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

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Background: Cartilage tissue engineering using synthetic scaffolds allows 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 polycaprolactone (PCL) scaffolds for the regeneration of articular cartilage in a rabbit model.

Study Design: Controlled laboratory study.

Methods: Five conditions were tested to attempt cartilage repair. 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 the latter condition, 1 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 months with 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 glycosaminoglycan. 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.

Conclusion: 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

Articular cartilage has very limited capacity for repair because of 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,[26] abrasion arthroplasty,[28] and microfracture.[49] Although microfracture works quite well for small lesions on the femoral condyles,[34] in other cases, the tissue formed by spontaneous healing is usually more fibrocartilage-like than hyaline-like and degenerates over a long term.[25,46] Other therapeutic strategies have been

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developed by orthopaedic surgeons—osteochondral transplantation (mosaicplasty),[19] and transplantation of periosteum or perichondrium to resurface the damaged cartilage.[43] Mosaicplasty may be limited by the size of the injured area and involves donor-site morbidity. Moreover, the difference in mechanical stress between the donor and receptor region has been related to degeneration of implants.[25] In 1994, Brittberg et al[6] reported the first clinical results of transplantation of human autologous chondrocytes covered with a periosteal flap (autologous chondrocyte implantation [ACI]). Although this technique has been applied successfully for more than a decade, it has 2 major disadvantages: a wide arthrotomy incision and the fact that the patient is submitted to 2 surgical procedures. Moreover, 2 studies demonstrated that clinical and histologic results obtained at 2 and 5 years after ACI repair were identical to microfracture results, although a trend toward better histologic results can be seen in the ACI group.[32,33]

In recent years, tissue-engineered cartilage using scaffolds and cells has been considered for improved treatment of cartilage defects.[4,44] 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[10]; in particular, the maintenance of chondrocyte phenotype and secretion of hyaline extracellular matrix (ECM) has been shown to be directly related to mechanical stimulation.[18] Moreover, chondrocyte phenotype may be lost during expansion in monolayer cultures,[47] but it has also been observed that dedifferentiated chondrocytes redifferentiate when returning to a 3-dimensional environment.[5] Solid scaffolds provide a 3-dimensional 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, polylactide, polycaprolactone (PCL), and their copolymers. 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.[1,8,27,39] Recent studies have demonstrated the biocompatibility, differentiation, and specific gene expression of rabbit chondrocytes seeded into scaffolds implanted subcutaneously or in cartilage defects.[14,29-31,48,50]

Here, we use PCL scaffolds and culture chondrocytes therein before implantation. Polycaprolactone is a semicrystalline material with good mechanical properties that degrades much more slowly than other polyesters.[35] Because of the degradation properties of PCL, it can be used for long-term in vitro cell culture before implantation into the injury site, as these scaffolds will maintain their architectural integrity and mechanical properties during the preimplantation 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.[36] 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 joint 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 to observe the healing response in the 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.

MATERIALS AND METHODS

Scaffold Fabrication

The PCL scaffolds were produced as described in our previous work.[36] In brief, porogen templates of the scaffolds were prepared by sintering acrylic resin microspheres (Elvacite 2043, Lucite International, Southampton, United Kingdom) at 140°C. The porosity of the templates was adjusted by submitting the templates to an appropriate degree of compression.

The PCL scaffolds were obtained by the injection of melted PCL (Polysciences Inc, Eppelheim, Germany; molecular weight = 50 000) at 110°C 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 200 μm. The elastic modulus of the dry, empty scaffold can be extrapolated from our earlier work[36] to be ~5 MPa.

Animals

Adult male New Zealand White rabbits weighing 2.5 to 3.0 kg were obtained from Granjas San Bernardo S.L. (Tulebras, 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 no. 273/06) according to 86/609/EEC (European Economic Community) 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 thiopental via intravenous injection. As a first step, 1 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 (with 100 U penicillin, 100 μg streptomycin and 0.4 % amphotericin B) Dulbecco's modified Eagle's medium (DMEM) (Invitrogen SA, Prat de Llobregat, Spain). Cartilage digestive enzymes were prepared with this medium. For chondrocyte isolation, the diced cartilage was incubated for 30 minutes with 0.5 mg/mL hyaluronidase (Sigma-Aldrich Química SA, Tres Cantos, Spain) in a shaking water bath at 37°C. The hyaluronidase was subsequently removed and 1 mg/mL pronase (Merck kGaA, Darmstadt, Germany) was added. After 60-minute incubation in a shaking water bath at 37°C, the cartilage pieces were washed with supplemented DMEM. After removal of the medium, digestion was continued by the addition of 0.5 mg/mL of collagenase-IA (Sigma-Aldrich) in a shaking water bath kept at 37°C overnight. The resulting cell suspension was filtered through a 70-μm–pore nylon filter (BD Biosciences Europe, Erembodegem, Belgium) to remove tissue debris. Cells were centrifuged and washed with DMEM supplemented with 10% fetal 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 (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 37°C in a 5% CO_2 humidified atmosphere. The medium was changed every 3 days. After 7 to 14 days, adherent cells were harvested by incubation with trypsin–ethylenediaminetetraacetic acid (Biological Industries, Kibbutz Beit Haemek, Israel) and resuspended with a minimum volume of culture medium. Polycaprolactone samples were placed on a 96-well polystyrene culture plate (Nunc A/S, Roskilde, Denmark) and moistened with Hanks' balanced salt solution (Sigma-Aldrich). After removal of the Hanks' solution, the cell suspension (0.5 to 1×10^6) 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% insulin-transferrin-selenium (ITS) (BD Biosciences) and 50 μg/mL ascorbic acid during 7 days. For each implant, 2 PCL samples were assayed: 1 was analyzed before implantation, and the other was implanted and then analyzed after implantation in 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 minutes at 1200 rpm. The resulting pellet was cultured with DMEM supplemented with 1% ITS and μg/mL ascorbic acid at 37°C in a 5% CO_2 humidified atmosphere for 7 days.

Two pellet samples were assayed at the same time to analyze the specimen before and after implantation to the rabbit articulation injury.

Engineered Cartilage Implantation

The rabbits were preanesthetized by subcutaneous injection of 25 mg/kg ketamine and 3 mg/kg xylacine. Before surgery, rabbits were prepared (washed, shaved, etc) and general anesthesia 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; nonsterile 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 to 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 periosteum transplantation, the periosteum was obtained from the medial metaphyseal tibial surface of the intervention knee. The specimens (PCL scaffolds or pellets-periosteum) were fixed into the holes with chondral stitches of 6/0 coated Vicryl (Ethicon, Johnson & Johnson, Somerville, New Jersey). 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. After removal of the conformed anesthesia 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 on the surgery day and the same 24-hour dosage for 3 days. At the end of surgery, 3 mg/kg intramuscular gentamicine was administered as antibiotic prophylaxis.

Animal Sacrifice and Tissue Retrieval

Rabbits were sacrificed after 3 months to assess cartilage repair. A 10-mm × 10-mm × 5-mm cut was made in the articulations with implanted engineered cartilage, being careful to keep the repaired defect at the center of the sample for purposes of compressive modulus analysis and histologic and immunohistologic 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 histologic and indentation tests.

TABLE 1
Number of Rabbits Used for Each Specimen Implantation[a]

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

[a]PCL, polycaprolactone; ACI, autologous chondrocyte implantation.

Moreover, more replicas of PCL/cells were performed to avoid possible variability when cells were seeded into the scaffold.

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 after implantation was monitored by Alcian blue staining, counterstained using Mayer's hematoxylin, and then analyzed by optical microscopy. The results were blind-evaluated with regard to the specimen implantation by 3 different individuals. Briefly, the scaffolds were embedded in optimum cutting temperature (OCT) compound (Tissue-Tek, Sakura Finetek Europe B.V., Zoeterwoude, the Netherlands) and cryosectioned (8-μm thick). Cryosections were air-dried and then fixed in acetone for 10 minutes at 4°C before staining or were stored at −20°C until use.

Rabbit articulation specimens were rinsed with phosphate-buffered saline (PBS) and fixed with 4% paraformaldehyde overnight at 4°C. 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 4°C. Finally, the specimens were embedded in OCT compound and cryosectioned (14-μm thick). Cryosections were air-dried before staining or were stored at −20°C until use.

Immunohistology

Immunohistologic analysis was used to detect the synthesis of type I and type II collagen and the expression of Ki-67 (a proliferation marker). Preimplanted 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-Aldrich), or 1 μg/mL of type II collagen antibody (Calbiochem, Merck), or 1:100 dilution of Ki-67 antibody (Dako Diagnósticos SA, Sant Just Desvern, Spain). Antigen-antibody complexes were detected colorimetrically using HRP-conjugated secondary antibody and diaminobenzidine (DAB) + chromogen (Dako Diagnósticos) and counterstained with Mayer's hematoxylin.

Indentation Tests

Mechanical properties were measured with Seiko TMA/6000 equipment (Seiko Instruments Inc, Chiba, Japan) in the compression mode, with a 1-mm 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 center of the sample, and symmetrically from the contralateral knee for use as a control. The probe was placed at the center 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 to 5 times per sample. The experiment was carried out at 38°C in a saline solution. The stress-strain curves of the loading run were constructed from these experimental data, and a linear fit of 10% of the initial curve was used to determine the Young modulus of all the cartilage samples. The slope of this line is related to the Young modulus (E) by the equation:

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

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).[20] Values of κ appear in Table 1 in the article by Hayes et al.[20]

The sample thickness was measured on a cross-sample section observed under the optical microscope; resolution was ~0.05 mm and the measurement was very repeatable. Thus, the sample strain was calculated as the displacement divided by the cartilage thickness.

Because we could not measure the Poisson ratio, we assumed $\nu = 0.4$ to compute E from our indentation tests, as done by other authors.[17,21,45] 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 that bone deformation was negligible in this procedure.

RESULTS

Histological and Immunohistological Analysis

Regeneration of articular cartilage after specimen implantation in the knee defect was evaluated by testing type I

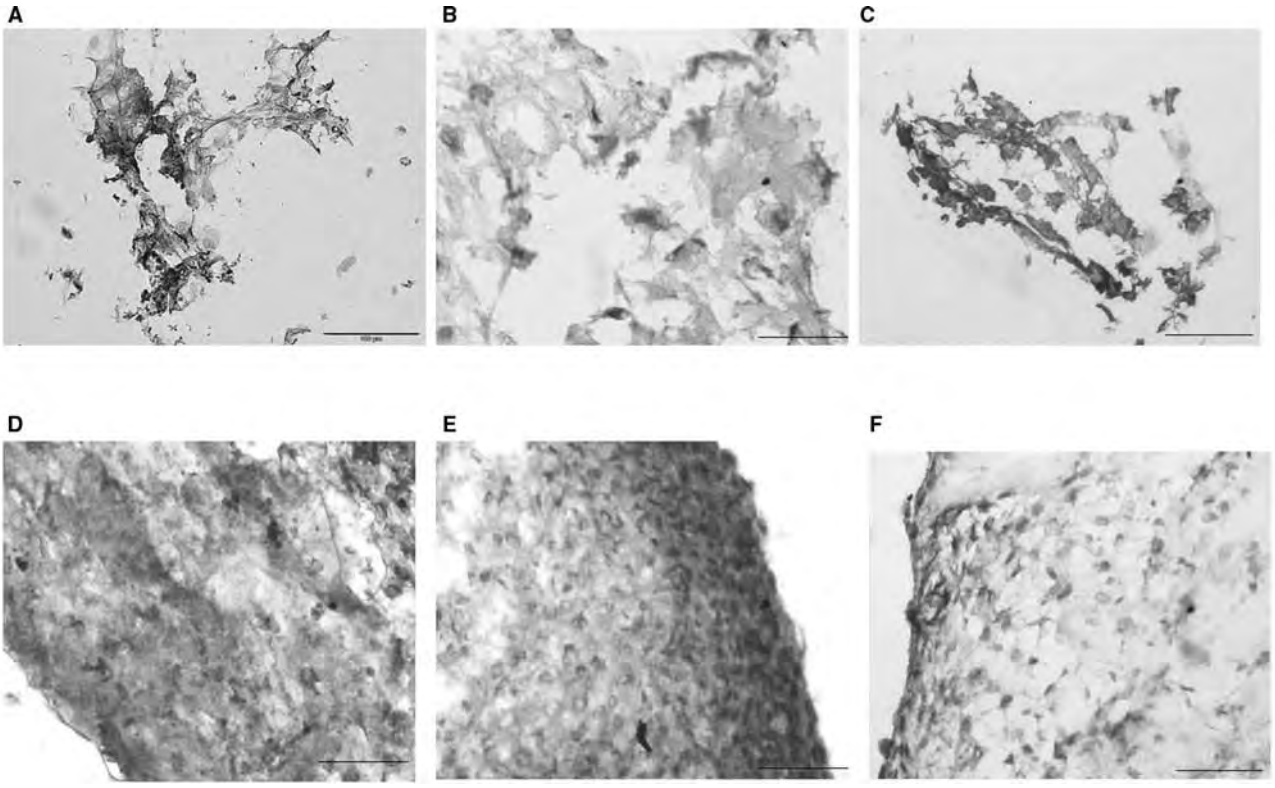

Figure 1. Type II collagen (A and D) and type I collagen (C and F) immunohistochemical staining and Alcian blue staining (B and E) of sections of rabbit chondrocytes cultured in polycaprolactone (PCL) scaffolds (A, B, and C) or in pellets (D, E, and F). Specimens were cultured for 7 days in insulin-transferrin-selenium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

and type II collagen and GAG synthesis. On the same basis, a previous histologic analysis of the cells/scaffold 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 ECM (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 ECM 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 S100, 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.

The macroscopic exploration showed good integration of the implanted specimen in the articular cartilage; the implant was firmly attached to the underlying bone and showed continuity with the surrounding cartilage. However, the implantation site looked different from the native surrounding region in all cases except when PCL was implanted alone (Figure 2D). 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 periosteum cover were implanted, the new cartilage differed in color from adjacent native cartilage. In some cases in which a PCL scaffold (with or without cells) was implanted, the aspect, including brightness, stiffness and color, 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 with the surrounding cartilage, although the defects were designed to provoke spontaneous healing by subchondral plate bleeding (Figure 2E).

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 (Figures 3A and 3B) showed better integration than bare PCL (Figure 3D). The letters in the micrographs designate, respectively, the bone (B), scaffold (S, appears as grey inclusions), native cartilage (N) and repaired cartilage (R). The 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

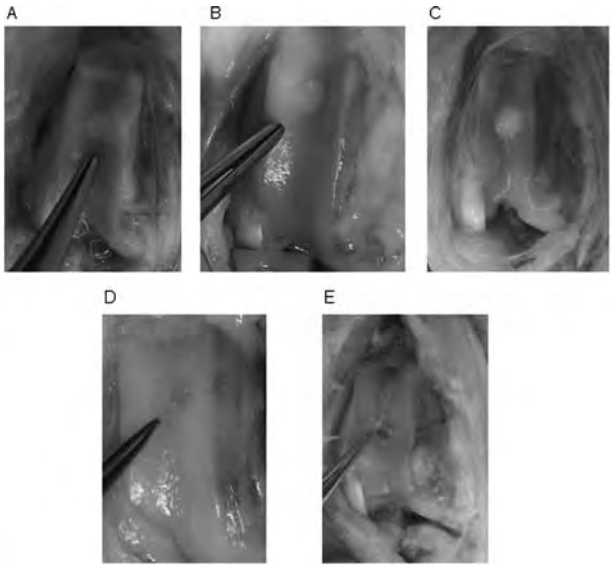

Figure 2. Macroscopic analysis of the repaired chondral defect 3 months after construct implantation. A, polycaprolactone (PCL)/cells; B, PCL/bone injury; C, pellet + inverted periosteum; D, PCL; and E, subchondral bone injury without PCL scaffold.

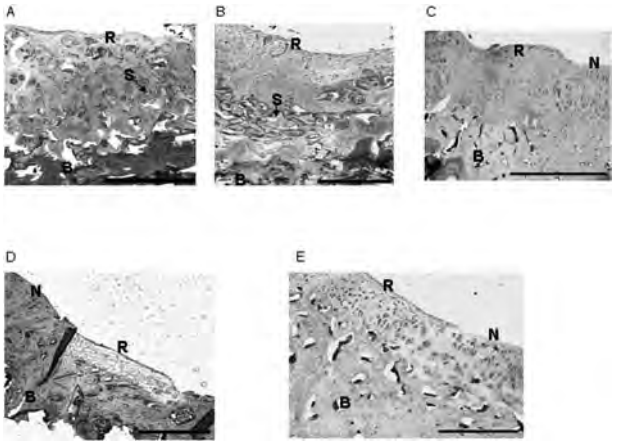

Figure 3. Hematoxylin staining for general observation of histologic sections of repaired rabbit articular joints. A, polycaprolactone (PCL)/cells; B, PCL/bone injury; C, pellet; D, PCL; and E, bone injury. Scale is 500 μm. B, subchondral bone; S, scaffold; N, native cartilage; R, repaired cartilage.

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 because of the blood flow that boosted the immune system response.

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 Figures 4A and 4B). 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 (Figures 4C and 4E).

High levels of GAG were detected in all cases tested (Figures 5A through 5E). 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 5G), along with stronger type II collagen expression (Figure 5L). 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 (Figures 5N and 5I, 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 collagen I secretion.

Indentation Tests

Figure 6 shows examples from 2 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. Because the Young 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%. As physiologic strains of cartilage lie below this limit, these discrepancies are of minor relevance for our purpose.[13] Pellet-repaired cartilage did not perform well; under low stress, high strain is observed (Figure 6B)—in other words, the observed Young's modulus is lower than for natural cartilage (Table 2).

The behavior 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 because of the contribution of PCL scaffold; for pellet-repaired cartilage, the Young 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,

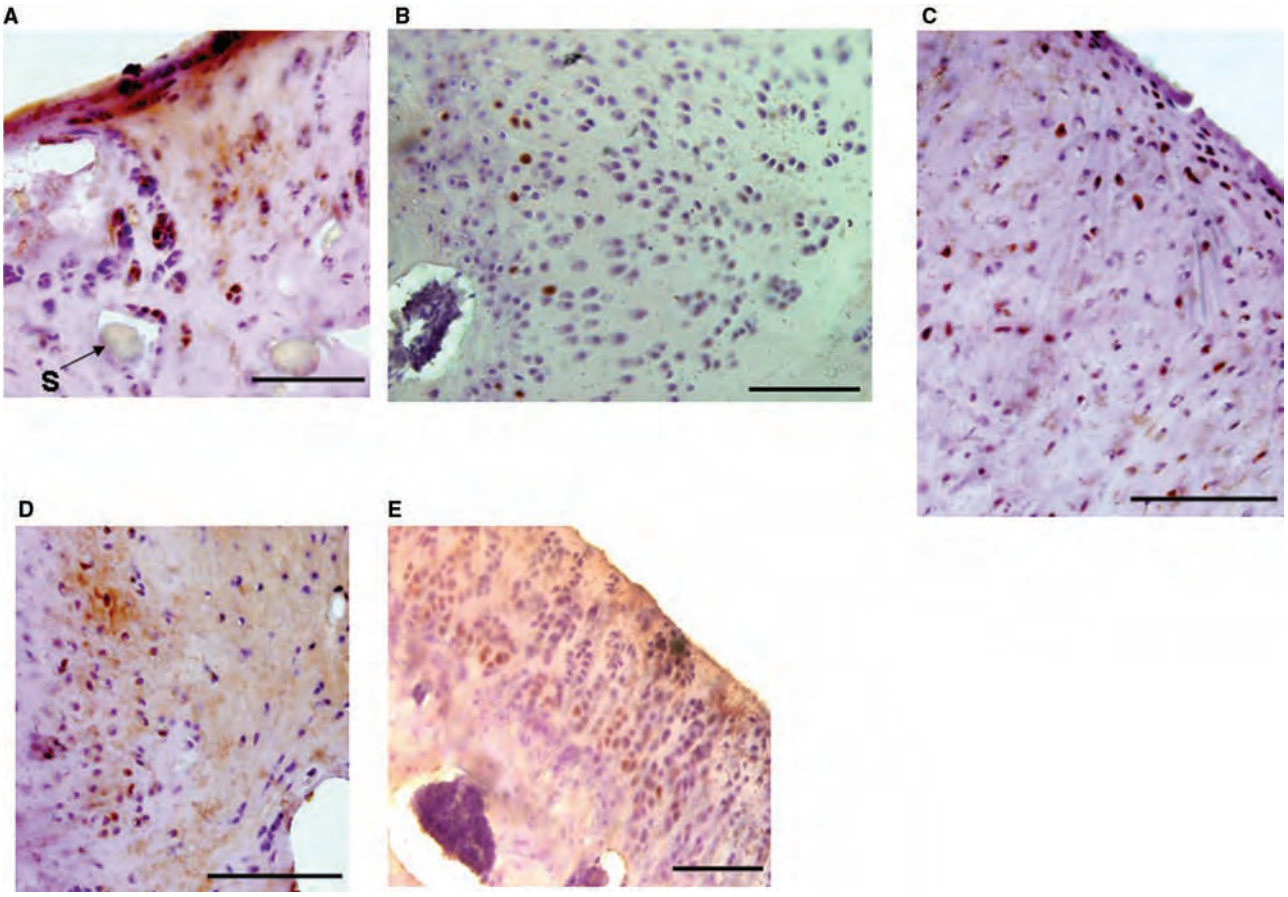

Figure 4. Immunohistochemical staining of Ki-67 on sections of repaired rabbit articular joints. A, polycaprolactone (PCL)/cells; B, PCL/bone injury; C, pellet; D, PCL; and E, bone injury. The brown color indicates the nuclei of proliferating cells. Scale is 100 μm. S, scaffold.

the New Zealand White rabbit was selected because of 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 immunologic response when standard rabbit allogeneic chondrocytes are transplanted.[37,40,42,51]

The morphologic 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.[12] The study of cartilage repair in a rabbit model has some limitations, as the rabbit has a much thinner hyaline cartilage layer than humans and the mechanical loads in rabbit joints are quite different from human joints. Nevertheless, the biological aspects (ultrastructure,[9] growth,[41] and progressive deterioration[24]) of rabbit articular cartilage are similar to those of human cartilage and the model was thus considered as suitable for our study. Finally, it should be borne in mind that chondrocyte density in rabbit cartilage is 10% higher than in human beings, which could speed up the repair process and/or improve the quality of the repaired tissue in some way.

In this work, 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 it representative of the avascular and thus hypoxic conditions in which cartilage formation is favored.[11] 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 before implantation. Some studies have demonstrated that cells are predominantly deposited in the superficial zone of scaffolds when static seeding is used.[22,23] In this manner, PCL/cells constructs were tested before implantation to ensure that cells colonized the scaffold pores and synthesized cartilage ECM (Figure 1). The PCL and pellet immunohistologic 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 after implantation, the rabbits were sacrificed and the implanted constructs macroscopically and microscopically explored. Macroscopically, partial repair to different degrees was observed in all cases.

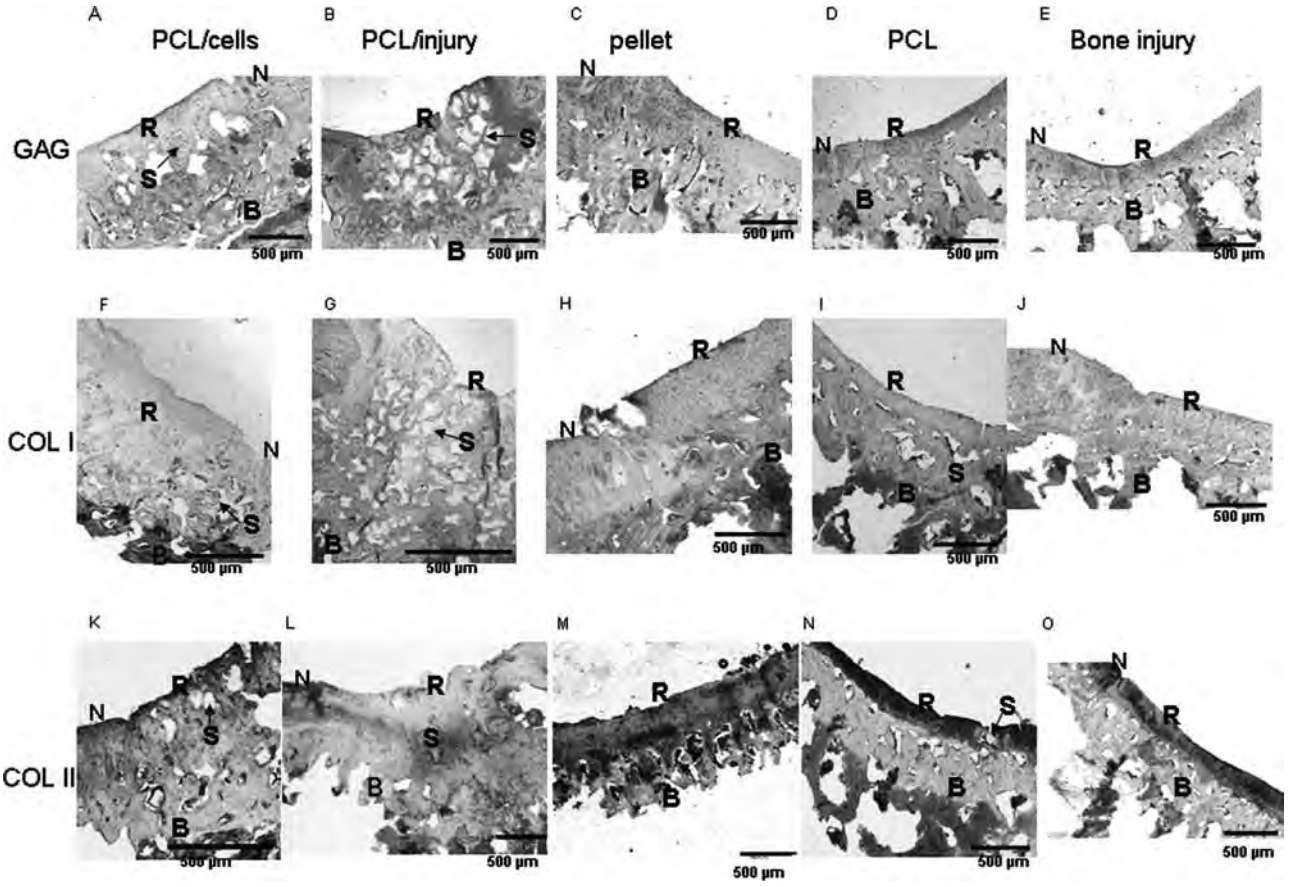

Figure 5. Histologic and immunohistochemical staining of repaired tissues. A, polycaprolactone (PCL)/cells; B, PCL/bone injury; C, pellet; D, PCL; E, bone injury. Scale is 500 μm. B, subchondral bone; S, scaffold; N, native cartilage; R, repaired cartilage.

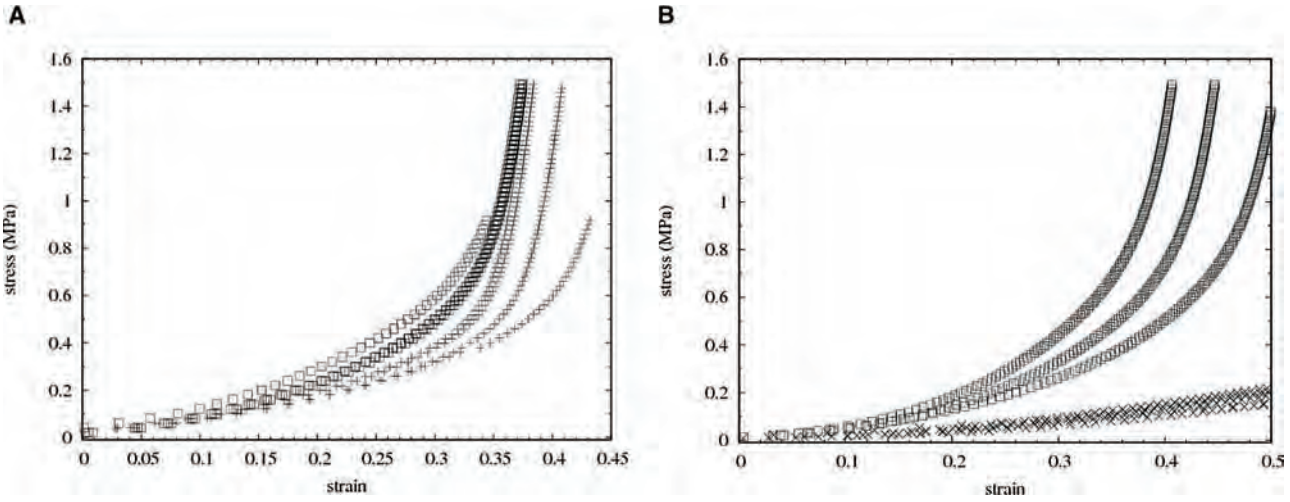

Figure 6. Stress-strain curves obtained in indentation tests. A, □ represents the contralateral knee and + represents the implanted polycaprolactone (PCL) knee replicates. B, □ represents the contralateral knee and × represents the pellet transplantation cartilage knee for the same specimen.

Moreover, it was difficult to differentiate the injury and the surrounded healthy cartilage by color and consistency in some joints repaired with PCL with or without cells. Alternatively, pellet-repaired regions showed whiter coloration than native cartilage but the stiffness was similar. In all cases, no macroscopic signs of infection,

TABLE 2
Young Modulus (E) and Standard Deviation (SD) Calculated From the Slope of the Stress-Strength Curves[a]

Specimen implant method	Implanted Knee		Contralateral Knee	
	E, MPa	SD, MPa	E, MPa	SD, MPa
Autologous chondrocyte implantation (pellet)	0.20	0.04	0.57	0.02
PCL/cells	0.09	0.03	0.29	0.05
	0.11	0.04	0.37	0.04
	0.39	0.28	0.57	0.55
	0.47	0.04	0.43	0.02
	0.28	0.04	0.26	0.03

[a]PCL, polycaprolactone.

synovial irritation, or biomaterial rejection were observed.

Some important properties, including integration with the 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 the preformed chondrocyte layer and the surrounding host cartilage.[50]

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 through 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 ACI, the gold standard in osteochondral repair. For this reason, the regenerated cartilage from the 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 (ie, 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 3 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 3-dimensional scaffolds seeded with allogeneic chondrocytes used to repair artificial chondral injuries in rabbit knees.[48,50]

As mentioned above, the kinetics of PCL bioresorption are quite slow and the material is still there after 3 months. Thus, during the whole experiment, the scaffold is biomechanically active throughout 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.[2,30,38] 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, which 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 morphologic characteristics of hyaline cartilage is formed on top of the scaffold upper surface. The histologic 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 toward the subchondral bone zone. This is probably because of faster tissue growth outside the scaffold than within the scaffold. The figures show that tissue ingrowth into the scaffold pores is deficient and thus poor interfacial adhesion between tissue and biomaterial is expected. This behavior 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 physiologic levels without subjecting repaired tissue to excessive stress during tissue growth. Improved wetability 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. Polycaprolactone degrades by hydrolysis in aqueous environments, and may be affected by different kinds of esterases. Breakdown product of PCL is known to be biocompatible, although its acidity may lead to some inflammation process if breakdown products accumulate, as shown for other polyesters like poly-L-lactic acid. Nevertheless, PCL resorption is slow, and breakdown products are likely to be eliminated through diffusion out of the joint progressively.

[correct word?]

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 made 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.

CONCLUSION

The PCL scaffold preseeded 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 ECM 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 because of 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 to the mechanical transduction operated by the scaffold that favors an adequate biomechanical environment of the repaired tissue. Worse results, comparable with the negative control, were obtained if the scaffolds were neither preseeded 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.

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