Repeated Platelet Concentrate Injections Enhance Reparative Response of Microfractures in the Treatment of Chondral Defects of the Knee: An Experimental Study in an Animal Model

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Purpose: To assess the histology and biomechanics of repair cartilage after microfractures with and without repeated local injections of platelet concentrate for the treatment of full-thickness focal chondral defects of the knee. **Methods:** A full-thickness chondral lesion on the medial femoral condyle was created in 30 sheep and treated with microfractures. Animals were divided into 2 groups, according to postoperative treatment: in group 1 we performed 5 weekly injections of autologous conditioned plasma, whereas group 2 did not undergo further treatments. Animals were killed at 3, 6, and 12 months after treatment. Macroscopic, histologic, and biomechanical evaluations were performed. Differences between groups at each time interval and differences over time within groups were analyzed for each outcome. Significance was set at $P < .05$. **Results:** Group 1 showed significantly better macroscopic, histologic, and biomechanical results than group 2 at each time interval. Analysis of time effect within groups showed that in group 1, quality of repair tissue significantly improved from 3 to 6 months after treatment and remained stable over time for all the outcomes; in group 2 a significant histologic and mechanical deterioration was observed between 6 and 12 months' follow-up. **Conclusions:** Five repeated local injections of autologous conditioned plasma after microfractures in the treatment of full-thickness cartilage injuries promoted a better and more durable reparative response than isolated microfractures, although they did not produce hyaline cartilage. **Clinical Relevance:** Periodical intra-articular injections of platelet concentrate after microfractures may improve cartilage repair and prevent further degenerative changes.

Arthroscopic microfractures represent the most widely used surgical technique for the treatment of full-thickness chondral defects of the knee.[1] Cartilage repair after microfractures is promoted by mesenchymal stem cells (MSCs), which migrate from bone marrow to the site of injury and differentiate into a chondrogenic cell line.[2] Despite a successful clinical outcome of the procedure,[3,4] several studies documented that microfractures cannot induce regeneration of hyaline cartilage and that repair tissue

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consists of fibrocartilage, containing a high quantity of type I collagen.[5,6] Furthermore, progressive impairment of clinical outcome, as well as increasing failure and revision rates 2 years after treatment, suggest that fibrocartilage deteriorates over time.[3] For these reasons, many researchers attempted to enhance cartilage repair by combining microfractures with scaffolds, chondrocyte implantation, and growth factors (GFs).[7-12]

Platelets play a crucial role in the normal healing response of connective tissues by local secretion of GFs and recruitment of reparative cells.[13] The use of platelet concentrate (PC) as the source of a high quantity of GFs was first popularized in maxillofacial and plastic surgery.[13,14] Its use in orthopaedics began later to augment bone healing.[15] However, studies on characterization of PCs showed a great variability in platelet concentration and GF content with different methods for PC preparation.[16-18] Therefore results on the efficacy of these blood-derived products are barely comparable.

Recently, the use of PC has been suggested to promote chondrocyte proliferation,[19] chondrogenic differentiation and proliferation of bone marrow stem cells,[20] and cartilage formation.[21] The combination of PC gel and microfractures has been shown to be effective in improving cartilage repair with respect to microfractures alone.[22] However, the use of PC gel implies a complex and time-consuming procedure, consisting of double centrifugation, clot formation by adding platelet activator, sizing, and placement of gel implant over the defect by use of a sealant, such as fibrin glue.[22] Moreover, it can require an open approach, thus increasing morbidity of the treatment. Therefore injectable treatment would be more advisable because it is easier to use and less invasive.

Autologous conditioned plasma (ACP) is an injectable blood-derived product containing a high concentration of platelets that can have a potential beneficial effect on chondral injuries by in situ releasing of platelet-derived GFs.[23]

Infiltrative therapy with PC has been successfully reported for the treatment of knee osteoarthritis (OA).[24-26] However, no previous studies investigated the effect of repeated intra-articular injections of PC on cartilage repair after microfractures.

The purpose of this study was to assess the histology and biomechanics of repair cartilage after microfractures with and without repeated local injections of ACP. Our hypothesis was that ACP can enhance reparative response after microfractures in comparison with isolated microfracture procedure.

METHODS

For this study, we used 30 adult sheep (Sarda ewes at dry off) that came from the same breeding and were homogeneous for age, size, and feeding. Age ranged from 33 to 46 months (mean, 41 months), and weight ranged from 35 to 44 kg (mean, 40 kg).

All the animals underwent a veterinary examination to evaluate general health status. Skeletal maturity was confirmed in all animals before the study by radiographic examinations to ensure the closure of the growth plates of the distal femur and proximal tibia. Degenerative changes of the stifle were assessed on radiographic examinations and intraoperatively. Animals showing systemic diseases, skeletal immaturity, and degenerative changes of the stifles were excluded from the study.

The research protocol was approved by the local ethics committee for animal experimentation.

Surgical Technique

Surgery was performed by use of sterile conditions and with sheep under general anesthesia by the same surgeon (G. Milano). The sheep were intubated after the administration of thiopentone (25 mg/kg) and ventilated with O_2 in N_2O by volume control. Anesthesia was maintained with 1.5 to 2% isoflurane; a bolus dose of 0.1 mg of fentanyl was given before surgery.

On each animal, a medial parapatellar arthrotomy was carried out on the right stifle. A full-thickness chondral lesion was created on the weight-bearing area of the medial femoral condyle. The lesion was 8 mm in diameter, and its size was standardized by use of a harvester instrument for osteochondral transplantation (OATS System; Arthrex, Naples, FL). The harvester was gently pushed to penetrate only cartilage, with care taken not to violate subchondral bone. A hand curette was used to remove noncalcified and calcified layers of cartilage from the lesion, leaving the subchondral plate intact. Four perforations were then performed with a 1.5-mm K-wire that was hammered into the subchondral bone. The depth of hole penetration was standardized by marking the K-wire at 5 mm from its tip. Accurate hemostasis and surgical wound closure were then performed, respecting anatomic layers.

Postoperative Regimen

After surgery, the animals were left free in their fencings without any immobilization of the operated limb. Full weight bearing was allowed as tolerated. No

specific exercise regimen was adopted. General health and weight-bearing status were monitored during recovery by a veterinarian (G. Masala).

Treatment Protocol

Animals were assigned to 2 groups (15 animals in each group), according to postoperative treatment. In group 1 we performed 5 intra-articular injections of PC (ACP) into the operated knee, according to the following scheme: first injection at 24 hours after surgery and then 1 injection every week for 4 weeks. Injections were performed through a superior-lateral approach with a 21-gauge needle by the same operator (E.S.P.). In group 2, operated knees did not undergo further treatment.

ACP Preparation

ACP was prepared with an apposite sterile double-syringe kit (Arthrex). Ten milliliters of autologous venous blood was withdrawn from the right external jugular vein into the outer syringe. Blood was centrifuged at 1,300 rpm for 5 minutes. The supernatant, consisting of about 3 mL of liquid ACP, was collected into the inner syringe and used for intra-articular injections.

In a previous pilot study, the centrifugation procedure of sheep blood was optimized to obtain the greatest platelet concentration. Five blood samples were withdrawn from 14 adult sheep not used for experimental studies, and ACP was prepared according the previously described method. Samples were randomly assigned to 14 groups consisting of 5 samples each, and a different centrifugation setting was used for each group. On the basis of this study, we chose the setting of group 3, which showed the greatest platelet concentration (Table 1). To confirm this result, on 5 animals enlisted for the study, 10 mL of autologous blood was harvested for ACP preparation 1 week before surgery, and platelet count analysis was performed. A 5-mL whole blood sample was then harvested from the same animals, and platelet count analysis was performed and compared with those shown by ACP samples. Each sample was tested by use of a digital hematology analyzer (Advia 120 Hematology Analyzer; GMI, Ramsey, MN), and mean platelet concentration ($\pm$ standard deviation) was assessed in ACP and whole blood. A nonparametric test (Mann-Whitney *U* test) was used to compare mean platelet concentration between ACP and whole blood. No differences in ACP volume were observed between samples. Data analysis showed a 2-fold greater platelet concentration in ACP ($868 \pm 112 \times 10^3$/mL) than in whole blood ($428 \pm 96 \times 10^3$/mL), with a highly significant difference between them ($P < .001$).

Table 1. *Results From Pilot Study on Different Centrifugation Settings for ACP Preparation (5 Samples per Group)*

	Centrifugation Setting			
Group	Rpm	Time (min)	Platelet Count ($\times 10^3$/mL)	ACP Volume (mL)
1	1,000	5	559 ± 106	1
2	1,200	5	554 ± 94	1
3	1,300	5	856 ± 97	3
4	1,400	5	347 ± 74	3
5	1,500	5	374 ± 81	3
6	1,700	5	468 ± 104	4
7	2,000	5	384 ± 108	5
8	1,000	10	578 ± 112	2
9	1,200	10	424 ± 101	3
10	1,300	10	572 ± 109	1
11	1,400	10	240 ± 92	5
12	1,500	10	184 ± 90	5
13	1,700	10	489 ± 104	4
14	2,000	10	259 ± 108	4

Randomization

Fifteen animals were randomly assigned to each group. Randomization was performed by a random number generator. The randomization list was then kept by an independent researcher, and the assignment code of each animal was shown to the investigators only at the end of surgery.

Outcome Measurements

Animals were euthanized by intravenous injection of 1 mL/kg pentobarbital sodium 3, 6, and 12 months after treatment (5 animals from each group at every time interval). At the time of death, the operated and contralateral stifles were harvested after removal of all peri-articular soft tissues. The primary outcome of the study was histologic assessment. A secondary outcome of the study was macroscopic evaluation of cartilage repair. Another secondary outcome was assessment of articular cartilage stiffness.

Macroscopic Evaluation

After the animals were killed, the macroscopic appearance of the repair site was evaluated by 3 different investigators (E.S.P., G. Masala, and

TABLE 2. *International Cartilage Repair Society Macroscopic Evaluation of Cartilage Repair*

Categories	Score
Degree of defect repair	
In level with surrounding cartilage	4
75% repair of defect depth	3
50% repair of defect depth	2
25% repair of defect depth	1
0% repair of defect depth	0
Integration to border zone	
Complete integration with surrounding cartilage	4
Demarcating border <1 mm	3
Three-quarters of graft integrated, one-quarter with a notable border >1 mm in width	2
One-half of graft integrated with surrounding cartilage, one-half with a notable border >1 mm	1
From no contact to one-quarter of graft integrated with surrounding cartilage	0
Macroscopic appearance	
Intact smooth surface	4
Fibrillated surface	3
Small, scattered fissures or cracks	2
Several small or few large fissures	1
Total degeneration of grafted area	0
Overall repair assessment	
Grade I: normal	12
Grade II: nearly normal	8-11
Grade III: abnormal	4-7
Grade IV: severely abnormal	1-3

A.M.) and rated according to the International Cartilage Repair Society evaluation score[27] (Table 2). This scoring system consists of 3 categories (degree of defect repair, integration to border zone, and macroscopic appearance) scored by a 5-point scale (from 0 to 4). Each observer was unaware of the other observers' evaluations.

Histologic Evaluation

Samples were fixed in 10% buffered neutral formalin for 7 days, decalcified in ethylenediaminetetraacetate-buffered saline solution (pH 7.4) (0.25 mol/L), dehydrated by serial ethanol washing, and embedded in paraffin wax. Five sagittal cuts (6 μm thick) from the central part of the defect were obtained, by use of a motorized microtome. Slices were stained with H&E and Safranin O/fast green and examined under a light microscope. Each slice was evaluated by 3 different investigators (L.D., R.P., and M.F.S.) well experienced in histologic assessment of musculoskeletal tissues and blind to treatment and was scored according to a modified O'Driscoll grading system[28,29] (Table 3).

TABLE 3. *Modified O'Driscoll Histologic Score*

Variable	Comment	Score
Tissue morphology	Mostly hyaline cartilage	4
	Mostly fibrocartilage	3
	Mostly non-cartilage	2
	Exclusively non-cartilage	1
Matrix staining	None	1
	Slight	2
	Moderate	3
	Strong	4
Structural integrity	Severe disintegration	1
	Cysts or disruption	2
	No organization of chondrocytes	3
	Beginning of columnar organization of chondrocytes	4
	Normal, similar to healthy mature cartilage	5
Chondrocyte clustering in implant	25%-100% of cells clustered	1
	<25% of cells clustered	2
	No clusters	3
Intactness of calcified layer, formation of tidemark	<25% of calcified layer intact	1
	25%-49% of calcified layer intact	2
	50%-75% of calcified layer intact	3
	76%-90% of calcified layer intact	4
	Complete intactness of calcified cartilage layer	5
Subchondral bone formation	No formation	1
	Slight	2
	Strong	3
Histologic appraisal of surface architecture	Severe fibrillation of disruption	1
	Moderate fibrillation or irregularity	2
	Slight fibrillation or irregularity	3
	Normal	4
Histologic appraisal defect filling	<25%	1
	26%-50%	2
	51%-75%	3
	76%-90%	4
	91%-110%	5
Lateral integration of implanted material	Not bonded	1
	Bonded at one hand/partially at both ends	2
	Bonded at both sides	3
Basal integration of implanted material	<50%	1
	50%-70%	2
	70%-90%	3
	91%-100%	4
Inflammation	No inflammation	1
	Slight inflammation	3
	Strong inflammation	5
Maximum total score		45

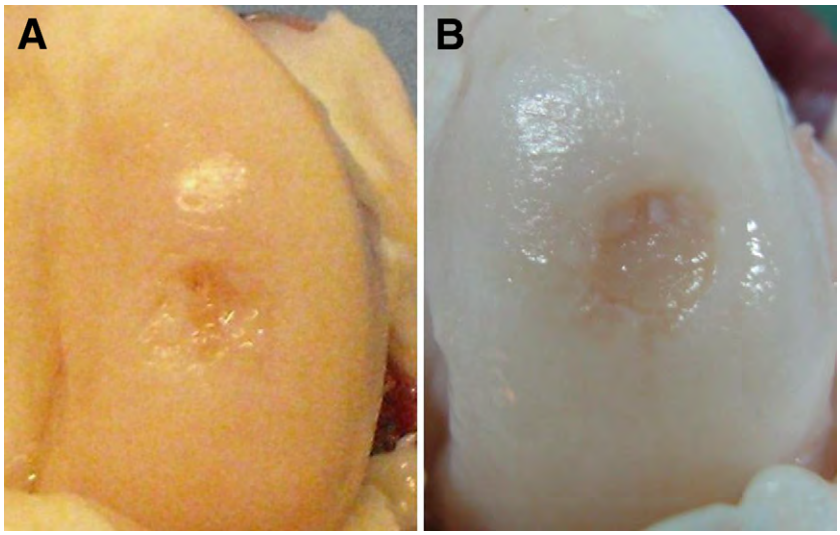

FIGURE 1. Macroscopic appearance at 3 months. (A) In group 1 repair tissue almost completely covered the defect, although it was thin and depressed in the central part, with a clear demarcation from the surrounding cartilage. Large and deep fissures were observed on the surface. (B) In group 2 a very thin layer of repair tissue covered the defect, without clear signs of integration with the surrounding cartilage.

Biomechanical Evaluation

After macroscopic evaluation and before tissue processing for histology, a biomechanical analysis was performed by use of the Artscan 200 electromechanical indentation probe (Artscan Oy, Helsinki, Finland). Consistent measurements were taken by applying a constant 10-N force manually to the cartilage surface to be tested for 1-second intervals over a period of 60 seconds under Artscan 200 software's control and recording the mean indenter force, which is a measure of stiffness. Each testing series was performed on the central part of the defect and approximately on the same area of the medial femoral condyle of the contralateral healthy stifle. Three repeated measurements of stiffness were recorded for each sample by the same operator (A.M.).

Data Analysis

All the outcome measurements were expressed as mean values ± standard deviations. Data were analyzed with statistical software (SPSS 19; SPSS, Chicago, IL). The null hypothesis of the study was that differences in cartilage repair after microfractures in isolation or combined with repeated local injections of PC were not significant.

Comparison between groups at each time period (3, 6, and 12 months after treatment) for histologic and macroscopic scores was performed with the Student *t* test. We calculated 95% confidence intervals around estimated differences between means for each comparison. Differences between experimental groups and normal cartilage samples for mean stiffness at each time period and between different time periods within groups for each outcome were assessed by use of analysis of variance. The Tukey post hoc test was performed for multiple pair-wise comparisons. Significance was set at $P < .05$. A post hoc power analysis was performed according to the primary outcome of the study.

RESULTS

No intraoperative or postoperative complications or adverse events related to treatment with ACP were reported. All animals were available at follow-up.

Power Analysis

Post hoc power analysis based on the primary outcome (histologic score) at final follow-up (12 months) showed an effect size of 2.83 and power ($1 - \beta$) equal to 0.97.

Macroscopic Evaluation

Macroscopic evaluation at 3 months (Fig 1) showed that in group 1 (microfractures + ACP), repair tissue almost completely covered the defect but was much thinner in the inner part; demarcation from surrounding cartilage was clearly evident, and large and deep fissures were observed. In group 2 (microfractures alone), a very thin layer of repair tissue covered the defect but without signs of integration with the surrounding cartilage.

At 6 months (Fig 2), repair tissue completely filled the defect in group 1, although it showed scattered fissures and cracks on the surface. Partial integration along the borders with surrounding cartilage was evident. In group 2, defect filling was more consistent

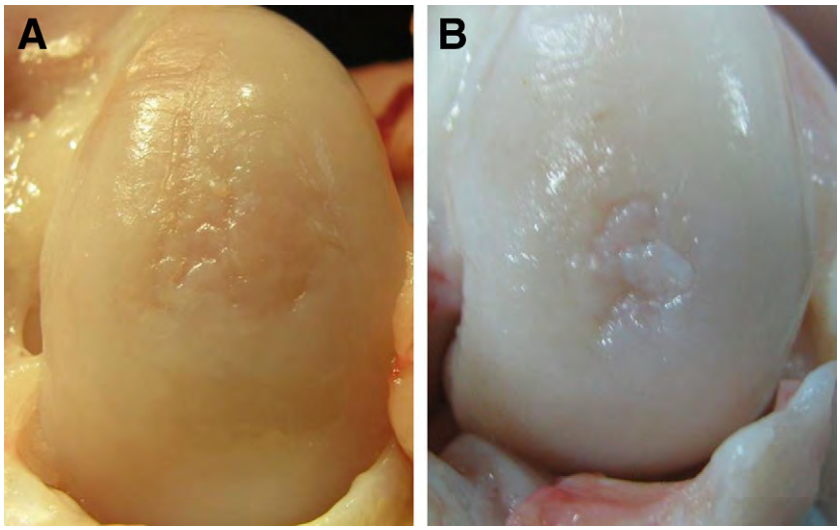

FIGURE 2. Macroscopic appearance at 6 months. (A) In group 1 repair tissue completely filled the defect, although it showed scattered fissures and cracks on the surface. Partial integration along the borders with surrounding cartilage was evident. (B) In group 2 repair tissue partially filled the defect, although large fissures and penetrating cracks were observed on the surface.

than in the samples at 3 months, although it never exceeded 50% of the defect depth; large fissures and penetrating cracks were observed in all samples.

At 12 months (Fig 3), samples from group 1 showed a further improvement of the macroscopic appearance, with the surface being rather smooth and flush with the surrounding cartilage; however, a slight demarcation was still evident along the borders. In group 2, no evident macroscopic changes were observed in comparison with previous observations at 6 months.

Macroscopic scoring analysis showed that the mean score in group 1 was significantly greater than that in group 2 at each time period (Table 4).

Comparison between different time periods within groups showed a significant difference in both groups ($P <$ .0001). The post hoc test showed that in group 1, the mean score at 3 months was significantly lower than the mean scores at 6 and 12 months ($P <$ .0001), whereas the difference between 6 and 12 months was not significant ($P =$ .465). Similarly, in group 2, the mean score at 3 months was significantly lower than the mean scores at 6 and 12 months ($P <$ .0001), whereas the difference between 6 and 12 months was not significant ($P =$ .533).

Histologic Evaluation

Histologic evaluation at 3 months (Fig 4) showed an intense proliferative activity in the subchondral bone under the defect area in group 1. Repair tissue almost completely covered the defect, contained a great num-

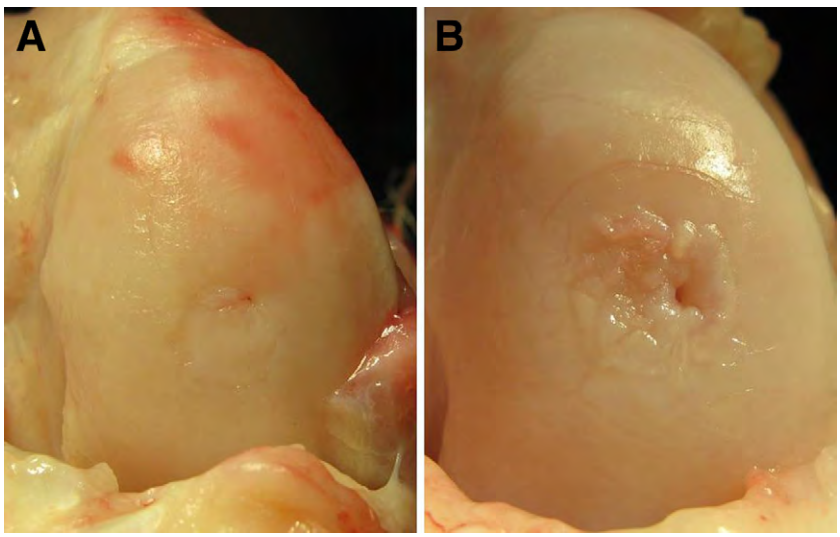

FIGURE 3. Macroscopic appearance at 12 months. (A) In group 1 the surface of repair tissue was rather smooth and flush with the surrounding cartilage, although a slight demarcation was evident along the borders. (B) In group 2 repair tissue was irregular, with fissures and cracks, as well as clear demarcation with the surrounding cartilage.

TABLE 4. *Results of Macroscopic Evaluation According to International Cartilage Repair Society Score (3 Observations per Sample)*

	International Cartilage Repair Society Score (Mean ± SD)			95% Confidence Interval	
Time Periods	Group 1 (n = 5)	Group 2 (n = 5)	*P* Value	Lower Limit	Upper Limit
3 mo	3.13 ± 1.06	2.07 ± 0.59	.003*	–1.72	–0.42
6 mo	7.33 ± 1.34	4.87 ± 2.06	.001*	−3.78	−1.15
12 mo	7.93 ± 1.67	4.27 ± 1.53	<.0001*	−4.86	−2.47

*Statistically significant difference.

ber of small rounded cells, and showed a partial integration with the surrounding healthy cartilage. Extracellular matrix (ECM) showed an intense Safranin O staining of ECM in some areas of the radial zone. Tidemark was completely absent in the entire defect area. In group 2 a thin and poorly organized repair tissue partially covered the defect. Some clusters of chondrocyte-like cells were observed in some areas showing a slight Safranin O staining. No consistent integration was observed with the surrounding cartilage, which showed an intense cell proliferation with formation of cell clusters. Tidemark was almost completely absent in the entire area of the defect.

At 6 months (Fig 5), in group 1, the defect area was almost completely filled with fibrocartilaginous tissue showing good lateral integration, although some clefts

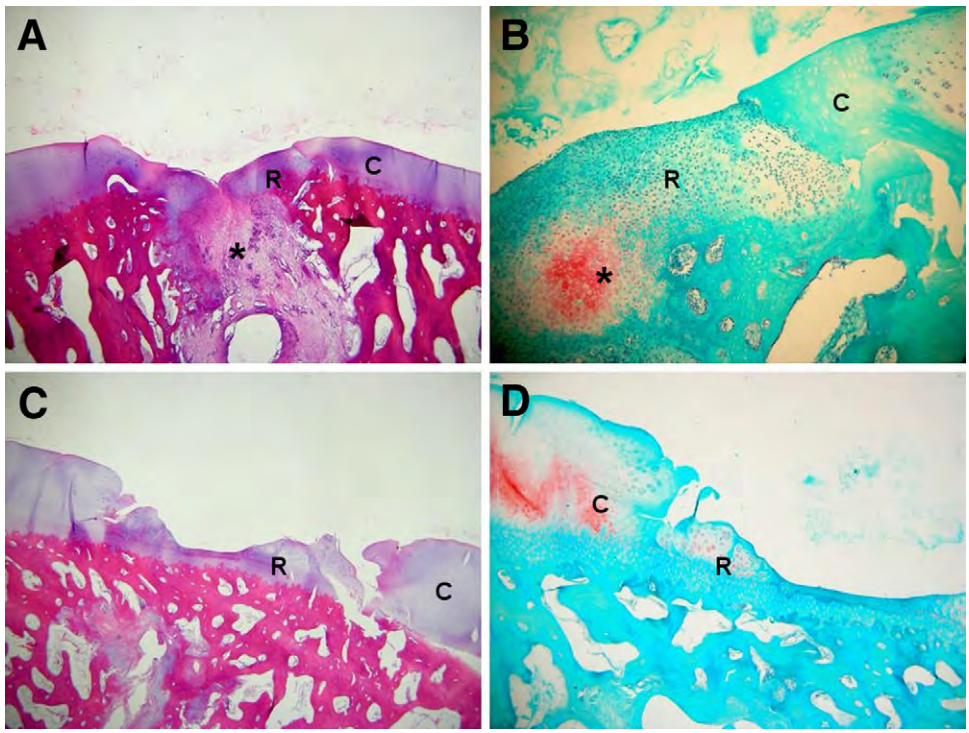

FIGURE 4. Histologic sections at 3 months. (A) In group 1 repair tissue (R) almost completely covered the defect, showing a slight integration with the surrounding healthy cartilage (C) and no tidemark formation. An intense cell proliferation from the subchondral bone consistently filled the defect (asterisk) (H&E staining, original magnification ×20). (B) At higher magnification, repair tissue showed a great number of small rounded cells with a slight Safranin O staining of the ECM in the radial zone (asterisk) (Safranin O staining, original magnification ×100). (C) In group 2 a thin and poorly organized repair tissue (R) partially covered the defect. No consistent integration was observed with the surrounding cartilage (C) (H&E staining, original magnification ×20). (D) At higher magnification, clusters of chondrocyte-like cells were observed in some areas of repair tissue (R), showing a slight Safranin O staining. Surrounding cartilage (C) showed an intense cell proliferation with formation of cell clusters (Safranin O staining, original magnification ×40).

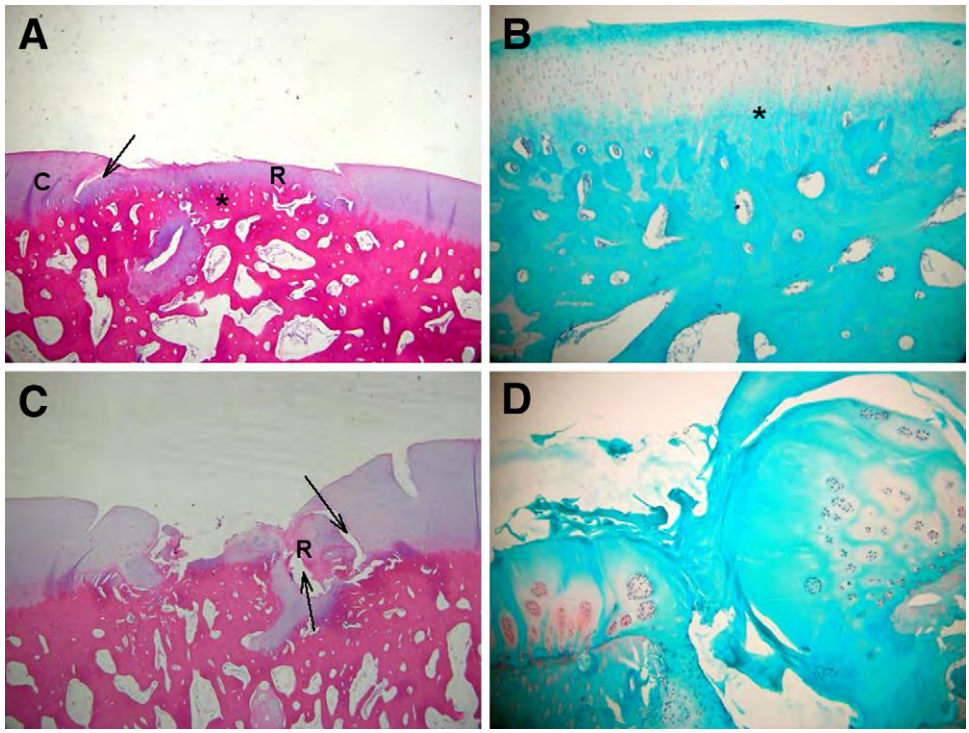

FIGURE 5. Histologic sections at 6 months. (A) In group 1 repair tissue (R) filled the defect for more than 50% of its volume. It showed a good basal integration, with evident overgrowth of subchondral bone (asterisk). Clefts along the borders with healthy surrounding cartilage (C) were evident (arrow) (H&E staining, original magnification ×20). (B) At higher magnification, a great number of rounded cells partially organized in columns were observed. Slight homogeneous Safranin O staining was evident in the ECM. Tidemark formation was occasionally observed (asterisk) (Safranin O staining, original magnification ×40). (C) In group 2 repair tissue (R) was thick but poorly organized, with fissures and penetrating clefts (arrows) (H&E staining, original magnification ×20). (D) At higher magnification, a great number of cell clusters were evident, with a slight Safranin O staining of ECM (Safranin O staining, original magnification ×100).

were observed at the interface with surrounding cartilage. Repair tissue was thinner than normal cartilage, and bone overgrowth was observed at the basal interface. A great number of rounded cells were partially organized in columns with some clusters. Slight homogeneous Safranin O staining was evident in the ECM. Tidemark formation was occasionally observed. In group 2 repair tissue was thicker than in the 3-month samples but still poorly organized and with penetrating clefts. A great number of cell clusters were evident, with a slight Safranin O staining of ECM. Tidemark formation was still poor.

At 12 months (Fig 6), in group 1, no evident changes were observed in comparison with the 6-month samples. Lateral integration was good, although a clear demarcation with the surrounding cartilage was still observed, with intense Safranin O staining of repair tissue along the borders. No complete tidemark formation was seen. In group 2 a deterioration of tissue quality was evident with respect to the 6-month samples. A central collapse of the repair tissue was observed, with a clear demarcation with the surrounding cartilage, presence of disorganized cell clusters, and slight Safranin O staining of ECM. Tidemark formation was poor.

Histologic scoring assessment at 3 months (Table 5) showed that group 1 had significantly greater scores than group 2 for all the variables, including total score, except for tissue morphology, matrix staining, surface architecture, and basal integration, which showed no significant differences between groups. Formation of tidemark and inflammation showed the lowest score in all observations of both groups.

At 6 months, group 1 showed significantly greater scores than group 2 for all the variables, including total score, except for subchondral bone formation, formation of tidemark, and basal integration, which showed no significant differences between groups. Inflammation showed the lowest score in all observations of both groups (Table 6).

At 12 months, group 1 showed significantly greater scores than group 2 for all the variables, including

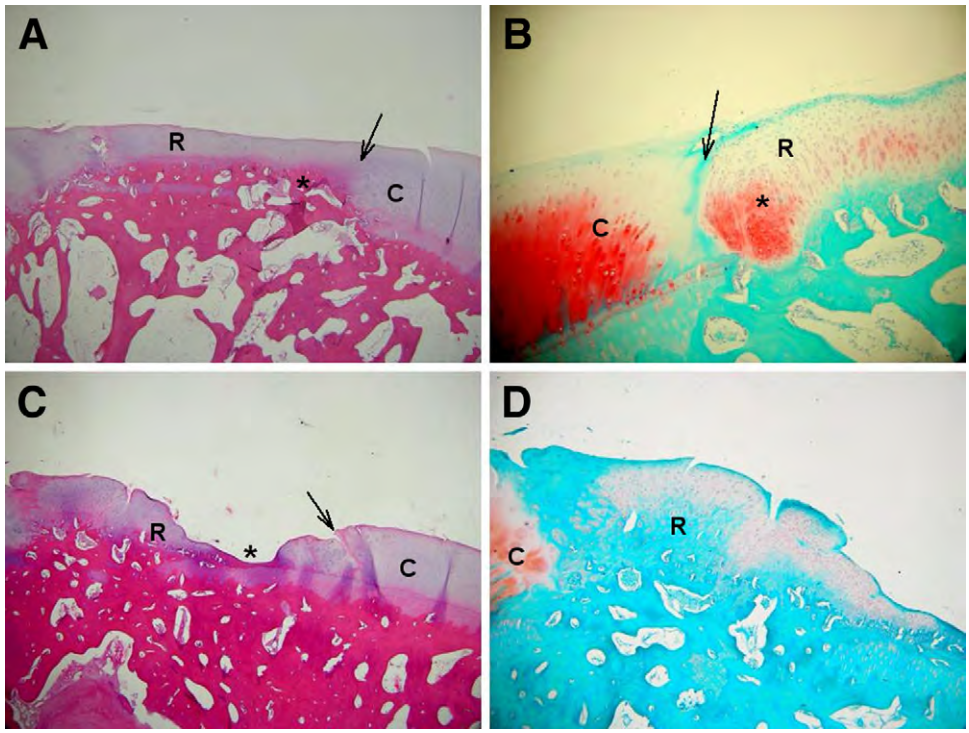

Figure 6. Histologic sections at 12 months. (A) In group 1 repair tissue (R) showed a regular surface, flush with that of healthy surrounding cartilage (C), and almost completely covered the defect. Lateral integration was almost complete, without a clear demarcation between normal and repair tissue (arrow). However, it was thinner than surrounding cartilage, because of strong formation of subchondral bone (asterisk) (H&E staining, original magnification ×20). (B) At higher magnification, a great number of small rounded cells showed a columnar arrangement similar to that of hyaline cartilage. ECM of repair tissue (R) showed an intense Safranin O staining (asterisk) at the lateral interface (arrow) with normal cartilage (C). No complete tidemark formation was seen (Safranin O staining, original magnification ×40). (C) In group 2 a central collapse (asterisk) of the repair tissue (R) was observed, with a clear demarcation (arrow) with the surrounding cartilage (C) (H&E staining, original magnification ×20). (D) At higher magnification, the presence of disorganized cell clusters and slight Safranin O staining of ECM were observed in the repair tissue (R). Tidemark formation was poor (Safranin O staining, original magnification ×40). (C, cartilage.)

total score, except for matrix staining and formation of tidemark, which showed no significant differences between groups (Table 7).

Comparison between different time periods within groups showed a significant difference in both groups ($P < .001$ for group 1 and $P = .01$ for group 2). Post hoc analysis showed that in group 1, the mean total score at 3 months was significantly lower than those at 6 and 12 months ($P < .001$), whereas the difference between 6 and 12 months was not significant ($P = .068$). In group 2 the mean total score at 6 months was significantly greater than those at 3 months ($P = .011$) and 12 months ($P = .018$), whereas the difference between 3 and 12 months was not significant ($P = .991$).

Biomechanical Evaluation

Biomechanical evaluation showed a significant difference between groups at each time period ($P < .0001$). Post hoc analysis (Table 8) showed that at 3 months, mean stiffness of group 1 was significantly greater than that of group 2, although the values in both groups were significantly lower than those in controls. At 6 and 12 months, group 1 showed significantly greater mean stiffness than group 2, whereas comparison with the control group did not show a significant difference. Group 2 values remained significantly lower than those in controls at 6 and 12 months.

Comparison between different time periods within groups showed a significant difference in both groups ($P = .003$ for group 1 and $P < .0001$ for group 2). The post hoc test showed that in group 1, mean stiffness at 3 months was significantly lower than that at 6 months ($P = .007$) and 12 months ($P < .010$), whereas the difference between 6 and 12 months was not significant ($P = .989$). In group 2 mean stiffness at 3 months

TABLE 5. *Results of Histologic Evaluation at 3 Months (15 Observations per Sample)*

Variable	Mean Score ± SD			95% Confidence Interval	
	Group 1 (n = 5)	Group 2 (n = 5)	*P* Value	Lower Limit	Upper Limit
Tissue morphology	3.87 ± 0.52	3.40 ± 0.91	.095	−1.02	0.09
Matrix staining	1.87 ± 0.64	1.80 ± 0.41	.737	−0.47	0.34
Structural integrity	2.20 ± 0.56	1.27 ± 0.59	<.0001*	−1.36	−0.50
Chondrocyte clustering	1.93 ± 0.26	1.60 ± 0.51	.031*	−0.63	−0.03
Formation of tidemark	1	1	—†		
Subchondral bone formation	2.67 ± 0.49	1.80 ± 0.41	<.0001*	1.20	0.53
Surface architecture	1.53 ± 0.52	1.20 ± 0.41	.061	−0.68	0.02
Defect filling	2.27 ± 0.70	1.47 ± 0.52	.001*	−1.26	−0.34
Lateral integration	2.27 ± 0.46	1.87 ± 0.35	.012*	−0.70	−0.09
Basal integration	1.20 ± 0.41	1.13 ± 0.35	.638	−0.35	0.22
Inflammation	1	1	—†		
Total	21.80 ± 2.65	17.53 ± 2.03	<.001*	−6.03	−2.50

*Statistically significant difference.
†We did not calculate *t* because SD equaled 0 in both groups.

was significantly lower than that at 6 months ($P < .0001$) and 12 months ($P = .16$), and mean stiffness at 6 months was significantly greater than that at 12 months ($P = .024$).

DISCUSSION

PCs can be classified depending on their leukocyte and fibrin content.[30] The most popular PC preparation is platelet-rich plasma (PRP), defined as an autologous blood component with concentrations of platelets above baseline values.[14] PRP is prepared by taking a sample of autologous, anticoagulated blood and using a centrifuge or filter to separate red blood cells from leukocytes and platelets. With further concentration, plasma is divided into platelet-poor and platelet-rich portions. Its combination with calcium chloride and/or thrombin immediately before injection initiates platelet activation, clot formation, and GF release at the injection site.[31]

GFs released upon platelet activation include platelet-derived growth factor (PDGF-$\alpha\alpha$, PDGF-$\beta\beta$, and PDGF-$\alpha\beta$ isomers), transforming growth factor (TGF-β, TGF-β1, and TGF-β2 isomers), platelet-derived angiogenesis factor (PDAF), vascular endothelial growth factor (VEGF), epidermal growth factor (EGF), platelet-derived endothelial growth factor (PDEGF), epithelial cell growth factor (ECGF), and

TABLE 6. *Results of Histologic Evaluation at 6 Months (15 Observations per Sample)*

Variable	Mean Score ± SD			95% Confidence Interval	
	Group 1 (n = 5)	Group 2 (n = 5)	*P* Value	Lower Limit	Upper Limit
Tissue morphology	3.95 ± 0.22	2.93 ± 1.03	<.0001*	−1.35	–0.68
Matrix staining	2.44 ± 0.78	1.67 ± 0.72	<.0001*	−1023	−0.31
Structural integrity	3.39 ± 0.99	2.13 ± 1.41	<.0001*	−1.93	−0.58
Chondrocyte clustering	2.07 + 0.26	1.07 ± 0.26	<.0001*	−1.16	−0.85
Formation of tidemark	2.05 ± 1.07	1.87 ± 1.19	.586	−0.85	0.48
Subchondral bone formation	2.49 ± 0.51	2.67 ± 0.49	.242	−0.12	0.48
Surface architecture	1.80 ± 0.56	1.27 ± 0.46	.002*	−0.86	−0.21
Defect filling	2.85 ± 1.19	1.40 ± 0.51	<.0001*	−2.09	−0.81
Lateral integration	2.17 ± 0.49	1.73 ± 0.46	.004*	−0.73	−0.14
Basal integration	2.22 ± 0.85	2.60 ± 0.51	.111	−0.09	0.85
Inflammation	1	1	—†		
Total	26.44 ± 3.56	20.33 ± 2.38	<.0001*	−8.10	−4.11

*Statistically significant difference.
†We did not calculate *t* because SD equaled 0 in both groups.

TABLE 7. *Results of Histologic Evaluation at 12 Months (15 Observations per Sample)*

Variable	Mean Score ± SD			95% Confidence Interval	
	Group 1 (n = 5)	Group 2 (n = 5)	*P* Value	Lower Limit	Upper Limit
Tissue morphology	3.50 ± 0.87	2.30 ± 0.67	<.0001*	–1.78	–0.62
Matrix staining	2.11 ± 0.76	1.60 ± 0.84	.054	−1.04	0.01
Structural integrity	2.93 ± 1.01	1.60 ± 0.52	<.0001*	−1.98	−0.68
Chondrocyte clustering	1.80 ± 0.48	1.10 ± 0.32	<.0001*	−1.01	−0.38
Formation of tidemark	2.02 ± 1.11	1.50 ± 0.85	.166	−1.25	0.22
Subchondral bone formation	2.95 ± 0.22	2.00 ± 0.67	<.0001*	−1.16	−0.73
Surface architecture	2.10 ± 0.77	1.10 ± 0.32	<.0001*	−1.50	−0.50
Defect filling	3.57 ± 1.27	1.60 ± 0.52	<.0001*	−2.78	−1.15
Lateral integration	2.75 ± 0.44	1.20 ± 0.42	<.0001*	−1.85	−1.25
Basal integration	3.50 ± 0.79	1.60 ± 0.52	<.0001*	−2.42	−1.38
Inflammation	1	1.80 ± 1.03	<.0001*	0.54	1.06
Total	28.23 ± 4.41	17.40 ± 3.24	<.0001*	−13.75	−7.92

*Statistically significant difference.

insulin-like growth factor (IGF-I).[32] These proteins may have therapeutic effects on chondral injuries through multiple mechanisms, including recruitment of chondrogenic cells (chemotaxis), stimulation of chondrogenic cell proliferation (mitogenesis), and enhancement of cartilage matrix biosynthesis.[32]

ACP differs from PRP for some procedural aspects. First, the amount of blood withdrawal (10 mL) is much less than that suggested for many PRP preparations (about 50 mL); second, preparation of ACP requires only 1 blood centrifugation, with lesser risk of blood contamination.

Potential benefits of PCs on cartilage repair have already been documented in the previous literature.[19-26,33-37] In vitro studies showed that PRP enhances proliferation and chondrogenic differentiation of MSCs,[20,33] as well as proliferation and anabolic activity of cultured chondrocytes.[19,34,35] In vivo animal studies confirmed a significant positive effect of PCs on cartilage repair and formation.[21,36,37] Frisbie et al.[36] administrated autologous conditioned serum (ACS) in horses with experimentally induced OA, obtaining significant clinical improvement in lameness, decreased synovial membrane hyperplasia, less gross cartilage fibrillation and synovial membrane hemorrhage, and an increased synovial fluid concentration of interleukin 1 receptor antagonist. Saito et al.[37] documented preventive effects against progression of OA with the administration of gelatin hydrogel microspheres containing PRP in a rabbit model. Wu et al.[21] investigated the feasibility of PRP to support chondrogenesis in vivo in an extra-articular environment. Clinical studies confirmed the efficacy of PCs on articular cartilage injuries.[24-26] Sánchez et al.[24] reported preliminary results about the effectiveness of intra-articular injections of an autologous preparation

TABLE 8. *Results of Biomechanical Evaluation (3 Measurements per Sample)*

Time Period	Stiffness (Indenter Force) (Mean ± SD) (N)			Differences Between Means		95% Confidence Interval	
	Group 1 (n = 5)	Group 2 (n = 5)	Control Group (n = 10)	Groups	*P* Value	Lower Limit	Upper Limit
3 mo	4.13 ± 0.92	2.46 ± 0.59	5.26 ± 0.19	1–2	<.0001*	1.18	2.17
				1–control	<.0001*	−1.55	−0.69
				2–control	<.0001*	−3.23	−2.37
6 mo	5.17 ± 1.18	3.54 ± 0.33	5.09 ± 0.24	1–2	<.0001*	1.08	2.17
				1–control	.910	−0.39	−0.55
				2–control	<.0001*	−2.02	−1.07
12 mo	5.12 ± 0.28	3.01 ± 0.61	5.29 ± 0.30	1–2	<.0001*	1.76	2.46
				1–control	.397	−0.47	0.14
				2–control	<.0001*	−2.58	−1.97

*Statistically significant difference.

rich in GFs for the treatment of knee OA, suggesting the safety and usefulness of this treatment approach. Baltzer et al.[25] evaluated 310 patients with knee OA in a prospective, randomized controlled trial, by comparing the clinical outcome of ACS, hyaluronic acid, and saline solution (placebo) intra-articular injections, and they observed considerably better results for the ACS-treated group at 2-year follow-up. Filardo et al.[26] reported on 90 patients affected by chronic degenerative condition of the knee and treated with PRP intra-articular injections. The clinical outcome remained stable from the end of the therapy to 6-month follow-up, whereas it progressively worsened up to 24 months after treatment, even if still significantly better with respect to the baseline evaluation.

The results of our study showed that macroscopic and histologic findings of repair tissue in the ACP-treated group were significantly better than those observed in the microfracture group at every time interval. Analysis of time effect on tissue repair within groups showed that in the ACP-treated group, repair progressed until 6 months and then a steady state was observed, without significant changes at 12 months. The microfracture group showed the strongest reparative response at 6 months as well. Thereafter histologic deterioration of tissue quality was observed. However, both treatments were unable to restore normal hyaline cartilage.

Histologic evaluation was considered the primary outcome of this study because it is the most frequently reported outcome measurement in animal studies on methods to improve cartilage repair. Previous studies using experimental models similar to that used in our study showed histologic improvement of repair tissue by combination of microfractures with synthetic scaffolds, free or seeded with cultured chondrocytes.[7-9,11,12] However, none of these studies reported formation of normal hyaline cartilage. Furthermore, the time effect on durability of repair tissue at follow-up longer than 6 months was not investigated.

Interpretation of results with respect to the functional requirements for repair cartilage is critical for the advancement of tissue engineering of articular cartilage. For this reason, biomechanical evaluation of repair tissue was also considered as a secondary endpoint of the study. Particularly, analysis of cartilage stiffness by use of Artscan 200 was previously reported as a stringent test to assess mechanical behavior of articular cartilage.[38] On analyzing data from the indentation test on normal cartilage, we observed greater values for mean stiffness than those reported in the same animal model by Lu et al.[38] However, variability in cartilage thickness of the sheep knee[39] and differences in race and size between our ovine model and that used by Lu et al. can justify this difference.

Results of the biomechanical evaluation showed that the use of ACP improved mechanical behavior of repair tissue, which reached mean stiffness similar to that of normal cartilage at 6 months, and then maintained the result at 12-month follow-up. On the contrary, in the microfracture group, stiffness progressively increased until 6 months and then decreased over time. These data can have consistent clinical relevance, because inferior biomechanical quality of repair cartilage contributes to tissue degradation over time and may be a factor in the functional decline and increasing failure rate observed in humans.[3] Discrepancies between histologic and biomechanical outcomes observed in our study can explain the lack of association between histologic repair tissue quality and functional outcome scores reported in human studies.[3] Indeed, better mechanical behavior observed in the ACP-treated group might depend on repair cartilage fill volume, which was significantly greater in the ACP-treated group at each time interval. However, potential assessment bias of these data could be related to the strong subchondral bone formation observed in the ACP-treated group at 6 and 12 months after treatment. According to this hypothesis, bone overgrowth contributed to defect filling, although repair cartilage was thinner than native tissue, thus producing high values for mean stiffness on mechanical testing. Although this phenomenon has been reported after microfractures when calcified cartilage is removed,[40] further investigations are mandatory on this issue, regarding potential risks of procedure-related adverse events.

According to the results of our study, it can be hypothesized that repeated intra-articular injections of ACP can improve the reparative response of focal full-thickness defects of articular cartilage after microfractures in comparison with an isolated microfracture procedure and also increase the durability of repair tissue over time.

The combination of microfractures and PRP was reported in a previous sheep study,[22] which showed that PRP had a positive effect on cartilage repair after microfractures and that the procedure was more effective when PRP was used as a gel in comparison with liquid intra-articular injection. However, differently from the present study, PRP was injected once at the end of the surgical procedure and was not repeated over time. This might have crucial clinical relevance, because local injection of ACP does not require any

additional surgical procedure, as needed with PRP gel. Furthermore, if repeated over time, injections of ACP could maintain reparative response and prevent further degenerative changes in the repair tissue.

Indeed, multiple intra-articular injections represent a relevant safety issue, because they imply an increased potential risk of infection. The choice to perform 5 injections was based on the scientific evidence that fibrocartilage is not evident in the defect until the fourth week after surgery.[5,6] Hence repeated postoperative treatment with ACP during the first month might induce earlier tissue formation and differentiation.

Nevertheless, it is difficult to generalize and compare results on efficacy of different PCs. Some authors showed noticeable variations in platelet concentration and GF content in different methods for PC preparation.[16-18] Although ACP used in this study showed a platelet concentration lower than that suggested as optimal to achieve the best efficacy of PRP,[14] it is still unclear whether effects of PC on cell proliferation and differentiation are dose dependent and related to platelet or GF concentration.[34,41]

Overall, inference of an animal study is limited because functional status cannot be assessed at baseline and follow-up evaluations. However, the animal model used in this study is considered suitable for cartilage defect testing.[39] Moreover, size and location of the defect were consistent with well-standardized models reported in previous studies.[39] Potential performance bias could be related to the variability of cartilage thickness in sheep.[39] Therefore volume of cartilage removed with respect to the defect size likely can be different between animals. For these reasons, depth of cartilage defect was not measured, and subchondral bone was used as the limit for tissue removal to obtain similar biologic effects in all the animals.[40]

This study has some other limitations. First, because the study design was aimed to verify the effect of ACP on stable knees with focal chondral defects, efficacy of the treatment on larger defects or extensive degenerative changes of articular cartilage, as observed in osteoarthritic knees, cannot be inferred from results of the present study. Second, no sham postoperative treatment (i.e., saline solution injection) was administered to the control group to provide baseline outcome measurements for the experimental protocol. Third, no analysis was performed on other joint structures, such as synovial lining, ligaments, menisci, and native cartilage, to investigate adverse effects of ACP on intact tissues. Fourth, although preliminary study to optimize the centrifugation setting provided a platelet concentration approximating 2-fold that of entire blood, we did not analyze GF content of ACP in animal blood. This is a relevant issue, because correlation between platelet concentration and in situ release of GFs is not clearly predictable.[41] Furthermore, even though we did not observe relevant variability in ACP volume and platelet concentration in the pilot study for the selected centrifugation setting, we did not repeat this analysis during treatments. Finally, we did not compare ACP with other PC preparations to find out whether eventual therapeutic effect on cartilage injuries varies according to differences in platelet concentration.

CONCLUSIONS

Five repeated local injections of ACP after microfractures in the treatment of full-thickness cartilage injuries promoted a better and more durable reparative response than isolated microfractures, although they did not produce hyaline cartilage.

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