IN A COMPOSITE SCAFFOLD FOR ONE STAGE OSTEOCHONDRAL REPAIR IN A GOAT MODEL

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Abstract

We propose a culture-free approach to osteochondral repair with minced autologous cartilage fragments loaded onto a scaffold composed of a hyaluronic acid (HA)-derived membrane, platelet-rich fibrin matrix (PRFM) and fibrin glue. The aim of the study was to demonstrate *in vitro* the outgrowth of chondrocytes from cartilage fragments onto this scaffold and, *in vivo*, the formation of functional repair tissue in goat osteochondral defects. Two sections were considered: 1) *in vitro*: minced articular cartilage from goat stifle joints was loaded onto scaffolds, cultured for 1 or 2 months, and then evaluated histologically and immunohistochemically; 2) *in vivo*: 2 unilateral critically-sized trochlear osteochondral defects were created in 15 adult goats; defects were treated with cartilage fragments embedded in the scaffold (Group 1), with the scaffold alone (Group 2), or untreated (Group 3). Repair processes were evaluated morphologically, histologically, immunohistochemically and biomechanically at 1, 3, 6 and 12 months. We found that *in vitro*, chondrocytes from cartilage fragments migrated to the scaffold and, at 2 months, matrix positive for collagen type II was observed in the constructs. *In vivo*, morphological and histological assessment demonstrated that cartilage fragment-loaded scaffolds led to the formation of functional hyaline-like repair tissue. Repair in Group 1 was superior to that of control groups, both histologically and mechanically. Autologous cartilage fragments loaded onto an HA/PRFM/fibrin glue scaffold provided a viable cell source and allowed for an improvement of the repair process of osteochondral defects in a goat model, representing an effective alternative for one-stage repair of osteochondral lesions.

Keywords: One-stage cartilage repair; cartilage fragments; hyaluronic acid-derived membrane; fibrin glue; platelet-rich fibrin matrix; scaffold; goat model; chondrocytes; osteochondral defects; nanoindentation.

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Introduction

Cartilage injuries are commonly found in orthopaedic surgery, and spontaneous healing of osteochondral lesions leads to the formation of fibrocartilage, a type of functional repair tissue that has different biochemical composition and inferior biomechanical properties from those of hyaline articular cartilage. Thus, finding a viable cell source and a suitable scaffold are significant milestones for cartilage repair, and the presence of chondrogenic cells in cartilage defects seems to be a key feature for good clinical and histological results. The most commonly available treatments to enhance the quality of repair of symptomatic deep cartilage and osteochondral lesions include: 1) bone marrow stimulation (microfractures, MF) (Mithoefer *et al.*, 2005); 2) osteochondral autograft transplantation (OAT) (Bobic, 1999) (Gudas *et al.*, 2012); and 3) autologous chondrocyte implantation (ACI) (Peterson *et al.*, 2010). MF and single-plug OAT are usually implemented for small chondral and osteochondral defects (< 1-2 cm²), while for larger lesions, multiple OAT and ACI are both preferable treatment options (Bekkers *et al.*, 2009; De Windt *et al.*, 2012).

Bone marrow stimulation through microfractures is the simplest and most economic one-step approach, but often results in the generation of mixed fibrocartilage tissue that tends to degenerate over time (Hunziker, 1999a; Shapiro *et al.*, 1993). Osteochondral autograft transplantation facilitates the formation of more homologous tissue at the lesion site, but raises concerns about donor-site morbidity, technical difficulties, and integration of the graft at the interface with the receiving site. Indeed, with OAT, mature hyaline cartilage is transferred from an area that bears less weight to the defect site, but the technical difficulties to obtain a careful restoration of the curvature of the articular surface are limiting factors. Moreover, in larger defects, multiple plugs are needed, and close contact with the surrounding host bone could be incomplete, compromising plug integration. Osteochondral allografts could overcome some of these limitation, but fresh graft availability and high costs are still limiting factors of this procedure (Gomoll *et al.*, 2010). Autologous chondrocyte

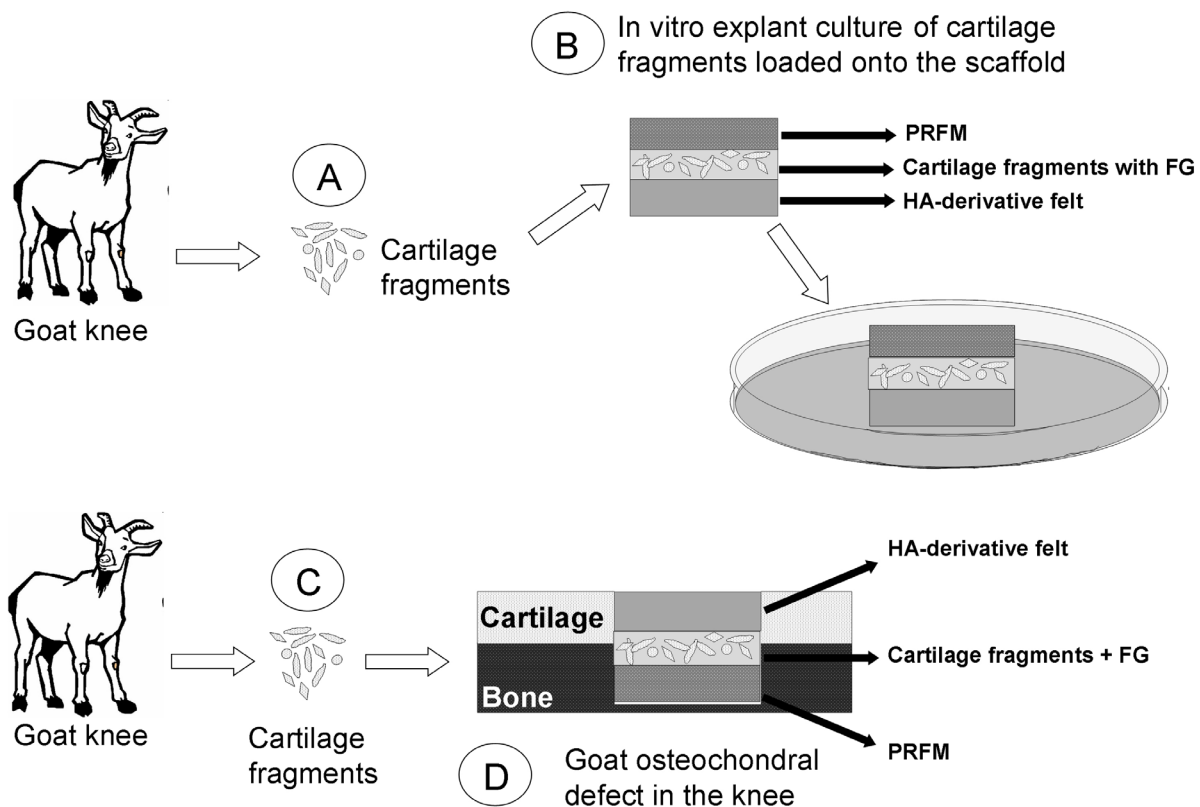


Fig. 1. Study design. *In vitro* study: cartilage fragments were harvested from goat knees (A); minced cartilage fragments were loaded onto the scaffold (B); *In vivo* study: cartilage fragments were harvested from goat knees (C), minced, loaded onto the scaffold (D) and used as a cell source for the repair of osteochondral defects in goat knees (study group A). FG, fibrin glue; HA, hyaluronic acid; PRFM, platelet-rich fibrin matrix.

implantation leads to a hyaline-like regenerative tissue with better histological and clinical results than microfractures as well as durable properties. It can be used both with a chondrocyte suspension or with a 3-D pre-loaded membrane (MACI, Hyalograft C and others), and combined procedures are being exploited for deeper defects, involving transplantation of trabecular bone in association with cell transplantation (Bartlett *et al.*, 2005). However, there are still several limitations to autologous chondrocyte implantation, as multiple surgical procedures are required and costly chondrocyte culture is needed in order to expand cells *in vitro* before implantation.

Recently, tissue engineering is developing new alternatives to overcome these limitations and new one-stage methods have been investigated in order to obtain controlled differentiation of repair tissue with good functional properties and valuable histological features, avoiding cell manipulation, multiple surgical procedures, high costs and major patient morbidity. The autologous matrix-induced chondrogenesis (AMIC) technique is a one-step procedure for focal chondral and osteochondral defects, combining subchondral microfractures with a collagen I/III membrane secured with fibrin glue or sutures. This enables bone marrow cell adhesion, optimising fibrocartilaginous repair (Benthien and Behrens, 2011); platelet-rich plasma gel can also be combined to enhance repair processes (Dhollander *et al.*, 2010). Maioregen

(Fin-Ceramica Faenza S.p.A., Faenza-Italy) is a thick, nanostructured biomimetic collagen and hydroxyapatite scaffold used for treatment of large osteochondral lesions. As in the AMIC technique, cell migration from the surrounding host bone and bone marrow is required in order to colonise the scaffold and achieve functional repair (Kon *et al.*, 2010a; 2010b). Osteochondral morselised grafts have been proposed as a “paste” without any scaffold for one-stage repair of full thickness articular cartilage lesions and osteochondral defects, both in animal and human studies (Stone *et al.*, 2006; Mahadev *et al.*, 2001). This solution implies an undifferentiated mixed cell source from cartilage, bone and blood for the repair process (Stone *et al.*, 2006). Bone marrow concentrate and platelet gel have also been recently combined with a hyaluronic acid membrane for one-step repair of osteochondral lesions of knees and ankles (Buda *et al.*, 2010; Giannini *et al.*, 2009). Results and effectiveness of all these procedures are promising, but still under investigation.

To preserve the advantages of chondrocyte therapy, whilst at the same time reducing cell manipulation, cartilage fragments have been also investigated in animal models as well as a human trial, as being a suitable cell source for one-step cartilage repair – in association with collagen membranes or polydioxanone mesh (cartilage autograft implantation system, CAIS) (Cole *et al.*, 2011; Frisbie *et al.*, 2009; Lu *et al.*, 2006). The rationale of this

repair technique is the spontaneous ability of chondrocytes to migrate from the cartilage chips and proliferate into the scaffold. Cartilage fragmentation greatly increases tissue surface area and maximises the number of cells that can then interact with the three-dimensional structure of the scaffold. Fragmentation also separates cell-matrix bonding and promotes a “motile phenotype”, eliciting cell outgrowth toward the surrounding environment (Reindel *et al.*, 1995).

This study proposes a one-stage culture-free chondrocyte approach to osteochondral repair, using autologous cartilage fragments as a cell source, loaded onto an absorbable scaffold composed of a hyaluronic acid-derived membrane (Hyaff-11), autologous platelet-rich fibrin matrix (PRFM, Cascade system) and injectable fibrin glue. Based on the results from previous investigators on the chondrogenic potential of fresh minced cartilage chips (Cole *et al.*, 2011; Frisbie *et al.*, 2009; Lu *et al.*, 2006), the present study aims to investigate (Fig. 1): (a) *in vitro* – the outgrowth of chondrocytes derived from chondral fragments, obtained by mechanical fragmentation of articular cartilage, loaded onto the aforementioned absorbable scaffold; and (b) *in vivo* – the repair efficacy of the experimental composite in goat trochlear lesions, a clinically relevant large animal model. For this purpose, deep osteochondral defects were treated with autologous cartilage fragments loaded onto the absorbable scaffold; other defects were either treated with the scaffold alone or left untreated for control groups.

Materials and Methods

Approvals were obtained both from the Ethical Committee of the MBC (Molecular Biotechnology Centre), University of Torino, and from the Animal Care and Use Centre (CISRA), Faculty of Veterinary Medicine, University of Torino.

Method for platelet-rich fibrin matrix (PRFM) preparation

A goat blood sample was collected preoperatively (10 mL) in order to obtain autologous PRFM (Cascade® Autologous Platelet System, MTF Sports Medicine, Cascade Medical Enterprises, Wayne, NJ, USA) (Hall *et al.*, 2009; Castillo *et al.*, 2011). Briefly, according to the manufacturer's instructions, 10 mL of peripheral goat blood were collected in a supplied sterile blood collection/separation tube (Tube 1). Mean concentration of original platelets was 348×10^3 platelets/ μL (range 166-504). Tube 1 was centrifuged at 1100 g for 6 min to obtain platelet rich plasma (PRP). Following centrifugation, the supernatant on top of the separation gel was composed of PRP, whereas red and white blood cell fractions were located below the gel. PRP supernatants were transferred to a second tube (Tube 2), containing a calcium chloride additive (0.25 mL 1 M CaCl_2). Tube 2 was centrifuged for 15 min at 1450 g, and a gel was obtained corresponding to a leukocyte-poor PRFM of 1.5 mL containing approximately 5x platelet/fibrin, according to the manufacturer (Simon *et al.*, 2009).

In vitro study

Constructs were prepared with goat cartilage fragments. Articular cartilage pieces were harvested from femoral trochleae of 7 adult female Roccaverano goats during the *in vivo* part of the experiment, from the animals assigned to control groups.

Care was taken in order to remove all debris of the calcified cartilage and the subchondral bone from the specimens. Cartilage pieces from both trochlear defects of the same animal were pooled together, rinsed with sterile saline, transferred to a Petri dish and manually minced into small cuboidal fragments (less than 1 mm³) in sterile conditions with a sharp No. 15 scalpel in the presence of phosphate-buffered saline (PBS) (Celbio, Pero, Milano, Italy). A non-woven esterified HA-derivative felt (Hyaff-11, FIDIA Advanced Biopolymers, Abano Terme, Italy) was trimmed to pieces of 0.7 cm². Two sheets were layered in a culture dish. Approximately 15 mg of cartilage fragments were evenly seeded onto the membrane and were retained with a thin coating of approximately 50 μL of fibrin glue (Tissucol, Baxter AG, Vienna, Austria) using thrombin (400-625 units/mL) that was previously diluted to 1:4 with calcium chloride (36-44 $\mu\text{mol/mL}$). A 200 μL volume of PRFM (Cascade) was then placed on top of the cartilage fragments. A total of 6 constructs per animal were prepared.

All constructs were cultured at 37 °C in a humidified atmosphere containing 5 % CO_2 . Culture medium contained high glucose (4500 mg/L) DMEM (Celbio, Pero, Milan, Italy) with 10 % foetal bovine serum (Sigma Chemical Co., St. Louis, MO, USA), HEPES (10 mM) (Sigma), nonessential amino acids (0.1 mM) (Sigma), L-proline (20 ng/mL) (Sigma), ascorbic acid (50 $\mu\text{g/mL}$) (Sigma), 10,000 units Penicillin, 10 mg Streptomycin in 0.9 % sodium chloride (Sigma). Culture medium was changed three times per week.

After 15 d, 1 month and 2 months, constructs were retrieved from cultures, fixed in formalin, embedded in paraffin, and processed for histological analysis. Tissue sections were stained for Haematoxylin/Eosin and Alcian Blue. Type I (MAB3391, Millipore, Milan, Italy) and II collagen (MAB-10200, Immunological Science, Rome, Italy) immunohistochemistry was also performed on the specimens retrieved after 1 and 2 months.

In vivo animal study

18 adult female Roccaverano goats were used in this study. 10 mL blood samples were collected preoperatively to obtain autologous PRFM (Cascade®), as described above. All animals were anaesthetised as follows: 10 mg/100 mL Acepromazine Maleate – Prequillan (Fatro S.p.A., Bologna, Italy) at the dosage of 0.1 mg/kg 1 h before surgery; 10 mg/20 mL Propofol – Rapinovet (Intervet Italia Srl, Milano, Italy) at the dosage of 7 mg/kg at the induction of anaesthesia. After induction, anaesthesia was maintained with isoflurane – Isoflurane-vet 250 mL (100 % isoflurane) (Merial Italia S.p.A., Padova, Italy) for a percentage of 1-2.5 % of isoflurane inspired during the procedure and buprenorphine – Temgesic iv (Shering Plough S.p.A., Milano, Italy) 0.3 mg/mL at the dosage of

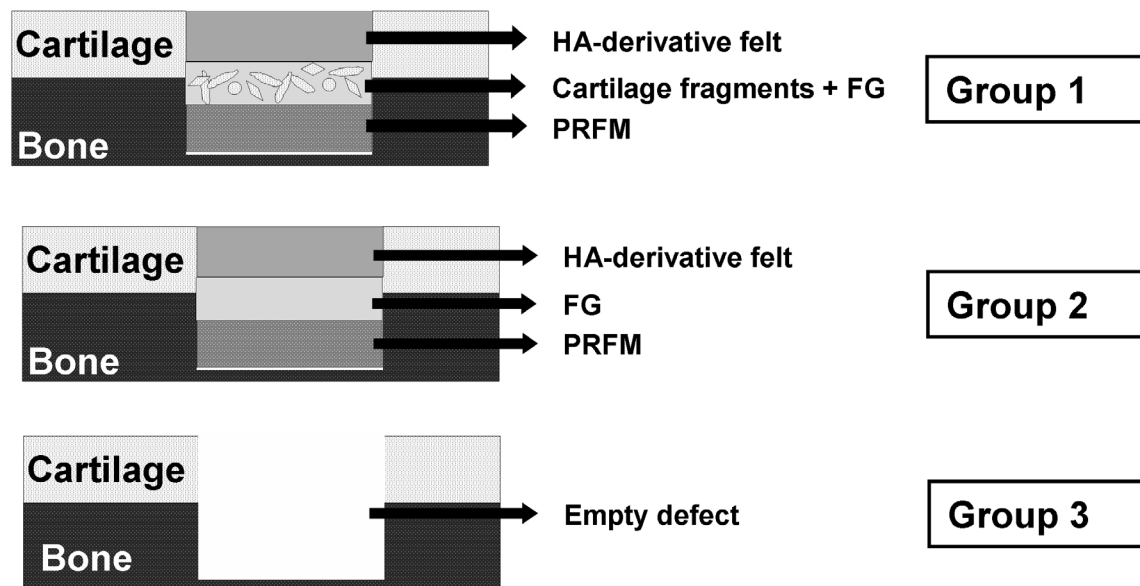


Fig. 2. Study groups. Experimental groups were prepared with cartilage fragments: Group 1) cartilage fragments loaded onto the scaffold; Group 2) scaffold alone; Group 3) empty defect. FG, fibrin glue; HA, hyaluronic acid; PRFM, platelet-rich fibrin matrix.

0.01 mg/kg. At the end of anaesthesia, postoperative pain management was instituted with buprenorphine – Temgesic im (Shering Plough S.p.A.) 0.3 mg/mL at the dosage of 0.01 mg/kg. Prophylactic antibiotic therapy was instituted as follows: tetracycline – Terramicina-ossitetraclina 92.7 mg/mL (Pfizer Italia SRL, Latina, Italy) at the dosage of 10 mg/kg the day before the operation and every 24 h up to the sixth postoperative day; cefazolin – Cefazolina im 1 g/4 mL (Mylan S.p.A., Milano, Italy) at the dosage of 20 mg/kg 1 h before surgery and every 12 h up to the second postoperative day.

Defects were prepared by exposing the femoral trochlea using a medial arthrotomy, thus dislocating the patella laterally. Unilateral trochlear models consisted of two large full-thickness osteochondral defects (7 mm diameter; 3.5 mm depth), created in the central trochlear area of the right stifle joints with a minimum of 5 mm of separation between the 2 defects. In each goat, cartilage was collected from the defect areas before defect creation and manually minced in the presence of PBS, obtaining fragments of less than 1 mm³. Cartilage fragments were then pooled together to be used both for the *in vitro* culture and for the *in vivo* autologous implant in the experimental group (Group 1). A 7 mm diameter manual drill was used to create the defects. A slow and progressive manual drilling was performed for each defect. Debris were removed by the advancing of the spiral drill and with the help of multiple lavages with physiological solution during and after the drilling procedure. This allowed for a controlled and standardised penetration of the drill and avoided any major heat generation around the defect surface and any debris build-up. Consequently, the structural integrity and vitality of the bone and vessels surrounding the defect site was preserved, as evidenced by the absence of any fracture of bone at the rim of the lesion and by the amount of blood

that appeared at the bottom of the defect site during the final drilling phase.

The dimensions chosen in this model were based on prior studies (Frisbie *et al.*, 2006; Jackson *et al.*, 2001) that showed that osteochondral defects of 6 mm diameter do not spontaneously heal. A depth of 3.5 mm enabled the removal of approximately 2-2.5 mm of underlying subchondral bone (Hunziker, 1999b; Ahern *et al.*, 2009).

A total of 36 defects (for all goats used in the study) were created. Animals were randomised into 3 different groups: cartilage fragments plus scaffold (Group 1, experimental group), scaffold alone (Group 2), empty unfilled defects (Group 3) (Fig. 2).

Goat defects in Groups 1 and 2 were filled as follows: the defect was dried with a sterile sponge, and 90 µL of autologous PRFM was manually placed at the bottom. In Group 1, autologous cartilage fragments, harvested from the two trochlear areas and pooled together, were evenly distributed at the surface of the autologous PRFM at a density of approximately 20 mg/cm², based on previous studies (Lu *et al.*, 2006; Frisbie *et al.*, 2009). Approximately 20 µL of fibrin glue (Tisseel, Baxter AG, Vienna, Austria; with thrombin previously diluted to 1:4 with calcium chloride) was added to stabilise the construct. A sheet of Hyaff-11 (diameter 7 mm) was then added to seal the defect. Manual pressure on the surface of the constructs was applied to further stabilise the scaffold until the fibrin glue polymerisation was completed and the implant surface was even with the surrounding trochlear area. The wound was closed and a soft bandage was bound on the wound for the first 4-5 d. Postoperatively, all animals were allowed free-loading activity and free cage activity, and they were kept in a properly cleaned animal care facility. A single stall (5 m x 5 m) was used to host each animal for the first 2 postoperative weeks; all animals were then kept

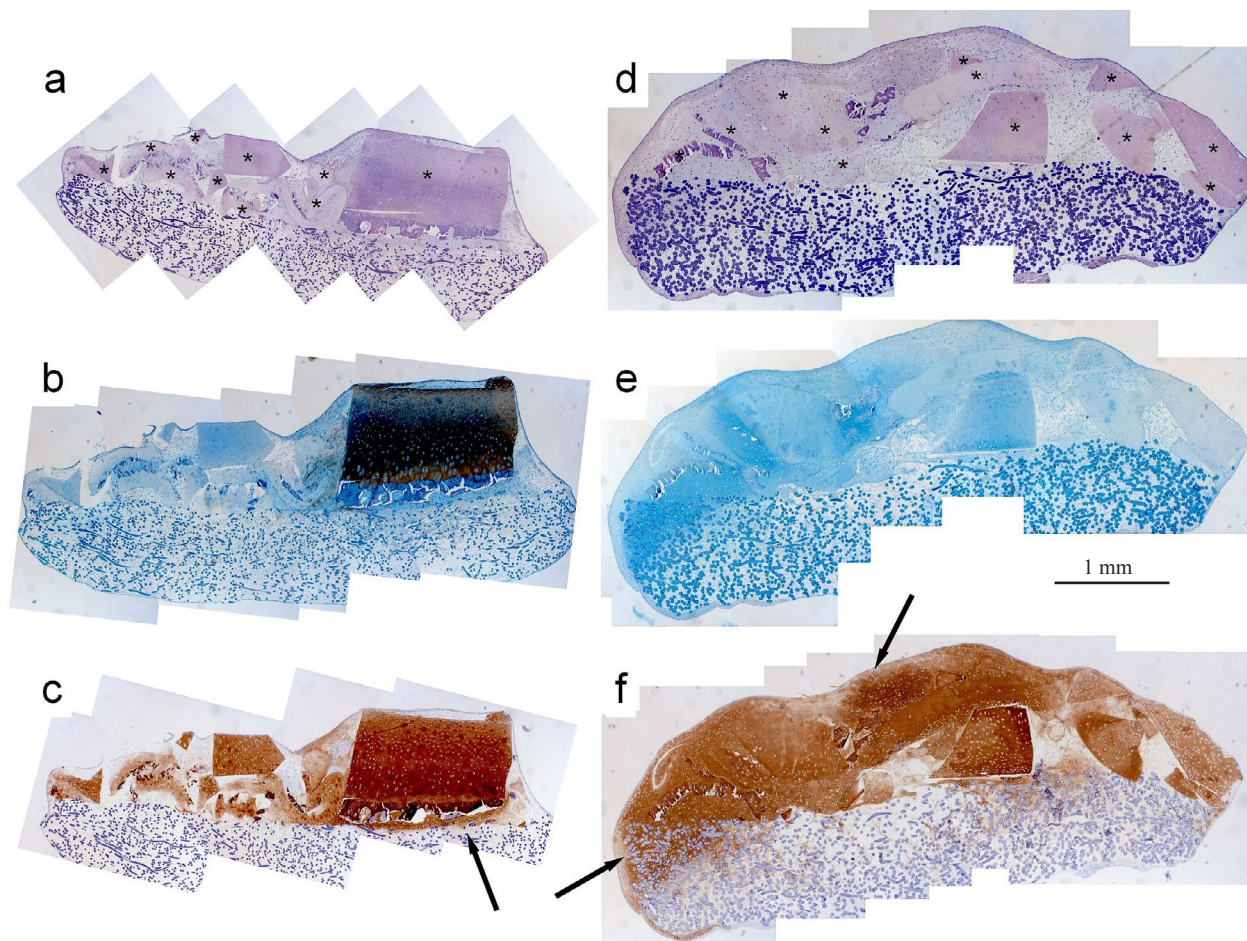


Fig. 3. Microscopic analysis of cartilage fragment-loaded constructs from goat femoral trochlea at 1 month (**a,b,c**) and 2 months (**d,e,f**); (**a,d**) haematoxylin-eosin staining; (**b,e**) Alcian Blue staining; (**c,f**) collagen type II immunostaining; the asterisk (*) marks the cartilage fragments in **a** and **d**; the arrows indicate the presence of neo-matrix positive for collagen type II, produced by the chondrocytes and located within the scaffold in **c** and **f**. Histological figures were obtained by combining different microscopic images.

together in a common environment (5 m x 15 m) up to the end of the follow-up period. Spontaneous rest and reduced movement of the animals were observed for the first 5-7 d. Generally, no sign of limping was observed after 8-10 d following surgery. We did not observe any difference of postoperative behaviour among the animals. Three goats died postoperatively and were excluded from the study. The remaining animals were humanely euthanised after 1 month (3 goats, 2 defects per group), 3 months (3 goats, 2 defects per group), 6 months (3 goats, 2 defects per group) and 12 months (6 goats, 4 defects per group). All animals were euthanised, following anaesthesia, using Tanax bottle 50 mL (Intervet Italia Srl) at the dosage of 0.1 mL/kg by intracardiac injection.

Total joint health was assessed by gross-morphological evaluation, by observing the repair tissue filling, the presence of osteophytes and the inflammatory changes of the synovial membrane. At 1 year, the International Cartilage Repair Society (ICRS) form (ICRS Cartilage Repair Assessment) was used to collect macroscopic characteristics of the repair tissue (Kaul *et al.*, 2012; Brittberg and Winalski, 2003) and each stifle joint was

evaluated with the macroscopic scores of Little (Little *et al.*, 2010).

For histological assessment, the distal femur was excised, fixed in 10 % formalin and decalcified. Samples were then paraffin-embedded, sectioned with a microtome (5 μ m) and stained with Haematoxylin/Eosin and Alcian blue. Samples were also processed for immunohistochemistry analysis for type I and II collagen. Type II collagen immunohistochemistry was performed with MAB-10200 (Immunological Science). Each slide was blindly examined under a light microscope by 2 blinded observers. Samples were semi-quantitatively graded using the modified ICRS score (Liu *et al.*, 2006), the modified O'Driscoll scale (Frisbie *et al.*, 2009), and the microscopic scores of Little (Little *et al.*, 2010). These scoring systems were based on the percentage of defect filling, articular surface continuity, restoration of osteochondral architecture, repair tissue integration, cellular morphology of articular cartilage regeneration, and matrix staining.

The superficial zone of repair tissue from 1 year specimens from Groups 1, 2 and 3, was biomechanically tested by nanoindentation (Franke *et al.*, 2007). For positive

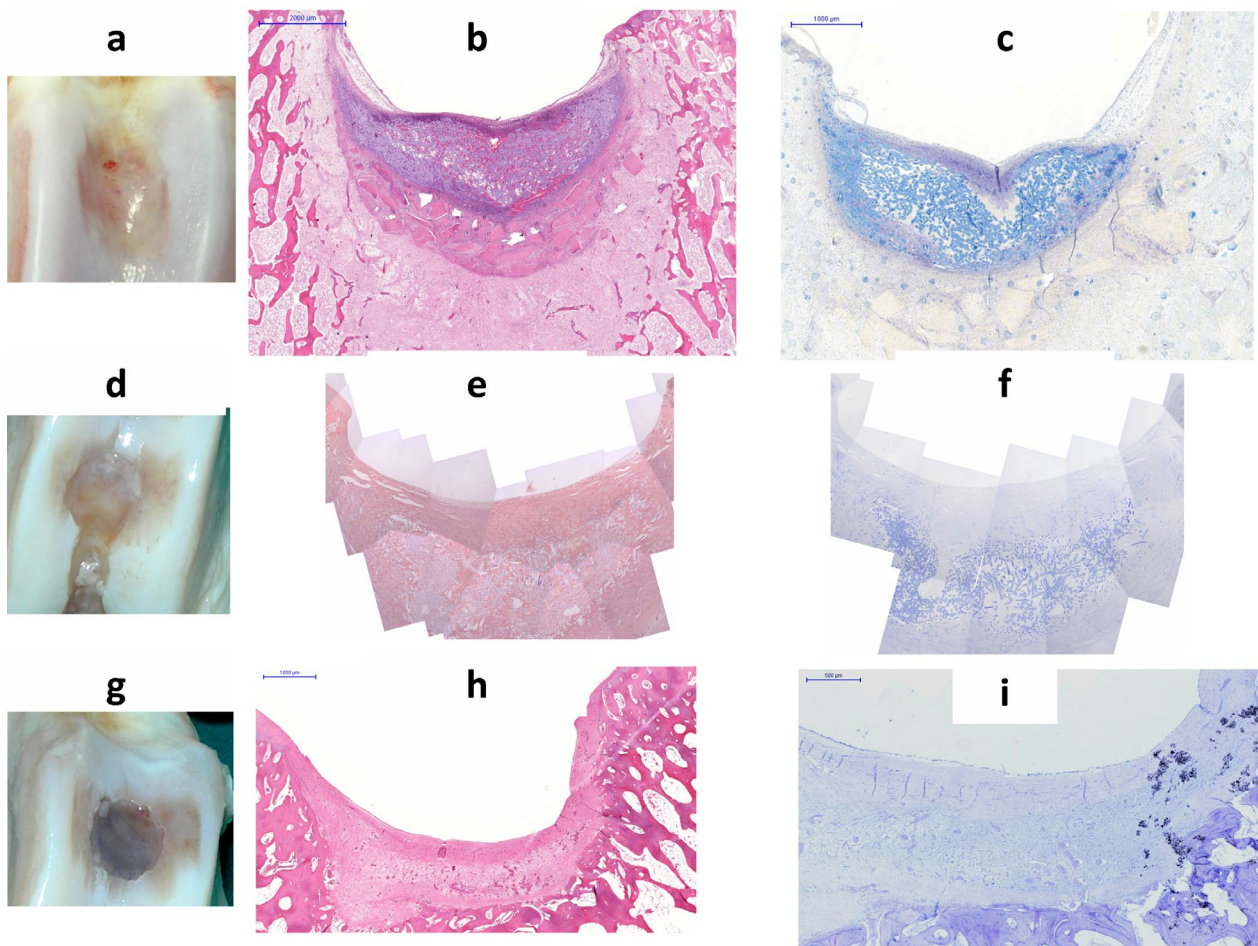


Fig.4. Macroscopic and microscopic views of repair tissue at 1 month post-implantation. Group 1: **a,b,c**; Group 2: **d,e,f**; Group 3: **g,h,i**. Macroscopic views (**a,d,g**); sections of the defect area stained with H/E (**b,e,h**), and Alcian Blue (**c,f,i**). Histological figures **e** and **f** were obtained by combining different microscopic pictures. Cell infiltration of the scaffold can be observed in all groups, more markedly in Groups 1 and 2. In Group 1, cells are visible around the cartilage fragments, which were located at the deeper zone of the defect under the HA sheet, suggesting the potential contribution of cartilage chips to the repair. Cells are also present at the upper layer of the defect, likely deriving from the surrounding synovial tissue and from the cartilage at the perimeter of the defect. Cells are also visible both infiltrating the bottom of the defect and inside the scaffold, likely deriving from the bone marrow underneath and around the defect walls. In all three groups, the lowest amount of cells corresponded to the central zone of the defects. No clear matrix deposition was observed in any group.

controls, mechanical tests were also performed on trochlear cartilage samples from the contralateral stifle joint of Group 1 goats. From treated goat stifle joint, a representative osteochondral sample corresponding to the inferior half of the defect area was prepared using a miniature rotating saw. The same area was isolated from the trochleae of 2 contralateral stifle joints of Group 1 goats. Samples were transferred to PBS to prevent drying and stored at 4 °C. Testing was performed within 72 h from harvesting. Nanoindentation was performed with a Nanoindenter XP (MTS, Eden Prairie, MN, USA) with a diamond indenter (Berkovich diamond tip) at room temperature. Samples were placed in a plastic chamber with the cartilage surface perpendicular to the indenter direction and secured to a metallic support. They were kept hydrated with saline prior to testing. The Berkovich tip was driven onto the specimen surface and the applied force and displacement obtained were recorded. Material properties were calculated from

the load and depth of indentation. Hardness (H) and Young's modulus (E) were calculated as a function of load and depth during the unloading phase of testing, with a maximal load of approximately 75 mN and a maximal depth of 100 μm. Sample fractures of Groups 2 and 3 were observed beyond 40 μm of Berkovich tip penetration; this compromised Young's modulus determination during the unloading phase for Groups 2 and 3. Mean values of Young's modulus (E) were then calculated for all samples on the loading curves, in a range of 1-40 μm depth. This allowed for a comparative evaluation between groups, although in a limited range of tip penetration.

Statistical analysis

All data in text and figures are provided as means ± SD. Results were analysed by a one-way Analysis of Variance (ANOVA) and Tukey's test. A $p < 0.05$ was considered statistically significant.

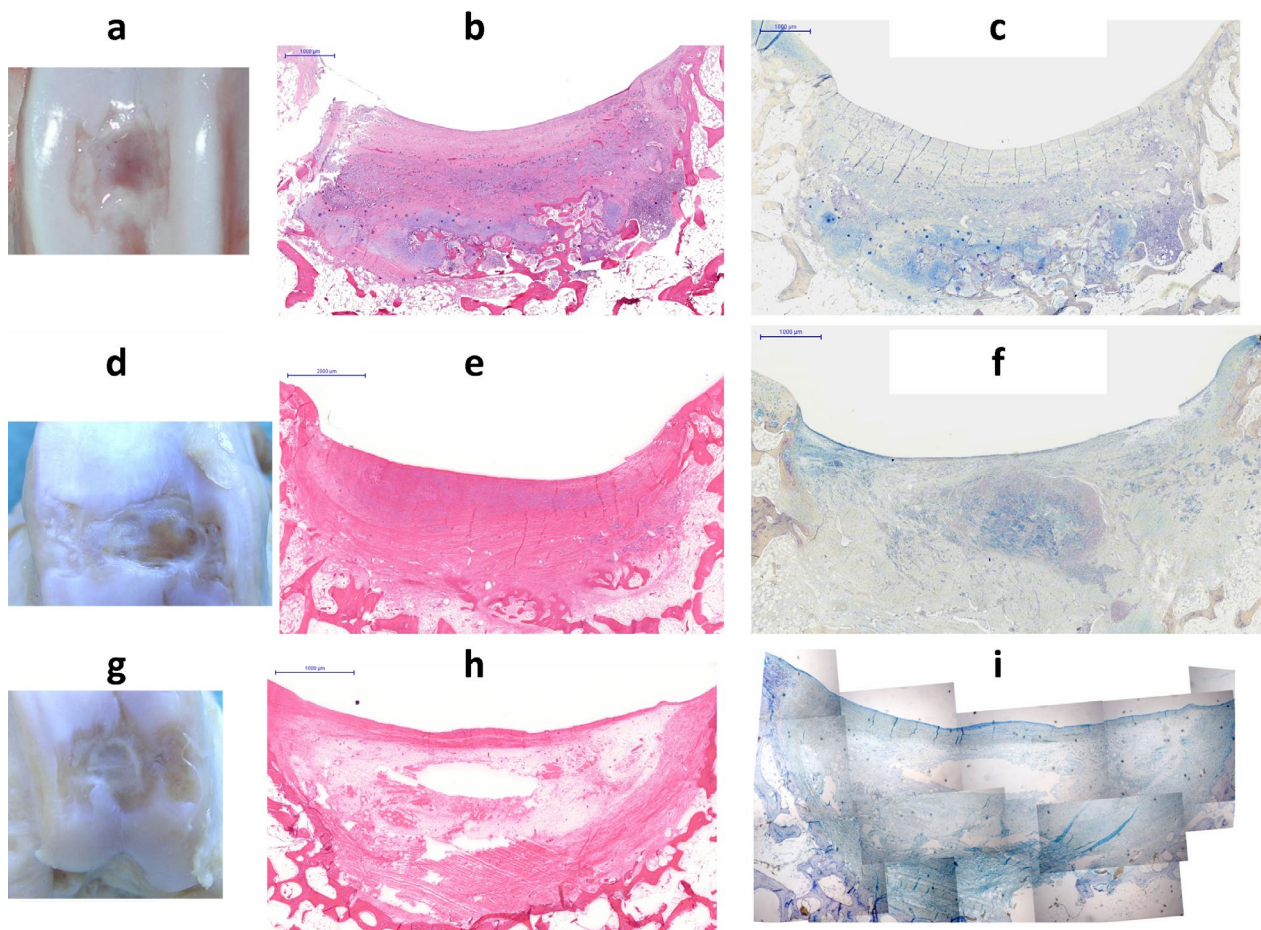


Fig. 5. Macroscopic and microscopic view of repair tissue at 3 months post-implantation. Group 1: **a,b,c**; Group 2: **d,e,f**; Group 3: **g,h,i**. Macroscopic views (**a,d,g**); sections of the defect area stained with H/E (**b,e,h**), and Alcian Blue (**c,f,i**). Histological figure **i** was obtained by combining different microscopic pictures. The repair tissue is irregular and poorly differentiated in all groups. A widespread cell infiltration is visible in all zones of the scaffold in Groups 1 and 2, the former showing higher cellularity at defect sites and inflammatory tissue around the defect area. Interposing areas of neo-matrix positive for type II collagen can be observed in Group 1 among the fragments.

Results

In vitro study

Chondrocyte outgrowth into scaffolds was evident at 15 d (data not shown) and increased at 1 and 2 months. Constructs loaded with cartilage fragments showed high cellularity after 1 month, while intense neo-matrix was evident within scaffold structures after 2 months. Migrated chondrocytes showed a rounded shape, surrounded by neo-matrix, after 2 months. The newly formed tissue between the fragments was positive for collagen type II immunostaining at 2 months (Fig. 3) and showed a weak positivity for collagen type I immunostaining.

In vivo animal study

Some major adverse events occurred during the *in vivo* study. Three animals died between day 30 and day 45 postoperatively due to acute infectious enteritis, despite the antibiotic prophylaxis, and were excluded from the study. Autopsies performed at the Animal Care Institute (CISRA) on the 3 goats did not show signs of thromboembolism in the mesenteric vessel. A total of 15 animals therefore completed the follow-up.

Three goats were euthanised at 1 month (with a total of 6 defects, 2 defects per group), 3 goats at 3 months (6 defects, 2 defects per group) and 3 goats at 6 months (6 defects, 2 defects per group) in order to qualitatively evaluate the repair process. Additionally, in order to perform semi-quantitative evaluation (ICRS, O'Driscoll and Little score) and mechanical testing (nanoindentation), 6 goats were euthanised at 1 year (12 defects, 4 defects per group).

At 1 month (Fig. 4), gross-morphological evaluation on specimens showed a depressed surface of repair tissue, pale in appearance, particularly marked in Groups 2 and 3. Gross appearances of Group 1 and 2 were similar due to the presence of the scaffold, still largely unresorbed at the surface of the defect site. Mild synovial changes were observed in all operated stifle joints. The irregular repair tissue corresponded histologically to a cell infiltration of the scaffold in Groups 1 and 2. Cells were scarce at the central zone of the defect in all three groups.

Various cells were observed as contributors to the repair. These were seen around the cartilage fragments, at the upper layer and at the bottom of the defect, along with cells visible inside the scaffold. No clear matrix

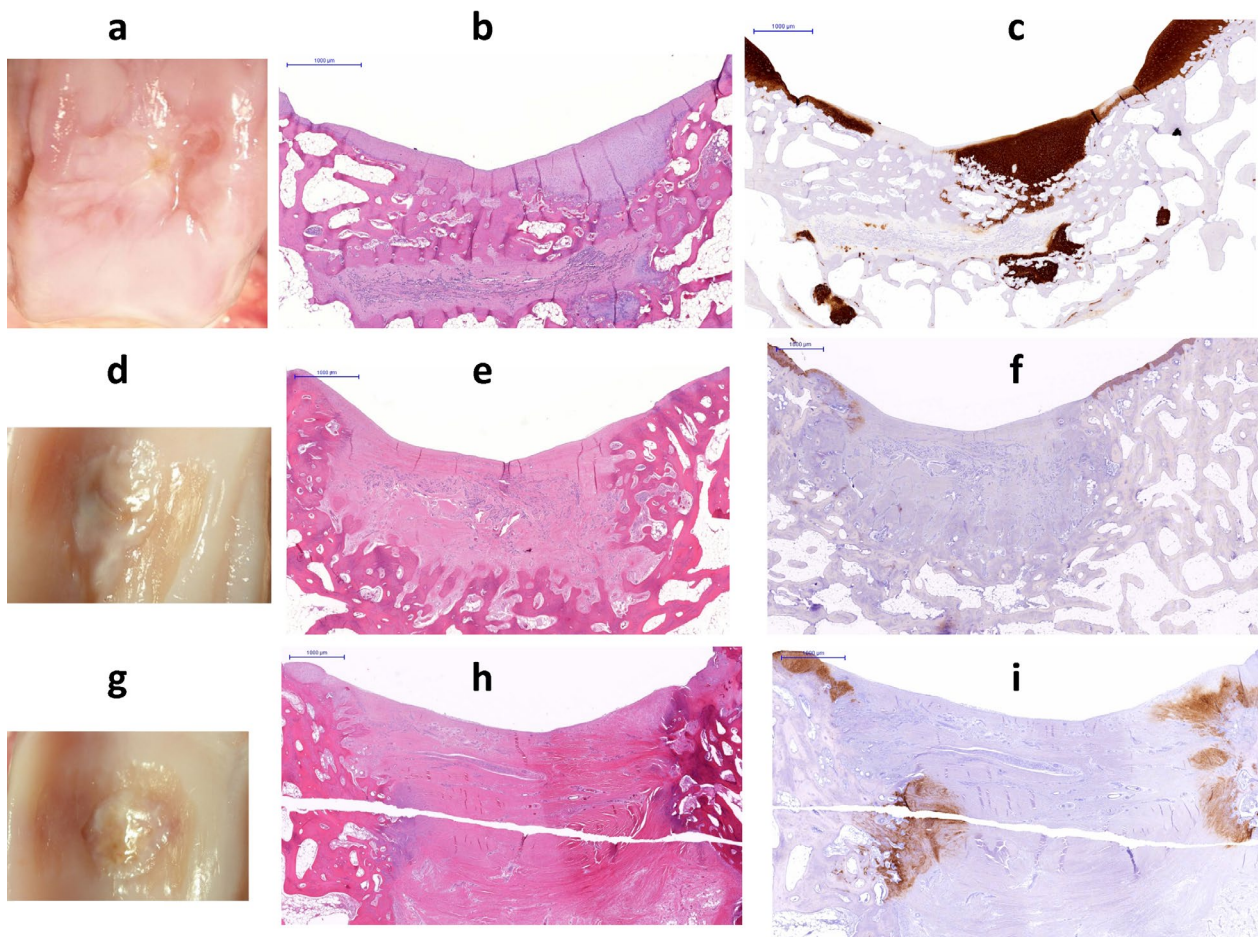


Fig. 6. Macroscopic and microscopic view of repair tissue at 6 months post-implantation. Group 1: **a,b,c**; Group 2: **d,e,f**; Group 3: **g,h,i**. Macroscopic views (**a,d,g**); sections of the defect area stained with H/E (**b,e,h**), and immunohistochemistry for collagen type II (**c,f,i**). Immature fibrous tissue and areas of hypercellularity are present in all groups. In Group 1, neo-matrix positive for type II collagen is visible at the superficial and central portions of the defects, along with remnants of the Hyaff membrane, while cartilage fragments are scarcely detectable.

deposition was observed in any group. In Group 1, cartilage fragments, which were located at the deeper zone of the defect, appeared under the HA sheet, surrounded by a large number of cells, infiltrating both the space between the fragments and the lower part of the HA sheet. Repair tissue of untreated defects was similar to the early healing process in deep osteochondral lesions in goat models, with the formation of fibrous tissue, as reported in the literature (Jackson *et al.*, 2001). Neither fracture sign nor major vascular compromise in the host bone were observed in any group.

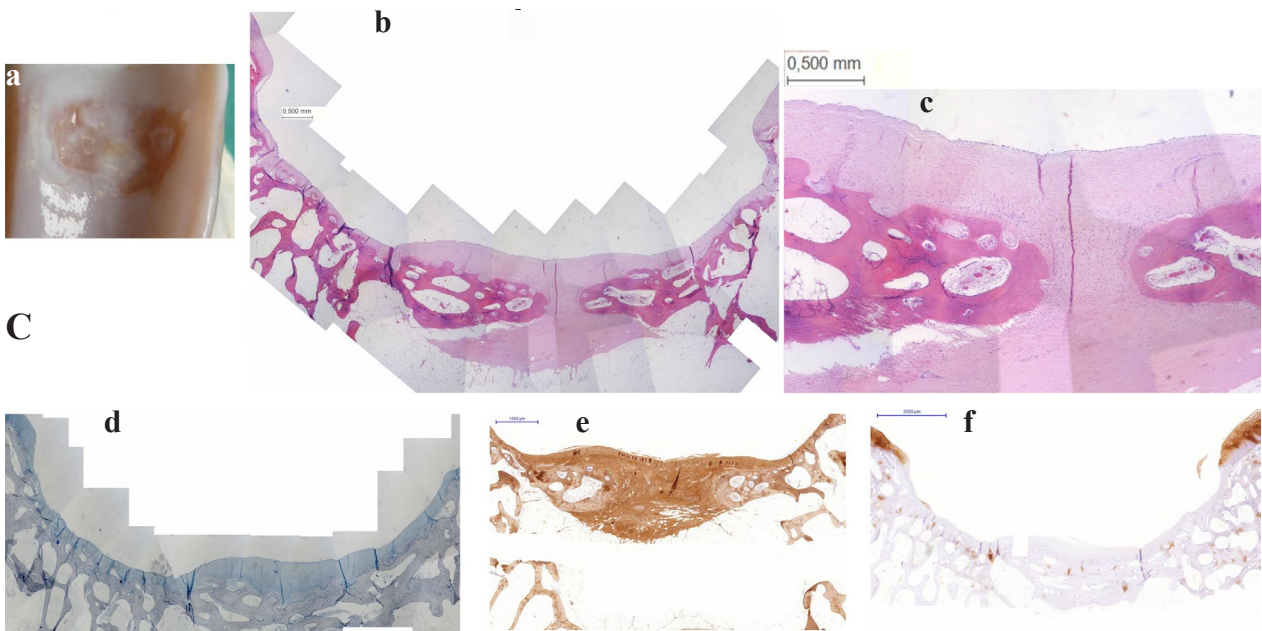
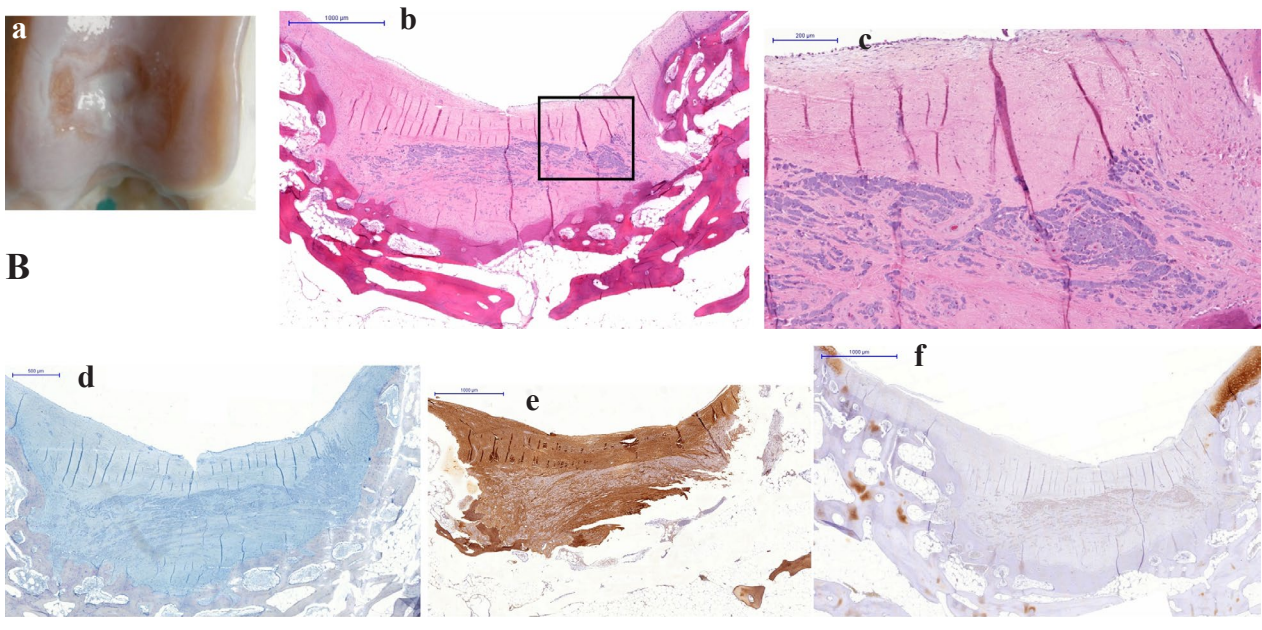
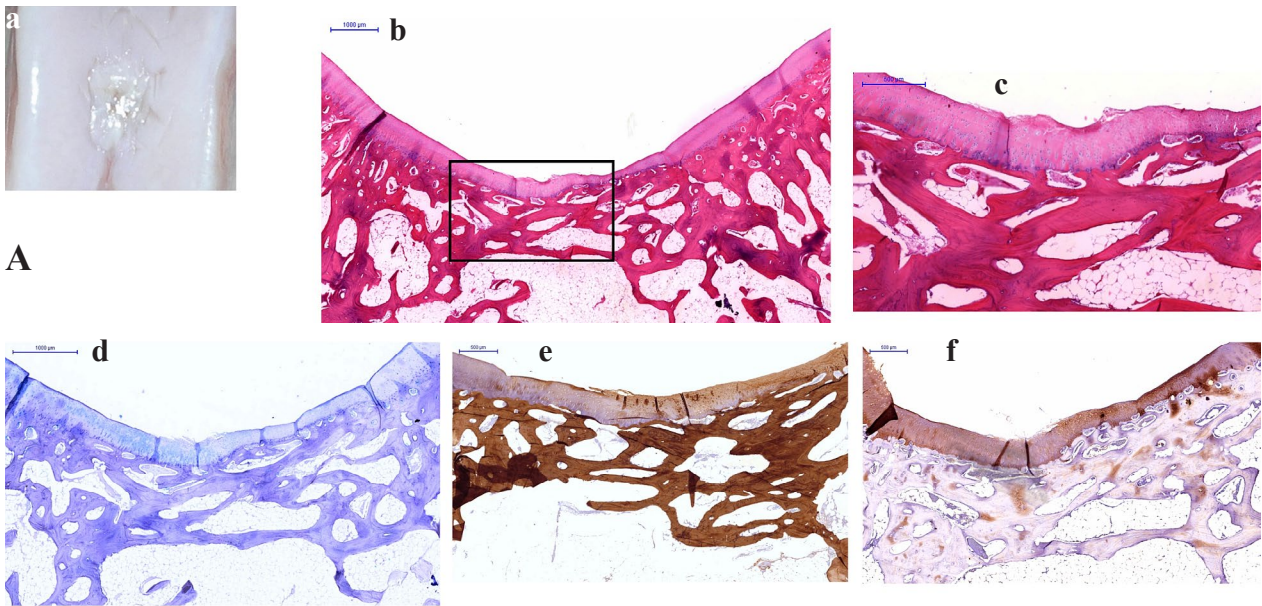
At 3 months (Fig. 5), gross-morphological evaluation in all groups continued to show an uneven and depressed layer of repair tissue. At the periphery of the defect, repair tissue was thicker in Group 1, showing a white colour similar to the surrounding cartilage. Minor synovial changes were observed in all groups. Histologically, the repair tissue was still irregular and poorly differentiated in all groups. A widespread cell infiltration was observed in all zones of the scaffold in Groups 1 and 2.

Group 1 showed high cellularity at defect sites, with some inflammatory tissue around the defect area; cartilage fragments were still detectable, although the cell migration created patchy over-stained interposing areas of matrix

positive for type II collagen among the fragments. In empty defects (Group 3) and in Group 2 samples, these areas were not detectable. Repair tissue showed a greater inflammatory reaction in Group 2 around the defect area, while in specimens of Group 3, a remodelling of the natural fibrin clot was observed, with formation, in some cases, of a central cyst-like structure, as also observed in previous studies (Jackson *et al.*, 2001).

At 6 months (Fig. 6), the repair process was still macroscopically irregular and depressed in all groups. Small osteophytes and surface irregularities were observed in all stifle joints along with mild macroscopic inflammatory changes of the synovial membrane. Immature fibrous tissue and areas of hypercellularity were observed at histological evaluation in all groups. In Group 1, cartilage fragments were scarcely detectable; areas of neo-matrix positive for type II collagen were observed at the superficial and central portions of the defects, with staining similar to that of the native cartilage at the border of the defect. In Groups 1 and 2, remnants of the Hyaff-11 membrane were still present. In the control Groups 2 and 3, no collagen type II-positive areas were observed.

After 1 year (Fig. 7), gross morphological evaluation showed better quality repair tissue in defects treated with



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Fig. 7. (previous page) Macroscopic and microscopic view of repair tissue at 1 year post-implantation. **A:** Group 1, **B:** Group 2; **C:** Group 3. Macroscopic views (**a**). Sections of the defect area stained with H/E (**b,c**), Alcian Blue (**d**), and immunohistochemistry for collagen type I (**e**) and type II (**f**). Histological figures **Cb** and **Cd** were obtained by combining different microscopic pictures. Some features of hyaline-like cartilage can be observed in repair tissue of Group 1, as the presence of type II collagen upon immunostaining in the superficial layer of the repair tissue, the presence of type I collagen in the newly formed subchondral bone, the restoration of osteochondral orientation, and, in some areas, the columnar arrangement of chondrocytes. Fibrous-fibrocartilaginous tissue is predominant in Group 2, along with remnants of the Hyaff-11 membrane in the central areas of the defect surrounded by inflammatory infiltration; incomplete subchondral bone restoration is also visible. Group C repair is mainly constituted by a homogeneous fibrocartilage repair tissue without columnar organisation surrounding a layer of newly formed subchondral bone; neomatrix in the upper layer lacks any type II collagen-positivity.

cartilage fragments along with scaffolds (Group 1). Repair tissue was white and mostly even with the surrounding cartilage, although some fissures and small depressed areas were observed at the periphery of the defects. In Groups 2 and 3, some even areas with whitish repair tissue were also present, but most of the defect site was filled with rough, uneven tissue. Synovial tissue proliferation and osteophyte formation were evaluated following the macroscopic scoring method of Little (Little *et al.*, 2010). No major synovial reactions (i.e. score > 1) were detected in any of the three groups, while mild osteophyte development (i.e. score = 1) was observed around the trochlear areas and around the medial femoral condyles in most of the specimens of the three groups. However, no significant differences were observed among all stifle joints of the experimental animals regardless of the treatment used.

Evaluation of the repair tissue showed higher scores for defects treated with cartilage fragments along with scaffolds (Group 1), both macroscopically and microscopically (Fig. 8). Macroscopic ICRS Cartilage Repair Assessment showed significant differences between Group 1 and both control groups in the “Degree of Defect Repair” and in the “Macroscopic Appearance”, while no significant differences were noticed between untreated defects (Group 3) and defects treated with scaffolds alone (Group 2). The same statistical trend was observed in the modified microscopic ICRS score, in the O’Driscoll microscopic score, and in the microscopic score of Little. In the latter, significant difference between Group 1 and both control groups was observed in section A (Structure), section D (Interterritorial Toluidine blue) and section E (Tidemark/calcified cartilage/subchondral bone), while in section B (Chondrocyte density) a significant difference was noticed between Groups 1 and 3 and between Groups 2 and 3.

Repair tissue of Group 1 showed: i) some features of hyaline-like cartilage with normal to moderate matrix staining (Fig. 7Ab and d); ii) the presence of type II collagen upon immunostaining in the superficial layer of the repair tissue (Fig. 7Af); iii) the presence of type I collagen in the newly formed subchondral bone, along with some traces also in the upper layer of the cartilaginous repair tissue (Fig. 7Ae); iv) restoration of osteochondral complexes and, in some areas, columnar arrangement of chondrocytes (Fig. 7Ac). Nevertheless, some irregular bonding adjacent to the healthy cartilage was observed.

Treatment with scaffolds alone (Group 2) mainly led to the formation of central fibrous-fibrocartilaginous

tissue with some remnants of the Hyaff membrane in the central areas of the defects (Fig. 7B). Peripheral areas of cartilage matrix were often present and were positive for type II collagen, suggesting a centripetal repair from surrounding healthy cartilage. Inflammatory infiltration was still detected around the Hyaff-11 membrane; and bone was present at the bottom of the defects, around the residual scaffold.

Empty defects were filled with a homogeneous fibrocartilage repair tissue without columnar organisation (Fig. 7C); some irregular gaps at defect borders were observed, occasionally with the exposure of subchondral bone. Group 3 fared better than Group 2 for subchondral bone restoration, but lacked any type II collagen-positive matrix at the peripheral areas.

Nanoindentation of goat samples after 1 year demonstrated interesting differences between groups (Fig. 9). Group 1 behaved in a considerably different manner from control Groups 2 and 3. From the unloading curve, hardness of Group 1 was greater than Groups 2 and 3, and mean values were closer to healthy trochlear cartilage than any of the control groups, although mean hardness (H) of healthy cartilage was significantly different from that of all groups ($p < 0.05$). Young’s modulus of Group 1 was similar ($p > 0.05$) to healthy cartilage. Mean Young’s modulus, as determined from the loading curves, revealed similar findings. A significant difference ($p < 0.05$) was still observed between mean values of healthy cartilage and mean values of all three Groups, but the group treated with cartilage fragments loaded onto scaffolds (Group 1) reached values superior to those of control Groups 2 and 3 ($p < 0.05$), and was the most similar to that of healthy cartilage (Fig. 9).

Discussion

In the present study, we have demonstrated that long-term osteochondral repair is made feasible using cartilage fragments embedded in a hyaluronic acid/PRFM/fibrin glue scaffold in a goat model. This method is distinct from aforementioned studies because it relies on a hyaluronic acid, fibrin glue and autologous PRFM scaffold to obtain chondrocyte migration from autologous cartilage fragments.

The hyaluronic acid-derived membrane, Hyaff-11, was chosen for its well-known chondrogenic and adhesive properties (Cavallo *et al.*, 2010; Kon *et al.*, 2009). In this

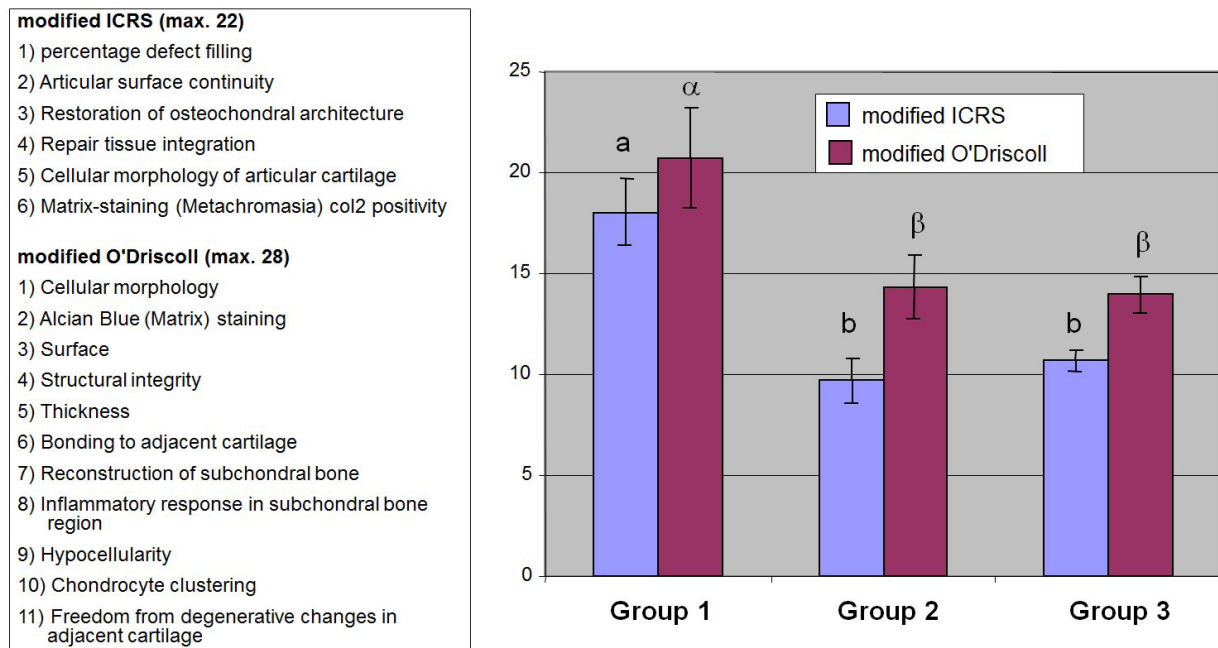


Fig. 8. Semi-quantitative histology scores using the modified ICRS score (Liu *et al.*, 2006) and the modified O'Driscoll scale (Frisbie *et al.*, 2009) at 1 year. Values with different superscripts differ at $p < 0.01$; in particular, normal letters a and b refer to ICRS score (blue bars), while Greek letters α and β refer to O'Driscoll scale (red bars).

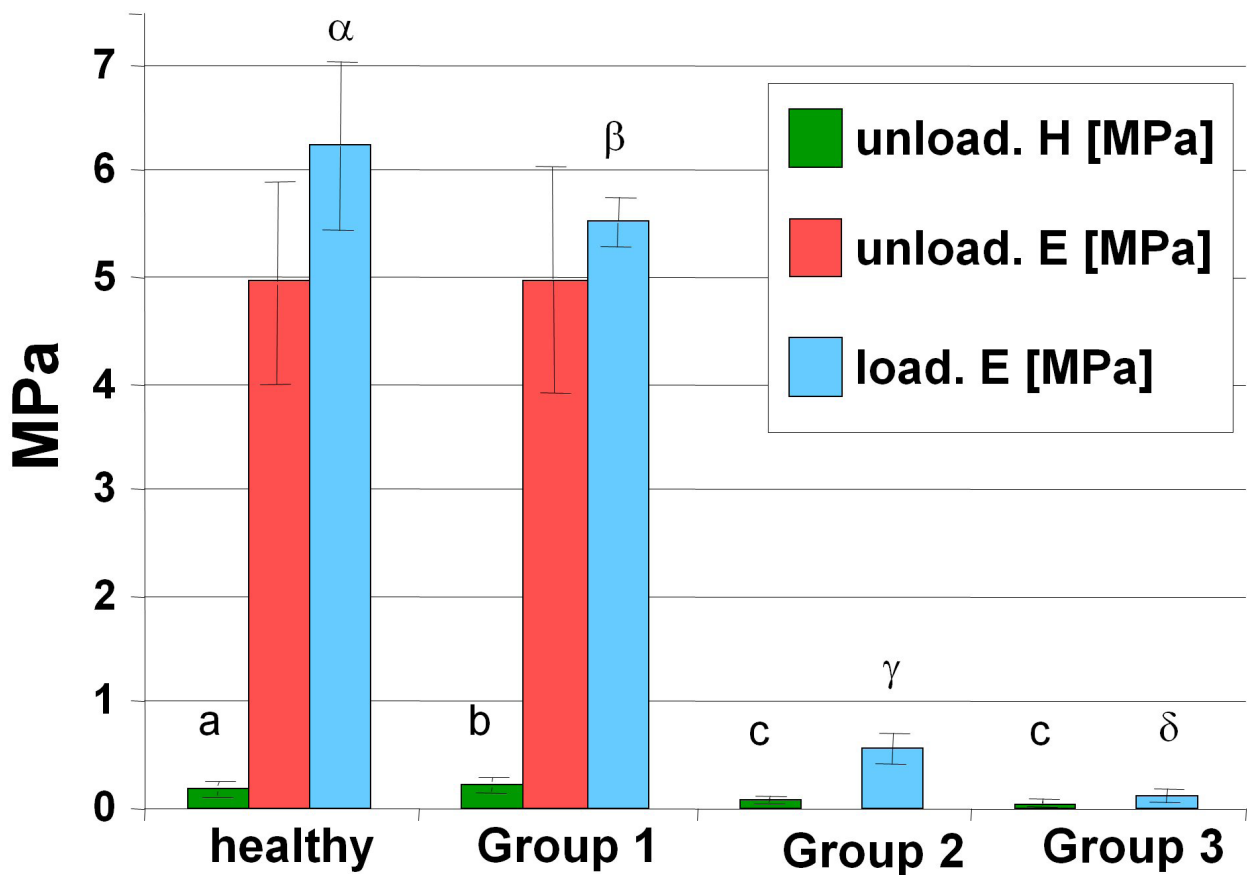
model, it also worked well in conjunction with fibrin glue for coverage of the defect, and no membrane dislodgements were observed. A single Hyaff-11 membrane was sufficient to cover the defects during the *in vivo* experiment. On the other hand, in the *in vitro* study, a single layer was inadequate to contain all migrating cells, as during the early set-up phase of the study we observed a considerable number of cells at 1 month attached to the plastic dishes. Therefore, two sheets of HA membrane were used as a barrier in the study design, in order to avoid any significant cell migration from the construct to the Petri dish.

The Cascade-PRFM was chosen as a bioactive mouldable component of the scaffold. The Cascade technique has several advantages. It enables reduction of the damage to platelets during extraction, due to the absence of bovine thrombin in the production process (Carroll, 2005), producing a gel-like matrix with significantly higher number of platelets (approximately 5x) (Simon *et al.*, 2009) in the end-product, compared to that of peripheral blood. The high capture efficiency of this commercial system was convenient for the design of the *in vivo* animal study, because it allowed for using smaller blood volumes compared to that of other commercial systems (i.e. Biomet GPS III) (Castillo *et al.*, 2011). This end-product is a platelet derivative with low content of leukocytes; this was also considered favourable, as data from the literature suggested that anabolism requires low levels of leukocytes and the presence of high leukocytes concentration may increase catabolic signalling molecules (Sundman *et al.*, 2011). Additionally, this PRFM contains higher concentrations of growth factors (e.g. TGF- β 1) compared to simple blood clots, and allows for temporary defect filling while delivering mitogenic factors from

autologous platelets up to several days after the preparation, as shown in previous studies (Visser *et al.*, 2010). These factors have also been shown to enhance tendon repair *in vivo* in animal models (Sarrafian *et al.*, 2010). Moreover, anti-inflammatory properties have been recently attributed to the presence of other agents, for example the hepatocyte growth factor (Bendinelli *et al.*, 2010). Furthermore, the end-product is a material that can be moulded in order to facilitate surgical application as the high fibrin content makes the matrix a natural scaffold with the ability to assist the ultimate repair of the lesions (Sánchez *et al.*, 2007).

For the scaffold preparation, a small quantity of commercial fibrin glue (Tisseel, Baxter) was applied in order to stabilise cartilage fragments under the HA membrane. This was supported by *in vitro* observations showing the effect of fibrin glue in facilitating the migration of chondrocytes out of cartilage fragments in the first phases of scaffold colonisation. A 1:4 thrombin dilution compared to the original commercial preparation was chosen in order to obtain a large pore three-dimensional structure; this created spatial continuity between fragments, thus enabling free movement of cells. The fibrin glue can therefore be considered as a wrapping material for the cartilage chips and a bridging material between the HA-felt and the PRFM. As shown in other *in vivo* studies (Deponti *et al.*, 2012; Lewis *et al.*, 2009; Peretti *et al.*, 2006; Peretti *et al.*, 2001; Peretti *et al.*, 2000), we did not observe any major inflammatory reactions in animal models, supporting the use of fibrin glue as a component of the scaffold.

In this study, cartilage fragments were harvested in each goat, during surgery, from the cartilage layer surrounding the two trochlear areas corresponding to the full-thickness osteochondral defect sites. They were pooled together and



from unloading curves				
	healthy	Group 1	Group 2	Group 3
H [MPa]				
Mean	0.18	0.23	0.08	0.05
SD	0.06	0.06	0.01	0.01
E [MPa]				
Mean	4.94	4.97	*	*
SD	0.92	1.06	*	*
from loading curves				
	healthy	Group 1	Group 2	Group 3
E [MPa]				
Mean	6.22	5.51	0.56	0.10
SD	0.82	0.23	0.17	0.09

Fig. 9. Nanoindentation of goat samples at 1 year. Healthy, healthy cartilage; unload. H, Hardness as measured during the unloading phase; unload. E, Young’s modulus as measured during the unloading phase; load. E, Young’s modulus as measured during the loading phase of testing; *, samples fracture during testing; SD, standard deviation. Values with different superscripts differ at $p < 0.05$; in particular, normal letters a, b, c refer to hardness “H” (green bars), while Greek letters α , β , γ and δ refer to Young’s modulus determined from the loading curves “load E” (blue bars).

then used for the *in vitro* explant culture and the *in vivo* implant in Group 1. Thus, the source for the repair was constituted of cartilage fragments originating from both the upper and the lower trochlear defect. This allowed for eliminating, both in the *in vitro* and the *in vivo* steps, any potential different contribution to the repair of the chips deriving from different trochlear sites, as pointed out by a recent study by Salzman *et al.* (2011).

The construct components were positioned in order to keep the same spatial orientation between the *in vitro* and *in vivo* part of the experiment, maintaining the cartilage chips and the fibrin glue in the middle of the construct and in contact with the PRFM on one side and with the HA felt on the opposite side. Indeed, *in vivo*, the PRFM was placed at the bottom of the goat trochlear osteochondral defects, the cartilage chips were wrapped in a small quantity of fibrin glue and placed in the middle, and the Hyaff-11 membranes at the top, due to the position of the defects and the gravity forces acting during the stance phase of the animal. For the *in vitro* part, an inverted spatial distribution was used, placing the Hyaff-11 membranes at the bottom of the Petri dishes, the mixture of chips and fibrin glue in the middle and the PRFM was placed at the top of the constructs.

The *in vitro* results showed the natural tendency of chondrocytes, once freed from the surrounding matrix, to migrate away from cartilage chips, in a standard non-chondrogenic culture medium. Indeed, our findings confirmed the initial hypothesis that the outgrowth of chondrocytes, derived from chondral fragments obtained by mechanical fragmentation of articular cartilage, allowed for the production of a newly synthesised matrix around the fragments and inside the Hyaff felt. This neofomed tissue was mostly originating from the migrated chondrocytes and showed features similar to those of articular cartilage, such as the overall predominance of collagen type II staining. These *in vitro* results are consistent with two previously published studies by Bonasia *et al.* (2011), in which adult and juvenile chondral fragments were cultured on a hyaluronic acid scaffold, and Lu *et al.* (2006). In the latter, human and bovine cartilage fragments were used both in explant cultures and in a heterotopic implant in SCID mice, after being seeded in scaffolds made of polyglycolide/non-woven felt or polyglycolide/polycaprolactone foam reinforced with polydioxanone mesh, with a coating of fibrin glue. These observations were the premise for developing an *in vivo* cartilage fragment-based technique for osteochondral repair.

Following previous evidence from preclinical animal models of trochlear defects in rabbits (Madry *et al.*, 2005; Marmotti *et al.*, 2012; Ueblacker *et al.*, 2007), goats (Lu *et al.*, 2006), pigs (Peretti *et al.*, 2006), and horses (Frisbie *et al.*, 2009), critically-sized trochlear osteochondral lesions were created to avoid spontaneous healing of smaller defects, as previously shown by Jackson and Ahern (Jackson *et al.*, 2001; Ahern *et al.*, 2009). This site has been widely used in the literature, and represents a valuable preclinical model being subjected both to shear forces, that may negatively influence the reparative process, and to compressive load during the stance and gait of animals, which have a prevalent flexed position of the stifle joint. Furthermore, it allows also for an easy surgical approach

to a large area where multiple defects could be created. Indeed, for each unilateral trochlear model two defects were created at the upper and lower trochlear areas. It is known that patellar excursion generates different load bearing between the upper and lower trochlear areas (Salzman *et al.*, 2011). Thus, the three treatment groups (1, 2 and 3) were randomised to the two defect positions during the surgical procedure, in order to lessen the bias that could be generated by the variable loads imposed on the repair tissue by the patella during the tracking along the femoral groove, and so avoiding the potentially different contribution of the two defect sites to the repair process.

A depth of 3.5 mm was chosen to simulate a human clinical setting of osteochondral lesions. As previously shown, lesions beyond 3.5 mm in goats would lead unquestionably to a violation of the subchondral bone (Hunziker, 1999b; Custers *et al.*, 2009; Ahern *et al.*, 2009) during defect drilling, with extensive bleeding and subsequent involvement of an abundance of different cell types, growth factors and signalling substances from bone, bone marrow space and its vasculature. In this regard, the methodology of creating the defect can have a significant impact on the potential of the biological response of the surrounding host tissue bed. In our study, we have chosen a manual drill with multiple lavages. With this technique, defects were created in a natural way by using a slow speed of penetration and by removing any visible debris during the defect creation through continuous irrigation with physiological solution. This allowed for a standardisation of the procedure of defect creation preserving the integrity of the surrounding tissue, avoiding major vascular occlusion by debris build-up and significant temperature increase during defect drilling. A possible alternative would have been to adopt a more physio-pathological “fracture method” as that used in the OATS procedure, in which an osteochondral bone plug is fractured off the subchondral bone. However, even if this “fracture technique” also represents a good biological duplication of osteochondral lesions, it would have presented more difficulties in an experimental setting related to the standardisation of the method.

In this large animal model, we verified the *in vivo* repair potential of autologous cartilage fragments embedded in hyaluronic acid, PRFM and fibrin glue scaffold. For control conditions, we used the whole scaffold alone and the empty defect. Although the scaffold itself was made by assembling different components, we have considered this as an entire element, and we did not further separate any of the single scaffold constituents into different control groups. Moreover, in a clinical setting, some of the components of the scaffold are often associated. This is valid, for example, for the fibrin glue with the HA membrane, as the fibrin acts as a biological glue for HA, thus representing a single complex unit. Additionally, we ran in parallel the *in vitro* and the *in vivo* study with the same design.

Generally, a slow and incomplete overall repair process was observed in all groups. This is consistent with the cartilage repair obtained with cell-based tissue engineering processes (Knutsen *et al.*, 2007; Brittberg *et al.*, 2001). Up to 6 months postoperatively, immature tissue filled the

major part of the defects, as confirmed by the persistent presence of the HA membrane in both Groups 1 and 2. Cartilage fragments in the experimental Group 1 were found to be located at deeper zones of the defects already at the earlier time points. This was presumably due to the mouldable and resorbable nature of PRFM, allowing for a deepening of the fragments inside the matrix following implantation. This also implied that the fragments at the lower defect zone underwent a slower absorption during the natural bone repair process. In this regard, further optimisation of the implant could involve the use of a stiffer matrix, to obtain a more rigid supporting structure for the cartilage fragments, thus allowing for their homogeneous distribution directly under the HA sheet at the superficial layers of the defects. Nevertheless, we can state that the fragments were positively involved in the cartilage repair process, as the samples of Group 1 achieved an improved repair compared to that of the other groups at 6 months and 1 year. However, we cannot identify the role of these fragments in the entire repair process. In fact they may represent the cell source for the repair process, or indeed they could be the releasing source of conditioning factors, guiding the migrating cells from the surrounding tissue to assume a chondrocyte phenotype, or a combination of both.

At the end of follow-up, morphological assessment showed no major synovial changes and only mild osteophyte formation in all groups, without any difference despite the treatment used. This suggests that the mild inflammatory process developed in the operated joints did not exert considerable influence in the osteochondral repair process. The end-term histological assessment of the defects demonstrated that cartilage fragment-loaded scaffolds led to a functional repair tissue with some hyaline-like features. Indeed, after 1 year, Group 1 showed an improved repair tissue with higher scores compared to those of the controls, with the presence of proteoglycans and areas of matrix positive for collagen type II. No relevant remnants of HA membrane were detectable at this period. Group 2 instead showed a persistent remodelling of immature repair tissue. There was, however, a slight chondrogenic potential of the scaffold alone, suggested by some small peripheral areas of matrix positive for type II collagen, but the overall histological appearance of the scaffold-alone samples at 1 year postoperatively was similar to that of the fibrocartilaginous healing of the empty defects. The difference between Group 1 compared with the controls suggests a key role of the minced cartilage fragments in the HA-PRFM-fibrin glue scaffold as being a source of chondrocytes and conditioning factors during the repair process. The better healing observed in Group 1, however, lacked the overall zonal organisation of hyaline cartilage, and showed some areas of incomplete repair, indicating that some improvement of the technique is still required. Nevertheless, histological results of the present study represent the basis for a suitable alternative to the new emerging one-stage methods. Additionally, we cannot exclude that further tissue remodelling may occur in a longer follow-up, although the final result of the experimental Group 1 is satisfactory for a functional repair tissue both histologically and biomechanically.

The biomechanical findings at 1 year showed a clear superiority of the Group 1 samples compared to the controls. A discrepancy can be noted by observing the greater difference between groups in the biomechanical tests compared to that measured by the histological scores. This apparent incongruence can be explained by the fact that the biomechanical analysis made by nanoindentation measured the superficial 100 μm of the samples, whereas the histological scores employed in this study related to the whole sample field. Nanoindentation was chosen to provide the experimental model with more sensitivity and resolution, compared to other clinical hand-held macroindentation devices. In light of these results, Group 1 showed also the closest mechanical properties to healthy cartilage, while both control groups fared significantly worse. Nevertheless, there was a significant difference ($p < 0.05$) in the unloading curve for Hardness between Group 1 and healthy cartilage, confirming the substantially different properties of hyaline articular cartilage compared to that of the repair tissue derived from cartilage fragment-loaded scaffolds at the longest time point tested. Analysis of Young's modulus (E) in the loading curve does not contradict these previous observations, although its value is limited to just a slight depth of indentation, and it has to be considered as only being partially representative of the mechanical behaviour of the tissue. Compared to other studies (Franke *et al.*, 2007), however, we observed overall greater values in H and E both for healthy cartilage and treated samples. This is most likely due to differences in hydrating conditions, sample preparation and storage of the specimens. This observation confirms that, in this model, biomechanical results should not be considered as absolute outcome measures for H and E, but as relative indicators of the quality of the different repair tissues in the different experimental groups compared to a corresponding area of healthy cartilage.

As with other one-stage techniques, the method described has several hypothetical advantages for clinical applications. Intra-operative construct preparation is relatively simple, and it eliminates multiple surgeries and the high costs of cell culture-expansion. From a small biopsy, large defects could be treated by uniform tissue distribution into the scaffold. Natural adhesive properties of Hyaff-11, PRFM and fibrin glue do not need any sutures or staples for fixation, simplifying the surgical procedure. Due to the mouldable properties of this scaffold, constructs with minced fragments could be prepared as "paste-like" structures and thus be implanted arthroscopically for small lesions, similarly to the Hyalograft C technique (Kon *et al.*, 2009).

Some observations should be noted with regard to the experimental study. Firstly, compared to a human setting, the situation described in the *in vivo* part can be considered a "worst case" scenario for the early postoperative period, as the goats were allowed to freely load on the operated limb. Although, in a quadruped such as the goat, it is likely that the load of the operated limb was partially reduced during the first postoperative period by the spontaneous transfer to the other three limbs, based on the pain. On the other hand, in human, patients are usually invited to reduce

the load-bearing with crutches for the first 4 weeks in order to protect the implants and the early phase of the repair. However, this aspect did not hamper the stability of the implants in the treated animals, as no sign of detachment was seen from the histology of the euthanised animals in the earlier experimental time (1 month). Secondly, as previously mentioned, 3 animals died during the first 45 days of follow-up and were thus excluded. However, this did not affect the results of the study. Indeed, the study itself was designed in order to describe the entire repair process throughout all time points, allowing for appreciating the differences at 1, 3, 6 and 12 months, especially for the experimental Group 1, and evaluating the final outcome of this new procedure at the longest time period. The number of animals and defects were sufficient to demonstrate a statistically relevant superiority of the experimental group at 1 year. Thirdly, we were not able to discriminate the cellular contribution to the repair process coming from the bone marrow, the synovial compartment and the cartilage surrounding the defects. In fact, due to the depth of the defects, cells from surrounding bone marrow could have migrated inside the scaffold, along with cells from synovial tissue and from the cartilage tissue at the perimeter of the defects, which could have adhered to the HA membrane. For this reason, different cell sources may have contributed to the final repair in all 3 groups. However, this confounding factor is common to all specimens and had therefore not jeopardised the differences between groups. A future study with a labelling technique could be employed to analyse this particular aspect.

Conclusions

These findings suggest that cartilage fragments loaded into a HA-PRFM-fibrin glue scaffold may facilitate the repair of osteochondral defects, and represent an effective alternative for a non-culture-based autologous one-stage repair technique. Both *in vitro* and *in vivo*, cartilage-like matrix was observed in the presence of chondral fragment-loaded scaffolds, suggesting that cartilage fragments represent a potential cell source for one-step procedures. Before clinical application, further studies are necessary in order to confirm the underlying mechanisms of this repair technique, both in a more mechanically challenging site, as in the medial femoral condyle, and in chronic lesions, which could respond differently to repair procedures and represent a more clinically relevant setting.

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Discussion with Reviewer

Reviewer II: Cells contributing to the *in vivo* repair could be recruited from the implanted scaffolds or the surrounding tissues or both – do you see ways of differentiating the roles of these cell populations?

Authors: This is an interesting question, focusing on one of the main observations of our *in vivo* study. Indeed, in the goat model the contribution to the repair may be derived both from the cells implanted by the scaffolds and from the surrounding tissues, such as the bone marrow, the synovial compartment and the cartilage surrounding the defects. In the literature, different studies have proposed a cell labelling technique to assess the location and the role of specific cell populations at the defect site, but this seems to be somehow impractical for our repair model, dealing with cartilage fragments embedded in a composite scaffold as a source of cells for the repair. On the other hand, a male source of fragments implanted in a female host could represent a possible solution to that issue, due to the possibility to detect cells carrying the Y gene sequence in the repair tissue by *in situ* hybridisation, as suggested by recent work of Dattena *et al.* (2009). Indeed, the use of scaffold with allogeneic chondral fragments transplanted to recipients of different gender may constitute an alternative to identify the position of the migrating chondrocytes in the repair tissue and to discriminate this particular cell population from the recipient cells of the surrounding tissue.

Reviewer II: The constructs consist of an array of components that due to their commercial source may have limited general and long-term availability. Can you speculate which components have the most favourable impact on healing and reversely which components could be left out or replaced with non-proprietary materials?

Authors: The use of a composite scaffold has several advantages linked to the different properties of the components, and the choice of a commercially available source for the materials has, potentially, the clinical relevance of a “direct” application of the same tools. The Reviewer’s observation is entirely appropriate because it is well known that commercial materials can be withdrawn for different reasons, spanning from commercial politics to emerging technologies that are offered in place of the previous components. We believe that two components are fundamental for the repair driven by cartilage fragments. The first is the fibrin glue. It allows the fragments to be bound together, stabilising the construct for the first phases of the repair, and facilitates the migration/outgrowth of chondrocytes toward the outer space through the fibrin net. This makes fibrin glue optimal for embedding cells for cartilage repair, also considering the possibility to modulate the formation of the “pores” simply by changing the concentration of the thrombin before the formation of the fibrin net. Besides, fibrin glue has also been proposed in the literature as a single scaffold for autologous chondrocyte implantation because of its peculiar properties (Bekkers *et al.*, 2013; Wysocka A *et al.*, 2010). However, the issue of the commercial availability of the fibrin glue could be simply solved in a potential clinical setting by preparing autologous fibrin glue from the blood of the patients or, as an alternative, by using allogeneic fibrinogen from voluntary donors stored in the local blood centre.

The second important component of the scaffold is certainly the HYAFF-11 membrane. The properties of a hyaluronic acid felt go beyond the simple action of “coverage” of a lesion, because the acid itself has chondrogenic properties and can positively influence the formation of cartilaginous tissue (Cavallo *et al.*, 2010). The ease of application of this membrane makes this component very useful, being an “active” coverage of the osteochondral defect. Moreover, in different clinical papers, HYAFF-11 has already been used as a scaffold for one-stage cartilage repair together with bone marrow and platelet-rich plasma (Giannini *et al.*, 2009; Buda *et al.*, 2010). However, in the unlikely event HYAFF-11 being

no longer available, another biological scaffold could be used as a substitute. The collagen I/III membranes used for the MACI technique (i.e. ChondroGide®) may perform as well for the coverage of the defects, although its structure is more rigid and less permeable than that of the HYAFF felt. The bottom of the goat defects in our study was filled with a platelet-derived gel classified as “Platelet-rich-fibrin-matrix” (PRFM). This is the third component of the construct. It was chosen to fill the bottom of the defects for the malleability associated with a short-term delivery of growth factors. This type of platelets derivative is largely used in clinical settings. Some impact, in the very first phases of the healing process, may derive from the release of the growth factors stored in the platelets. However, the main action of this type of PRFM lies probably on the tri-dimensional structure of the fibrin network that is slowly resorbed and substituted by the host bone. If not available, it can be easily substituted for by more rigid components such as the demineralised bone matrix chips or, in case of superficial defects not involving major portions of the subchondral bone, it could be left out – provided that a sufficient filling is guaranteed by the other components of the constructs.

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