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In Vivo Evaluation of 3-Dimensional Polycaprolactone Scaffolds for Cartilage Repair in Rabbits

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Abstract

Background: One of the challenges of a scaffold in cartilage tissue engineering is maintaining mechanical integrity and withstanding stress loads in the body, as well as providing a temporary substrate to which transplanted cells can adhere.

Purpose: This study evaluates the use of poly(ε-caprolactone) (PCL) scaffolds for the regeneration of articular cartilage in a rabbit model.

Study Design: Controlled Laboratory Study

Methods: Five conditions were tested to attempt to cartilage repair: In order to compare spontaneous healing (from subchondral plate bleeding) and healing due to tissue engineering, the experiment considered the use of osteochondral defects (to allow blood flow into the defect site) alone or filled with bare PCL scaffold and the use of PCL-chondrocytes constructs in chondral defects. For latter condition, one series of PCL scaffolds was seeded *in vitro* with rabbit chondrocytes for 7 days and the cell/scaffold constructs were transplanted into rabbits' articular defects avoiding compromising the subchondral bone. Cell pellets and bare scaffolds were implanted as controls in a chondral defect.

Results: After 3 month of *in vivo* implantation of PCL scaffolds or cells/PCL constructs, defects were filled with white cartilaginous tissue; integration into the surrounding native cartilage was much better than control (cell pellet). The engineered constructs showed histologically good integration to the subchondral bone and surrounding cartilage with accumulation of extracellular matrix including type II collagen and GAG. The elastic modulus measured in the zone of the defect with the PCL/cells constructs was very similar to that of native cartilage, while that of the pellet-repaired cartilage was much smaller than native cartilage.

Conclusions: The results are quite promising with respect to the use of PCL scaffolds as aids for cartilage tissue engineering.

Keywords : PCL scaffold; chondrocytes; articular cartilage; tissue engineering.

Introduction

Articular cartilage has very limited capacity for repair due to its poor cellularity and absence of vascularization. Spontaneous repair of cartilage only takes place when the damage reaches the subchondral bone and mesenchymal stem cells are released from bone marrow. Some techniques stimulate this repair capacity from bone marrow, for example drilling [24], abrasion arthroplasty[26] and microfracture.[46] Although microfracture works quite well for small lesions on the femoral condyles (Kreuz P.C.), in other cases, the tissue formed by spontaneous healing is usually more fibrocartilage-like than hyaline-like and degenerates on a long term. [23, 43] Other therapeutic strategies have been developed by orthopaedic surgeons: osteochondral transplantation (mosaicplasty)[19], and transplantation of periosteum or perichondrium to resurface the damaged cartilage.[40] Mosaicplasty may be limited by the size of the injured area and involves donor site morbidity. Moreover, the difference in mechanical stress between donor and receptor region has been related to degeneration of implants.[23] In 1994, Brittberg et al [6] reported the first clinical results of transplantation of human autologous chondrocytes covered with a periosteal flap (ACI, autologous chondrocyte implant). Although this technique has been applied successfully for more than a decade, it has two major disadvantages: a wide arthrotomy incision and the fact that the patient is submitted to two surgical procedures. Moreover, two studies demonstrated that clinical and histological results obtained at two and five years after ACI repair were identical to microfracture results. [31, 30]

In recent years, tissue-engineered cartilage using scaffolds and cells has been considered for improved treatment of cartilage defects.[4] In particular, a scaffold can play an important role in cartilage tissue engineering by maintaining mechanical integrity, withstanding stress loads in the body and thereby providing an adequate mechanical

environment to cells. Mechanical environment is crucial to cell fate [13] , and in particular the maintenance of chondrocyte phenotype and secretion of hyaline extra-cellular matrix (ECM) has been shown to be directly related to mechanical stimulation.[18] Moreover, chondrocyte phenotype may be lost during expansion in monolayer cultures [44] , but it has also been observed that dedifferentiated chondrocytes redifferentiate when returning to a 3D environment.[5] Solid scaffolds provide a 3D environment where cells can spread out, redifferentiate and synthesize hyaline-specific ECM proteins.

A wide range of biodegradable polyesters have been tested as scaffolding materials, including polyglycolide (PGA), polylactide (PLA), poly(ε-caprolactone) (PCL) and their co-polymers. These materials have been shown to support cell attachment, proliferation, and matrix production for a variety of cell types, including chondrocytes, osteoblasts and mesenchymal stem cells.[1, 25, 35] Recent studies have demonstrated the biocompatibility, differentiation and specific gene expression of rabbit chondrocytes seeded into scaffolds implanted subcutaneously or in cartilage defects. [28, 27, 47, 45, 29, 14]

Here we use polycaprolactone (PCL) scaffolds and culture chondrocytes therein before implantation. PCL is a semi-crystalline material with good mechanical properties that degrades much more slowly than other polyesters.[32] Owing to the degradation properties of PCL, it can be used for long-term *in vitro* cell culture prior to implantation into the injury site, as these scaffolds will maintain their architectural integrity and mechanical properties during the pre-implantation period while chondrocytes are both redifferentiating and synthesizing a new cartilage matrix (as seen in our previous work[16]). Moreover, the scaffolds used have been shown to have an elastic modulus close to the values measured for cartilage and are thus susceptible to provide an adequate mechanical environment to cells.[33] The aim of the present study was to evaluate if PCL engineered constructs are able to regenerate a high quality articular

cartilage and support the join mechanical loads in an *in vivo* system. For this purpose, we implanted the engineered constructs in rabbit knee joints to evaluate the influence of PCL scaffold on the observed response, comparing it with a chondrocyte pellet transplant (as a model for ACI). We also use void scaffolds in osteochondral defects and chondral defects in order to observe the healing response in presence of a scaffold without cells and compare it with the tissue engineered constructs, as well as an osteochondral defect without any implant as a negative control.

Material and methods

Scaffold fabrication

The PCL scaffolds were produced as described in [28] . Shortly thereafter, porogen templates of the scaffolds were prepared by sintering acrylic resin microspheres (Elvacite 2043, Lucite International) at 140ºC. The porosity of the templates was adjusted by submitting the templates to an appropriate degree of compression.

PCL scaffolds were obtained by the injection of melted PCL (Polysciences, Mw=50000) at 110ºC into the templates using a custom-made injection device. The pressure of nitrogen gas in the device forced the PCL melt to efficiently fill the template's pores. Thereafter, the porogen was leached out by repeated washings in ethanol. The PCL scaffolds had a porosity of 70±2%, with good interconnected morphology and homogeneity. The pore size is equal to the diameter of the porogen particles used, around 200μm. The elastic modulus of the dry, empty scaffold can be extrapolated from [28] to be ~5MPa.

Animals

Adult male New Zealand white rabbits (NZW) weighing 2.5-3.0 kg were obtained from Granjas San Bernardo S.L. (Spain) and kept under conventional housing conditions. Quarantine lasted 7 days. The animals were housed with appropriate bedding and provided free access to drinking water and food. Rabbits were kept in standard single cages under controlled temperature and light conditions.

The study protocol was approved by the Ethics Committee of our center (Permission Nos 273/06) according to 86/609/EEC law and decrees 214/1997 and 164/1998 of the local government.

Rabbit chondrocyte harvesting

Articular cartilage was obtained from knee joints after rabbit sacrifice with a high dosage of Tiopental (Tiobarbital®, Braun laboratories) via intravenous injection. As a first step, one rabbit was sacrificed to obtain enough chondrocytes to seed biomaterials or pellets implanted as an allograft in the first set of *in vivo* experiments. After sacrifice, articular cartilage from healthy contralateral knees of this first set of specimen-implanted rabbits was used to obtain chondrocytes for subsequent experiment sets.

The cartilage was dissected from subchondral bone, finely diced and then washed with supplemented 100 U penicillin, 100 μg streptomycin (Biological Industries) and 0.4 % fungizone (Gibco) Dulbecco's modified Eagle's medium (DMEM) (Life Technologies). Cartilage digestive enzymes were prepared with this medium**.** For chondrocyte isolation, the diced cartilage was incubated for 30 min with 0.5 mg/ml hyaluronidase (Sigma-Aldrich) in a shaking water bath at 37ºC. The hyaluronidase was subsequently removed and 1 mg/ml pronase (Merck, VWR International SL) was added. After 60 min incubation in a shaking water bath at 37ºC, the cartilage pieces were washed with supplemented DMEM. After removal of the medium, digestion was

continued by addition of 0.5 mg/ml of collagenase-IA (Sigma-Aldrich) in a shaking water bath kept at 37ºC overnight. The resulting cell suspension was filtered through a 70 µm pore nylon filter (BD Biosciences) to remove tissue debris. Cells were centrifuged and washed with DMEM supplemented with 10% foetal bovine serum (FBS) (Invitrogen SA). Finally, the cells were plated in tissue culture flasks for immediate chondrocyte culture or cryopreserved in liquid nitrogen with DMEM containing 20% FBS and 10% dimethyl sulphoxide (DMSO) (Sigma-Aldrich**)** until use.

Cell culture in PCL scaffolds

After isolation or thawing, cells were plated in culture flasks at high density in culture medium DMEM supplemented with 10% FBS and 50 µg/ml ascorbic acid (Sigma-Aldrich) at 37ºC in a 5% CO_2 humidified atmosphere. The medium was changed every 3 days. After 7-14 days, adherent cells were harvested by incubation with trypsin-EDTA (Biological Industries) and resuspended with a minimum volume of culture medium. PCL samples were placed on a 96-well polystyrene culture plate (Nunc A/S) and moistened with Hanks' Balanced Salt Solution (Sigma-Aldrich). After removing the Hanks' solution, cell suspension ($0.5\text{-}1x10^6$) was poured onto the scaffolds to allow infiltration of the cells into the porous structure. After 1 hour, culture medium was gently added to ensure that the material was covered. The biomaterials were changed to a new well after 1 day and cells were cultured with DMEM supplemented with 1% ITS (BD Biosciences) and 50 µg/ml ascorbic during 7 days. Two PCL samples were assayed in parallel to analyse the specimen pre- and post-implantation to the rabbit articulation injury.

Pellet preparation

After harvesting the cells from the culture flasks, resuspended cells were transferred to a 15 mL polystyrene centrifuge tube (1,000,000 cells per tube) to which culture medium was added until a total volume of 1 mL was reached. The cell suspension was centrifuged for 4 min at 1,200 rpm. The resulting pellet was cultured with DMEM supplemented with 1% ITS and 50 µg/ml ascorbic acid at 37ºC in a 5% CO_2 humidified atmosphere for 7 days.

Two pellet samples were assayed in parallel to analyse the specimen pre- and post-implantation to the rabbit articulation injury.

Engineered cartilage implantation

The rabbits were pre-anaesthesized by subcutaneous injection of 25 mg/kg ketamine (Ketolar® Pfizer Laboratories) and 3 mg/kg xylacine (Xilagesic 2% ® Calier S.A.Laboratories)). Before surgery, rabbits were prepared (washed, shaved, etc.) and general anaesthesia was induced by 4% isofluorane using a specially designed mask and maintained by administration of 2% isofluorane with O_2 at 2 l/min. The surgical site was sterilized using iodine solution; non-sterile parts of the rabbit were covered with sterile drapes. All instruments were sterilized and kept sterile during the operation.

An arthrotomy of the knee joint was performed through a medial longitudinal parapatellar incision. The medial capsule was incised and the patella laterally dislocated. A 3-mm steel trephine was used to create the chondral or osteochondral defect (depending on the case studied), 3 mm in diameter and 1-2 mm in depth, in the central articulating surface of the trochlear groove. The defect was cleaned and rinsed with sterile saline. In the case of inverted periostium transplantation, the periostium was

obtained from the medial metafisarium (metaphyseal) tibial surface of the intervention knee. The specimens (PCL scaffolds or pellets-periostium) were fixed into the holes with chondral stitches of 6/0 Coated Vicryl ® (Johnson-Johnson Intl). The specimens were held in place within the defects by repositioning the patella within the trochlear groove. Arthrotomy and skin were sutured with continuous stitches of 4/0 Coated Vicryl ® (Johnson-Johnson Intl). After removal of the conformed anaesthesia mask, all rabbits were returned to their cages and allowed free cage activity. All rabbits wore a soft knee bandage in flexion position for 3 days. Postoperative analgesia consisted of 3 mg/kg intramuscular dexketoprofen (Enantyum ® Menarini Laboratories) on the surgery day and the same 24h dosage for 3 days. At the end of surgery, 3mg/kg intramuscular Gentamicine 3mg/kg (Genta-Gobens ® Normon Laboratories) was administered as antibiotic prophylaxis.

Animal sacrifice and tissue retrieval

Rabbits were sacrificed after 3 months to assess cartilage repair. A 10x10x5 mm cut was made in the articulations with implanted engineered cartilage, being careful to keep the repaired defect at the centre of the sample for purposes of compressive modulus analysis and histological and immunohistological analyses. Table 1 shows the total number of rabbits used in the study, the majority of them in PCL/cells and pellet implantations because these specimens were used in histological and indentation tests. Moreover, more replicas of PCL/cells were performed to avoid possible variability when cells were seeded into the scaffold.

Table 1: Number of Rabbits Used for Each Specimen Implantation (CL, polycaprolactone; ACI, autologous chondrocyte implantation).

Implant	Subchondral Injury	Role	Rabbits
None	Yes	Negative control	2
PCL, void	Yes	Control spontaneous healing in presence of scaffold	4
PCL, void	No	Negative control for scaffold/cells constructs without spontaneous healing	4
PCL/cells	No	Specimen tested	10
ACI (pellets)	No	Positive control	6

Histology

The ability of chondrocytes to synthesize glycosaminoglycan (GAG) in the porous PCL scaffold and pellet before after in vitro culture (previous to implantation) and at 3 months post implantation was monitored by Alcian blue staining, counterstained using Mayer's haematoxylin and then analysed by optical microscopy. The results were evaluated by three individuals who were blinded with the specimen implantation. Briefly, the scaffolds were embedded in optimum cutting temperature (OCT) compound (Tissue-Tek, Sakura Finetek) and cryosectioned (8 μm thick). Cryosections were air-dried and then fixed in acetone for 10 minutes at 4ºC before staining or were stored at -20ºC until use.

Rabbit articulation specimens were rinsed with PBS and fixed with 4% paraformaldehyde overnight at 4ºC. Then, samples were rinsed with PBS and decalcified with osteosoft (Merck) from 48 to 72 hours at room temperature. Then, samples were rinsed with PBS and soaked in 20% sucrose overnight at 4ºC. Finally, the specimens were embedded in OCT compound and cryosectioned (14 μm thick). Cryosections were air-dried before staining or were stored at -20ºC until use.

Immunohistology

Immunohistological analysis was used to detect the synthesis of type I and type II collagen and the expression of Ki-67 (a proliferation marker). Pre-implanted scaffolds and rabbit articulation specimens were manipulated as in the histology procedure. Sections were incubated for 1 hour at room temperature with a 1:1000 dilution of type I collagen antibody (Sigma), or 1 μg/ml of type II collagen antibody (Calbiochem), or 1:100 dilution of Ki-67 antibody (Dako Cytomation). Antigen-antibody complexes were detected colorimetrically using HRP-conjugated secondary antibody and the DAB+chromogen (Dako Cytomation) and counterstained with Mayer's haematoxylin.

Indentation tests

Mechanical properties were measured with Seiko TMA/6000 equipment in the compression mode, with a 1mm diameter stainless steel probe. Samples were selected that contained both the cartilage and a section of bone from the knee, with prismatic geometry and approximate dimensions of 10×10×5 mm. Samples were obtained from the site of the initial incision in the femoral trochlea, being careful to keep the repaired defect at the centre of the sample, and symmetrically from the contralateral knee for use as a control. The probe was placed at the centre of the repaired cartilage and at the same position on the contralateral knee specimen.

A load ramp was applied at a constant speed of 10 g/min. After that, the load was removed at the same speed and the sample recovered for 10 minutes. The process was repeated 3-5 times per sample. The experiment was carried out at 38ºC in a saline solution. The stress-strain curves of the loading run were constructed from these experimental data (Figure 6), and a linear fit of 10% of the initial curve was used to

determine Young's modulus of all the cartilage samples. The slope of this line is related to Young's modulus (E) by the equation [20] :

$$\frac{dT}{d\varepsilon} = \frac{2\kappa E}{\pi a\left(1-\nu^2\right)}\omega_0$$

where T is the applied stress, a is the probe radio, ν is the Poisson ratio, ω_0 is the cartilage thickness, ε is the sample strain, and κ is a correction factor that accounts for the finite layer effect, depending upon ν and the ratio a/h (h is the sample thickness). Values of κ appear in Table 1 of reference. [20]

The sample thickness was measured on a cross-sample section observed under the optical microscope. Thus, the sample strain was calculated as the displacement divided by the cartilage thickness.

Since we could not measure the Poisson ratio, we assumed ν=0.4 to compute E from our indentation tests, as done in references.[17, 21, 42] This assumption is not very restrictive here, given that E is relatively insensitive to small changes in ν.[21] As the surrounding bone is much more rigid than cartilage, we also assumed that all the deformation was produced in the rabbit cartilage and bone deformation was negligible in this procedure.

Results

Histological and immunohistological analysis

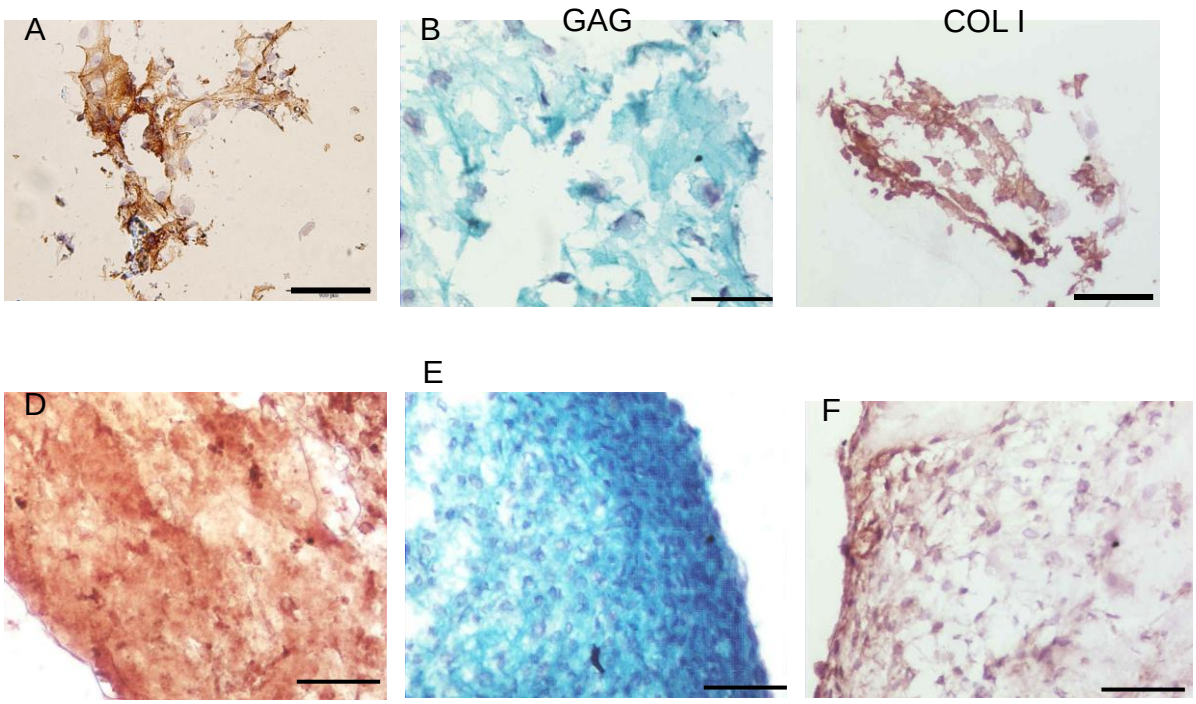

Figure 1. Type II collagen (A and D) and type I collagen (C and F) immunohistochemical staining and Alcian blue staining (B and E) of sections of rabbit chondrocytes cultured in polycaprolactone (PCL) scaffolds (A, B, and C) or in pellets (D, E, and F). Specimens were cultured for 7 days in insulin-transferrin-selenium (ITS) and ascorbic acid. Scale is 100 μm.

Regeneration of articular cartilage after specimen implantation in knee defect was evaluated by testing type I and type II collagen and GAG synthesis. On the same basis, a previous histological analysis of the cell-scaffolds constructs was performed before implantation to assess the level of cell colonization and ECM production within the constructs as well as adequate phenotypic expression. At the time of implantation, all the scaffold-cells constructs showed high cell density in the pores, and the cells were surrounded by a hyaline-like extracellular matrix (type II collagen and GAG expression) (Figure 1A and B). Additionally, some type I collagen synthesis was detected (Figure 1C). Results for pellets and PCL scaffold-cells constructs were similar in terms of

chondrocyte differentiation and extracellular matrix synthesis (Figure 1D and E). Type I collagen was expressed only in peripheral cells of pellets with a fibroblastic-like shape (Figure 1F). Cells in both PCL scaffold and pellet samples expressed S-100, suggesting that cells were well differentiated to chondrocyte phenotype (data not shown).

After specimen implantation into the cartilage defect, the rabbits were stabled with free activity for 3 months and then sacrificed to permit macroscopic and microscopic exploration of the implanted joint.

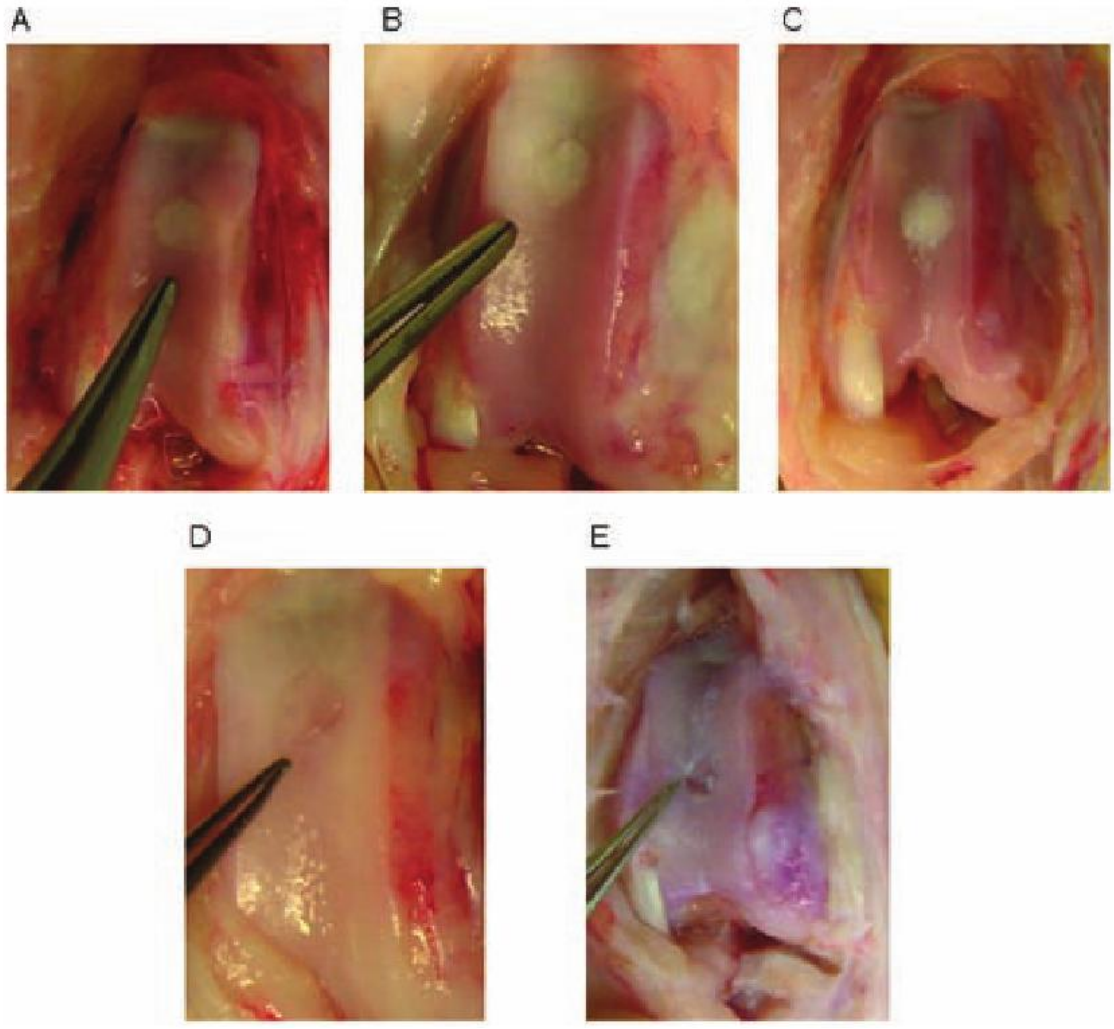

Figure 2. Macroscopic analysis of the repaired chondral defect 3 months after construct implantation. A, polycaprolactone (PCL)/cells; B, PCL/bone injury; C, pellet 1 inverted periosteum; D, PCL; and E, subchondral bone injury without

PCL scaffold.

The macroscopic exploration showed good integration of the implanted specimen in the articular cartilage. However, the implantation site looked different from the native surrounding region in the majority of cases (Figure 2). The regions repaired by PCL+cells (Figure 2A) and pellets (Figure 2C) showed better border regularity and smooth continuity with the surrounding native cartilage. When pellets with an inverted periostium cover were implanted, the new cartilage differed in colour from adjacent native cartilage. In some cases in which a PCL scaffold (with or without cells) was implanted, the aspect, including brightness, stiffness and colour, was very similar to the adjacent cartilage. In the cases in which neither chondrocytes nor scaffolds were implanted (negative control), the defect was evident after 3 months and showed clear differences compared to the surrounding cartilage although the defects were designed to provoke spontaneous healing by subchondral plate bleeding (Figure 2E).

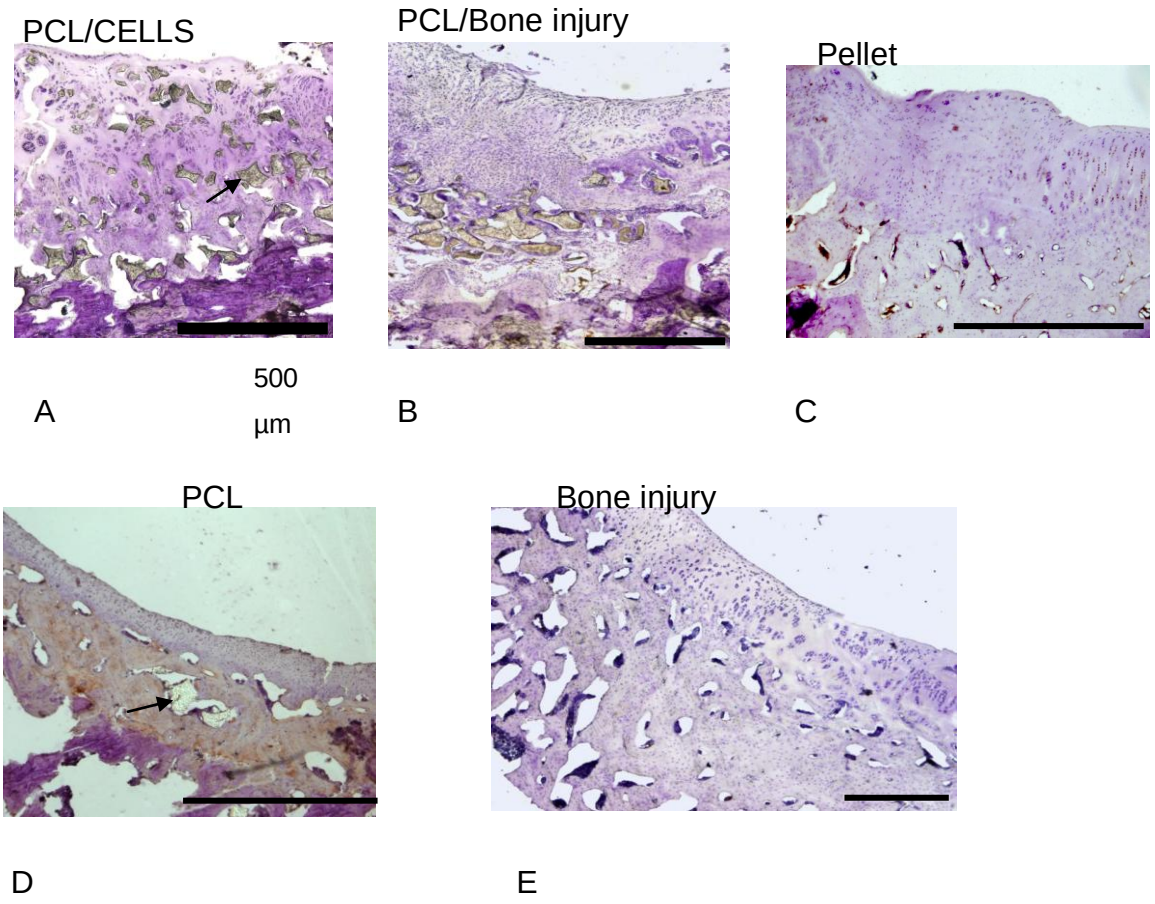

Figure 3. Hematoxylin staining for general observation of histologic sections of repaired rabbit articular joints. A, poly- caprolactone (PCL)/cells; B, PCL/bone injury; C, pellet; D, PCL; and E, bone injury. Scale is 500 µm. B, subchondral bone; S, scaffold; N, native cartilage; R, repaired cartilage.

Microscopic analyses showed that all implanted specimens were in the implantation site and had good integration in the subchondral bone and surrounding cartilage (Figure 3). However, PCL/cells construct and PCL/bone injury (figure 3A and B) showed better integration than bare PCL (figure 3 D). The letters in the micrographs designate respectively the bone (B), scaffold (S, appears as grey inclusions), native cartilage (N) and repaired cartilage (R). PCL material clearly appears as it had not degraded at 3 months after implantation; more time is needed to assess the final outcome of the

cartilage regenerated with these scaffolds if total degradation of the scaffold is desired. As can be seen, the scaffolds that were implanted alone do not appear at the surface of the defect, but seem to have been compressed in the deep of the defect, whereas in this case the repaired cartilage appears on top of it. However, only the defects repaired with PCL/cells (Figure 3A) or PCL/bone injury (Figure 3B) showed a repaired tissue with a substantial thickness and a structure comparable to native cartilage. In all cases studied, a small amount of inflammation was observed, mainly limited to the external surface of new synthesized cartilage. Higher inflammation levels were detected when subchondral bone was damaged than with subchondral bone preservation (data not shown) likely due to the blood flow that boosted the immune system response.

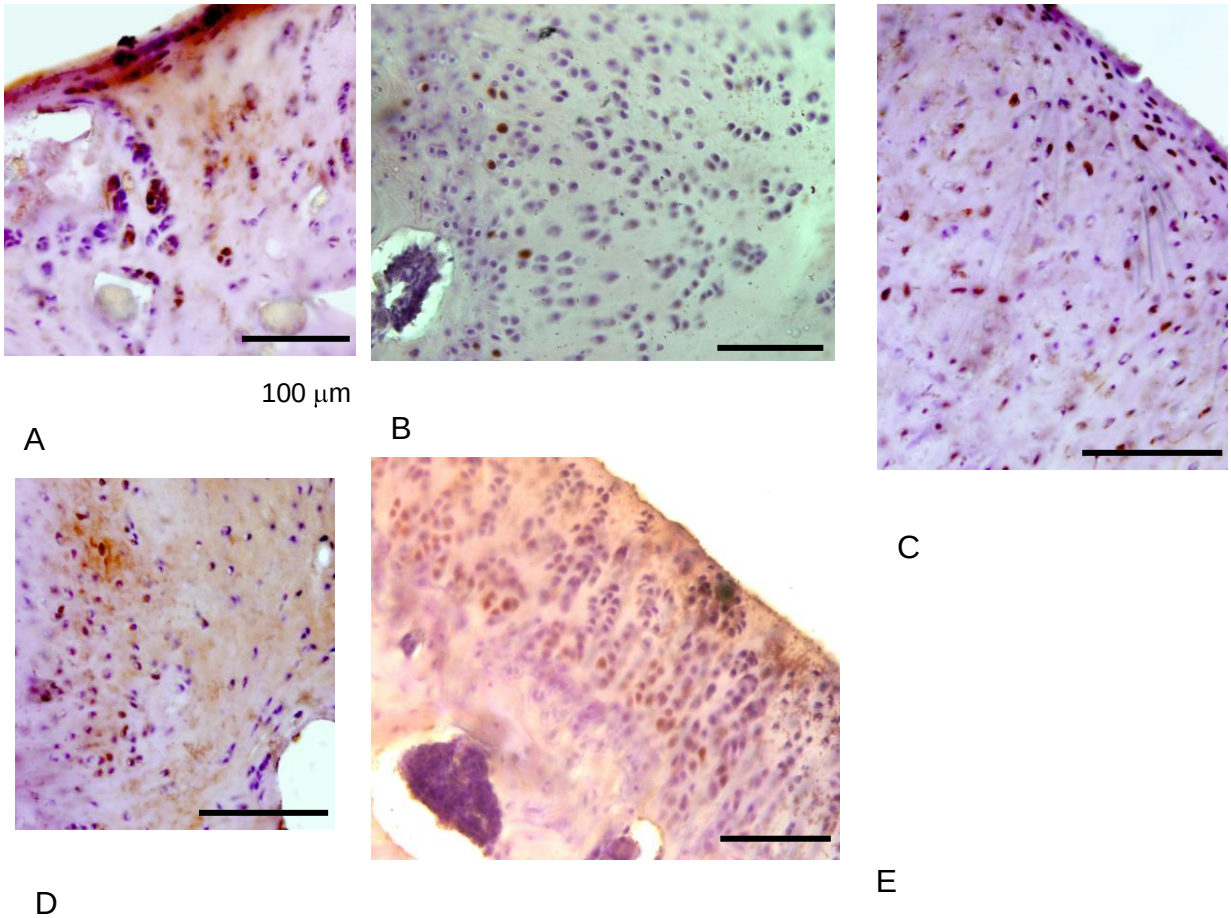

Figure 4. Immunohistochemical staining of Ki-67 on sections ofB, PCL/bone injury; C, pellet; D, PCL; and E, bone injury. The100 lm. S, scaffold. repaired rabbit articular joints. A, polycaprolactone (PCL)/cells; brown color indicates the nuclei of proliferating cells. Scale is 100 μm. S, scaffold.

Low levels of proliferation were detected in all specimens and reparation procedures tested (Figure 4). In defects repaired with PCL/cells and PCL/bone injury, Ki-67 staining showed some proliferation foci but the proliferation was generally insignificant (brown stained nuclei in Figure 4A and B). Samples repaired with pellet (positive control) and samples with no implant (bone injury without scaffold, negative control) seem to have higher rates of cells proliferating than other specimens tested (Figure 4C and E).

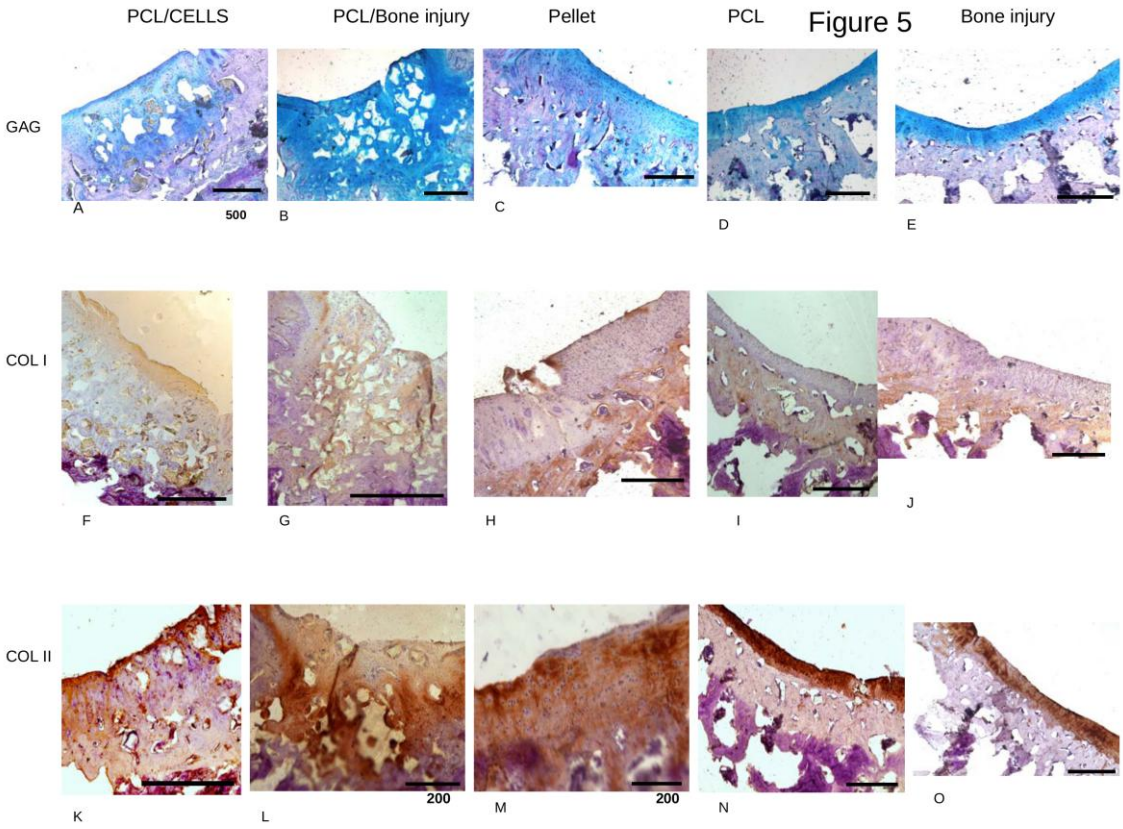

Figure 5. Histologic and immunohistochemical staining of repairedtissues. A, F, and K, polycaprolactone (PCL)/cells; B, G, and L, PCL/bone injury; C, H and M, pellet; ; D, I, and N, PCL; E, J, and O, bone injury. Scale is 500 μm. B, subchondral bone; S, scaffold; N, native cartilage; R, repaired cartilage.

High levels of GAG were detected in all cases tested (Figure 5A to E). Immunohistochemistry of PCL/cell-repaired cartilage shows an early stage of type II collagen synthesis, with a distribution similar to native cartilage (Figure 5K). On the other hand, no type I collagen synthesis was detected (Figure 5F). Weak type I collagen expression was detected in the PCL/bone injury repaired cartilage (Figure 5 G), along with stronger type II collagen expression (Figure 5 L). However, the type II collagen levels were lower than in native cartilage. Cartilage repaired with void scaffold and no bone injury had high levels of type II collagen and low type I collagen (Figure 5 N and I, respectively), but this new cartilage was very thin and fragile.

Similar results were obtained in subchondral bone injury repair, with high type II collagen and low type I collagen synthesis (Figure 5) but only in a superficial, thin layer. Pellet repair resulted in high type II collagen secretion in a wider thickness, and low col I secretion.

Indentation tests

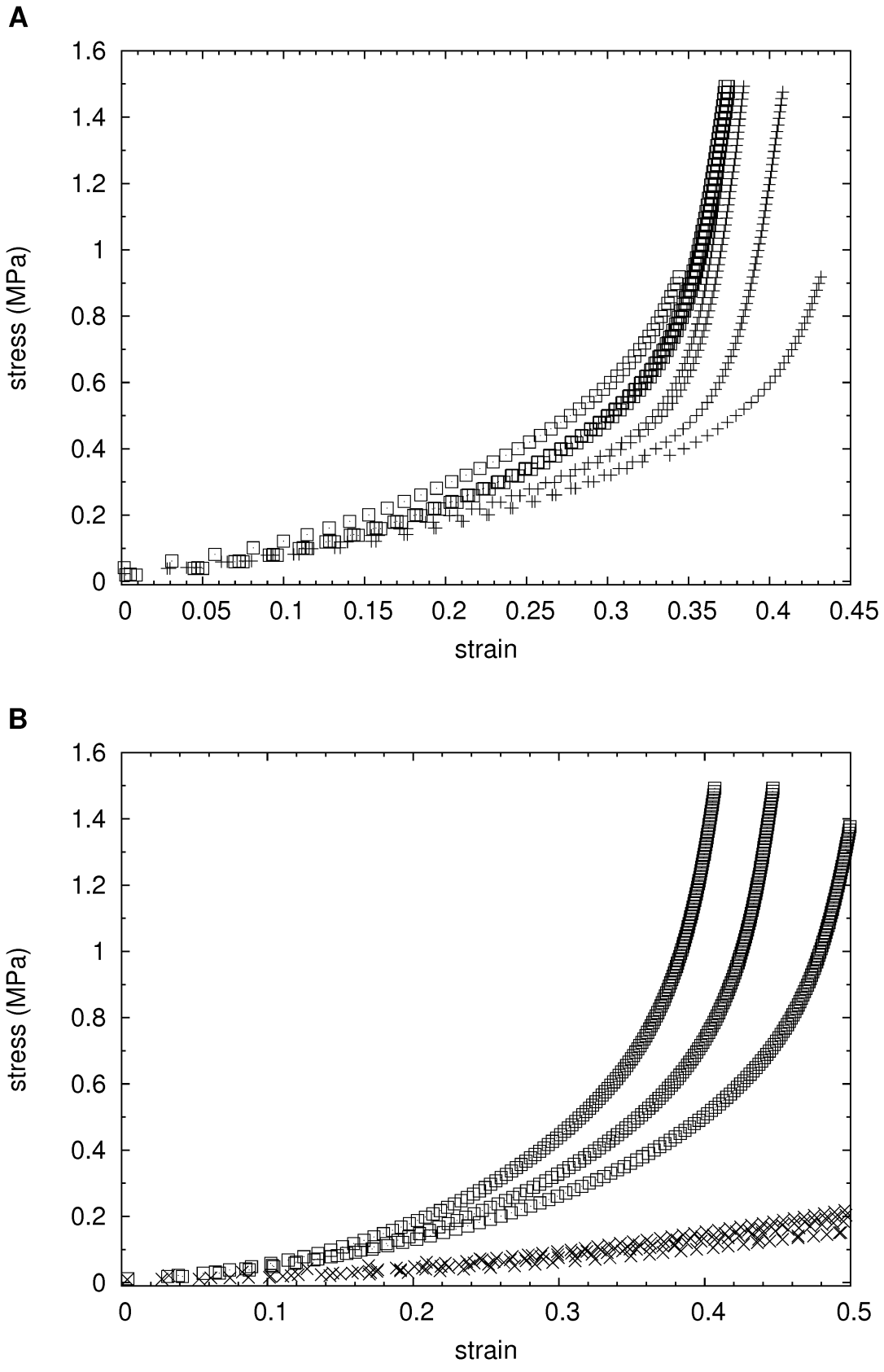

Figure 6. Stress-strain curves obtained in indentation tests. A, □ represents the contralateral knee and + represents the implanted polycaprolactone (PCL) knee replicates. B, □ represents the contralateral knee and × represents the pellet transplantation cartilage knee for the same specimen.

Table 2: Young Modulus (E) and Standard Deviation (SD) Calculated From the Slope of the Stress-Strength Curves (PCL, polycaprolactone).

	Implanted knee		**Contralateral knee**	
Specimen implant method	***E (MPa)***	***SD (MPa)***	***E (MPa)***	***SD (MPa)***
Autologous chondrocyte transplantation (pellet)	0,20	0,04	0,57	0,02
	0,09	0,03	0,29	0,05
	0,11	0,04	0,37	0,04
PCL/cells	0,39	0,28	0,57	0,55
	0,47	0,04	0,43	0,02
	0,28	0,04	0,26	0,03

Figures 6A and 6B show examples from two different specimens of the stress-strain curves obtained in these experiments.

Figure 6A shows that for strains less than 20%, the repaired PCL had a response very similar to that of control cartilage. Since the Young's modulus was calculated from the initial part of the stress-strain curve (up to 10% strain), the results are similar to Young's modulus for PCL and for control cartilage, as seen in Table 2. Small differences appeared only for deformations greater than 20%. Since physiological strains of

cartilage lie below this limit, these discrepancies are of minor relevance for our purpose.[12] Pellet-repaired cartilage did not perform well: under low stress, high strain is observed (Figure 6 B), or in other words, the observed Young's modulus is lower than for natural cartilage (Table 2).

The behaviour noted so far, and shown in Figure 6, is similar for all studied samples. Table 2 shows the calculated Young modulus for all samples. In all cases, the Young modulus for PCL-repaired cartilage is very similar to control cartilage, likely due to the contribution of PCL scaffold; for pellet-repaired cartilage, the Young's modulus is much smaller than that of control cartilage.

Discussion

This study evaluated regeneration of articular cartilage *in vivo* using a PCL scaffold. As in a great number of studies, the New Zealand rabbit was selected due to its docile nature and medium size, thus facilitating care and handling. Moreover, the repair processes produce results relatively quickly as rabbits have a short half-life. Finally, several authors have demonstrated a minimal immunological response when standard rabbit allogeneic chondrocytes are transplanted. [39, 48, 36, 37]

The morphology and biomechanical characteristics of rabbit knees have some differences with respect to human knees[3] and no animal models have been validated to perform biomechanical experiments.[11] There were some limitations in rabbit model to evaluate cartilage repairing. The rabbit has a much thinner hyaline cartilage layer than human does and it support different mechanical strengths than human joints. On the other hand, the biological aspects (ultra structure[9] , growth[38] and progressive deterioration[22]) of rabbit articular cartilage make it suitable for our study, as they are similar to those of human cartilage. However,

In this study, implantation into rabbit articular cartilage injuries was chosen rather than subcutaneous implantation because the latter does not characterize the physiopathology of human chondral damage in joint loading regions nor is representative of the avascular and thus hypoxic conditions in which cartilage formation is favoured.[10] Inducing rabbit chondral injuries in the femoral trochlea and studying their repair is an accepted method as it allows getting closer to the environment and biomechanical characteristics of human cartilage defects.

The first step was to evaluate the quality of the engineered cartilage specimen pre–implantation. Some studies have demonstrated that cells are predominately deposited in the superficial zone of scaffolds when static-seeding is used. [Holy CE, Shoichet MS, Davies JE. Engineering three-dimensional bone tissue in vitro using biodegradable scaffolds: investigating initial cell-seeding density and culture period. J Biomed Mater Res. 2000 Sep 5;51(3):376-82.; Holy, C. E.; Shoichet, M. S.; Davies, J. E. Bone marrow cell colonization of, and extracellular matrix expression on biodegradable polymers. *Cells Mater.* **1997**, *7*, 223-234.]. In this manner, PCL/cells constructs were tested before implantation to ensure that cells colonized the scaffold pores and synthesize cartilage ECM (figure 1). PCL and pellet immunohistological results obtained in this study were consistent with the results obtained in previous studies by our group.[16] In all cases, specimens showed the capacity to synthesize specific articular cartilage proteins (Figure 1).

At 3 months post-implantation, the rabbits were sacrificed and the implanted constructs macroscopically and microscopically explored. Macroscopically, partial repair to different degrees was observed in all cases. Moreover, it was difficult to differentiate the injury and the surrounded healthy cartilage by colour and consistency in some joints repaired with PCL with or without cells. Alternatively, pellet-repaired regions showed

whiter colouration than native cartilage but the stiffness was similar. In all cases, no macroscopic signs of infection, synovial irritation or biomaterial rejection were observed.

Some important properties including integration with host environment and the ability to regenerate cartilage and tolerate mechanical loads are necessary. In our study, the PCL/cells constructs showed histologically good integration to the subchondral bone and surrounding cartilage. A probable cause may be the fusion of preformed chondrocyte layer and the surrounding host cartilage. [47]

Cartilage regeneration was good in the cases of PCL/cells and PCL/bone injury, and was less efficient in the remaining cases (pellets and PCL without cells and bone injury without PCL), Figures 3, 4 and 5. Some degree of inflammation was found in the bone injury cases (data not shown). Our specimens repaired only with pellets can be considered as closest to autologous chondrocyte transplantation, the gold standard in osteochondral repair. For this reason, the regenerated cartilage from pellet-repaired injury case was selected for mechanical evaluation together with the PCL/cells-repaired cartilage, which was the most successful regeneration obtained in our study (Figure 6)**.**

Figures 3A and 3B show that tissue regeneration is quite different when the implanted scaffold is seeded with adult chondrocytes avoiding blood flow to the zone of the implant, (i.e., cutting off the supply of mesenchymal cells to the implant zone), and when it is implanted empty and regeneration is expected to come from the stem cells supplied by blood flow into the injured zone (spontaneous healing). In the former case, after three months the pores of the scaffold appear *in vivo* to be filled with cartilaginous tissue produced by the implanted cells; the scaffold maintains its original shape and position with the top face aligned with the articular surface. These results are in line with other studies that have demonstrated good repair results with three-dimensional

scaffolds seeded with allogeneic chondrocytes used to repair artificial chondral injuries in rabbit knees.[47, 45]

As mentioned above, the kinetics of PCL bioresorption is quite slow and the material is still there after three months. Thus, during the whole experiment the scaffold is biomechanically active all through the regeneration process. The scaffold's reinforcing effect surely contributes to the mechanical characteristics of the regenerated tissue, similar to that of healthy cartilage (Figure 6). In this way the cells within the scaffold pores are subjected to compression stresses similar to those suffered by the chondrocytes of the healthy tissue.[34, 28, 2] On the other hand, the indentation experiment performed on the regenerated tissue from pellet repair reveals a much smaller elastic modulus of the new tissue. This low mechanical capacity indicates that neotissue organization is poor; there may be a lack of proteoglycans in comparison with native tissue (proteoglycans are responsible for compressive strength, due to their water binding nature[15]), and in this case there is no scaffold to contribute to bear stresses. It means that the regenerated tissue is supporting abnormal stresses, that may cause collagen disruption, proteoglycan loss, and deviation in cell phenotypic expression (collagen function, metabolic activity).[18] This could further lead to a degeneration of hyaline-like tissue into fibrocartilage-like tissue; as a matter of fact, different studies have addressed the role of applied compression forces in the differentiation process of chondrocytes previously expanded in monolayer culture to acquire the phenotype of the cells of hyaline cartilage.[7] Figure 3B shows the structure of regenerated tissue when an empty scaffold initially occupies all the volume of the defect and bone injury allows blood to flow to the zone of the injury. Tissue regeneration in this case must come from mesenchymal stem cells supplied by the blood. As can be observed in Figure 3B, regenerated tissue with the morphological characteristics of hyaline cartilage is formed

on top of the scaffold upper surface. The histological aspect of this tissue closely corresponds to hyaline cartilage and clearly shows columns of chondrocytes perpendicular to the articular surface. The growth of this tissue layer has pushed the scaffold down towards the subchondral bone zone. This is probably because of faster tissue growth outside the scaffold than within the scaffold. The pictures show that tissue in-growth into the scaffold pores is deficient and thus poor interfacial adhesion between tissue and biomaterial is expected. This behaviour might be explained by the hydrophobic character of PCL affecting the intrusion of blood flow into the pores during implantation. The role of the scaffold in this case seems to be simply providing the newly formed tissue with a substrate that regulates the compression levels and can be deformed to physiological levels without subjecting repaired tissue to excessive stress during tissue growth. Improved wettability of the scaffold material is needed when the cell supply comes entirely from the subchondral bone injury.

Although the PCL is biodegradable, its degradation rate was very slow and the scaffold retained its structural integrity and provided sufficient mechanical support during this study's relatively long tissue repair period of at least 3 months.

Experiments with longer regeneration periods after implantation should be performed to evaluate the degradation rate and determine the time required to eliminate the entire scaffold or modifications of the scaffolds may be met in order to adequate the degradation rate to tissue repair rate. Further modifications of the material surface in order to facilitate the blood inflow may lead to efficient scaffold colonization when implanted and eventually facilitate spontaneous repair on the only basis of an adequate mechanical environment provided by the scaffold.

The results obtained are encouraging as they prove that the scaffold seeded with autologous chondrocytes can host viable cartilaginous tissue. Experiments with longer

regeneration periods after implantation are needed to determine the complete degradation time *in vivo* of the scaffolds and the characteristics of the repaired tissue after complete resorption of the entire scaffold.

Conclusions

PCL scaffolds pre-seeded with allogeneic chondrocytes achieved full thickness cartilage repair in the rabbit articular cartilage defect model over a period of 3 months. The engineered cartilage showed a smooth integrated surface and good cellular and extracellular matrix distribution, with a hyaline-like histology. The PCL scaffolds maintained their mechanical integrity after implantation and guided cartilaginous tissue growth *in vivo*. The mechanical properties of the regenerated tissue matched those of normal articular cartilage, and were better than the mechanical properties of regenerated cartilage with a cell pellet in the absence of scaffold, likely due to the contribution of the scaffolding material. The performance of the scaffolds implanted without cells was also good, with hyaline-like characteristics, if cells supplied by blood from the bone injury were allowed to reach the empty scaffold during implantation. The relative good results are thought to be related with the mechanical transduction operated by the scaffold that favours an adequate biomechanical environment of the repaired tissue. Worse results, comparable with negative control, were obtained if the scaffolds were neither pre-seeded nor allowed to be in contact with blood during implantation. These results demonstrate that a porous PCL scaffold is a suitable material for cartilage tissue engineering. Moreover, the results strongly support the hypothesis that efficient mechanical transduction of stimuli is crucial during tissue regeneration.

Legends

Figure 1: Type II collagen (A and D) and Type I collagen (C and F) immunohistochemical staining and Alcian blue staining (B and E) of sections of rabbit chondrocytes cultured in PCL scaffolds (A, B, and C) or in pellets (D, E and F). Specimens were cultured for 7 days in ITS and ascorbate. Scale is 100 µm.

Figure 2: Macroscopic analysis of the repaired chondral defect 3 months after construct implantation. A) PCL/cells; B) PCL/bone injury; C) pellet+inverted periostium; D) PCL; E) subchondral bone injury, without PCL scaffold.

Figure 3: Haematoxylin staining for general observation of histological sections of repaired rabbit articular joints. A) PCL/Cells; B) PCL/ Bone injury; C) Pellet; D) PCL; E) Bone injury. The arrows point to the translucence PCL framework. Scale is 500 µm.

Figure 4: Immunohistochemical staining of Ki-67 on sections of repaired rabbit articular joints. A) PCL/Cells; B) PCL/ Bone injury; C) Pellet; D) PCL; E) Bone injury. The brown colour indicates the nuclei of proliferating cells. Bar scale is 100 µm.

Figure 5: Histological and immunohistochemical staining of repaired tissues. R: repaired cartilage; N: native cartilage; B: subchondral bone. Scale is 500 µm. A) PCL/Cells; B) PCL/ Bone injury; C) Pellet; D) PCL; E) Bone injury.

Figure 6: Stress-strain curves obtained in indentation tests. A) • represents the contralateral knee and + represents the implanted PCL knee replicates. B) • represents

the contralateral knee and X represents the pellet transplantation cartilage knee for the same specimen.

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