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The effect of platelet rich plasma combined with microfractures on the treatment of chondral defects: an experimental study in a sheep model

G. Milano †*, E. Sanna Passino ‡, L. Deriu †, G. Careddu ‡, L. Manunta ‡, A. Manunta §, M.F. Saccomanno †, C. Fabbriciani †

† *Department of Orthopaedics, Catholic University, Rome, Italy*
‡ *Department of Veterinary Surgery, University of Sassari, Sassari, Italy*
§ *Department of Orthopaedics, University of Sassari, Sassari, Italy*

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SUMMARY

Objective: To evaluate the effect of autologous platelet rich plasma (PRP) combined with microfractures on the treatment of chondral defects. The hypothesis of the study was that PRP can enhance cartilage repair after microfractures.

Methods: A chronic full-thickness chondral lesion of the medial femoral condyle was performed in 15 sheep. Animals were divided into three groups, according to treatment: group 1: microfractures; group 2: microfractures + PRP and fibrin glue gel; group 3: microfractures + liquid-PRP injection. Animals were sacrificed at 6 months after treatment. Macroscopic appearance was evaluated according to International Cartilage Repair Society (ICRS) score; cartilage stiffness was analyzed with an electromechanical indenter (Artscan 200); histological appearance was scored according to a modified O'Driscoll score. Comparison between groups for each outcome was performed with Kruskal–Wallis test, and Tukey's test for pairwise comparisons.

Results: Macroscopic ICRS score of group 2 was significantly better than those of the other groups, and score of group 1 was significantly lower than those of the other groups. Scores of group 1 and 3 were significantly lower than that of normal cartilage. Mean cartilage stiffness of groups 1 and 3 was significantly lower than that of normal cartilage. Histological total scores of group 2 and 3 were significantly better than that of group 1.

Conclusions: PRP showed a positive effect on cartilage repair and restoration after microfractures. The procedure was more effective when PRP was used as a gel in comparison with liquid intra-articular injection. Histological analysis revealed that none of experimental treatments produced hyaline cartilage.

Introduction

Microfracture procedure is one of the most frequently used techniques for the treatment for focal defects of articular cartilage. Biological rationale of microfractures is based on stimulation of bone-marrow stem cells (BMSCs) to migrate to the site of injury and differentiate in a chondrogenic cell line. However, several clinical and animal studies showed that repaired tissue obtained after microfractures is fibrocartilage, containing a high quantity of type I collagen[1–6]. This could be due to an insufficient local stimulation necessary to modulate chondrogenic differentiation of BMSCs[7].

It is well known that some platelet-derived growth factors (GFs) can stimulate chondrogenic differentiation of BMSCs and enhance chondrocyte proliferation and metabolism[8–17].

Platelet rich plasma (PRP) is defined as a volume of the plasma fraction of autologous blood having a platelet concentration above baseline[18]; consequently, it contains a high concentration of platelet-derived GFs with a potential promotive effect on tissue healing and regeneration[19]. Recent *in-vitro* studies showed that PRP stimulates *in-vitro* chondrocyte proliferation and extra-cellular matrix (ECM) biosynthesis and enhances proliferation and chondrogenic differentiation of BMSCs[20–24]. However, no previous studies tested the *in-vivo* efficacy of PRP in enhancing reparative response of chondral defects induced by marrow stimulating techniques, such as microfractures.

The purpose of the present study was to evaluate the effect of PRP combined with microfractures on healing of focal chondral defects of the knee. The primary objective of the study was to

* Address correspondence and reprint requests to: Giuseppe Milano, Department of Orthopaedics, Catholic University, Largo A. Gemelli 8, 00168 Rome, Italy. Tel: 39-06-3015-4353; Fax: 39-06-3051-161.
E-mail address: giuseppe.milano@rm.unicatt.it (G. Milano).

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compare repaired tissue after microfractures as isolated treatment and combined with PRP. The secondary objective was to compare efficacy of PRP used as gel or liquid intra-articular injection. The hypotheses of the study were that PRP can enhance reparative response of chondral defects after microfractures, and that efficacy of PRP is greater when used as gel placed over the lesion than liquid intra-articular injection. The null hypotheses of the study were that there are no significant differences on chondral defect healing after microfractures isolated or combined with PRP, and that there are no significant differences between gel and liquid PRP.

Materials and methods

For the present study, we used 15 sheep (sarda ewes at dry-off) that came from the same extensive farming system. Age ranged from 32 to 42 months (average: 38 months) and weight ranged from 35 kg to 42 kg (average: 40 kg). Exclusion criteria for enrolment of animals were: skeletal immaturity and degenerative changes of the stifle joint. Skeletal maturity was confirmed by stifle radiographs to ensure the closure of the growth plates of the distal femur and proximal tibia (physeal scar but no open physis). Degenerative changes were assessed on radiographic exams, and intraoperative macroscopic appearance of the articular cartilage. All the animals underwent a veterinary examination to evaluate general health status. Research protocol was approved by Ethic Committee for Animal Experimentation of the University of Sassari, where treatments were performed.

Surgical treatment

Surgery was performed using sterile conditions and under general anaesthesia. On each animal, we performed a medial parapatellar arthrotomy on the right stifle. A full-thickness articular cartilage lesion on the weight-bearing area of the medial femoral condyle was performed using an 8-mm harvester instrument for osteochondral transplantation (OATS System; Arthrex, Naples, FL). As the tip of the core harvester was sharp, difference between inner and outer diameter of the instrument was negligible, so that the exact diameter of the lesion was 8 mm. Noncalcified cartilage was removed from the defect using a hand curette, taking care of retaining calcified cartilage in the base of the defect. Accurate haemostasis and surgical wound closure were then performed, respecting anatomical layers. After surgery, the animals have been 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.

A second surgery on the operated stifle was performed after 12 months on each animal. After a medial parapatellar incision, the lesion performed during the previous surgery was identified. As a mild reparative response from surrounding cartilage was observed along the borders of the lesion (Fig. 1), the margins of the defect were debrided to restore the original diameter (8 mm), that was checked with an appropriate 8-mm sizer/tamp from OATS instrumentation set. The margins and the base of the lesion were debrided using a small curette. Care was taken to remove the calcified layer of the cartilage leaving intact the subchondral plate, and four perforations were then performed using a 1.5-mm K-wire that was hammered into the subchondral bone. The depth of holes penetration was standardized by marking the K-wire at 5 mm from its tip.

Animals were assigned to three groups (five animals for each group), according to the repair technique. In group 1, isolated microfractures were performed. In group 2, a PRP and fibrin glue (Tissucol; Baxter BioSurgery, Baxter International Inc., Deerfield, IL) gel was placed over the lesion where microfractures were previously performed. Gel perfectly matched the size of the defect and was sealed along the borders with fibrin glue. In group 3, after microfractures, 5 ml of liquid PRP was injected into the joint after closing the surgical wound.

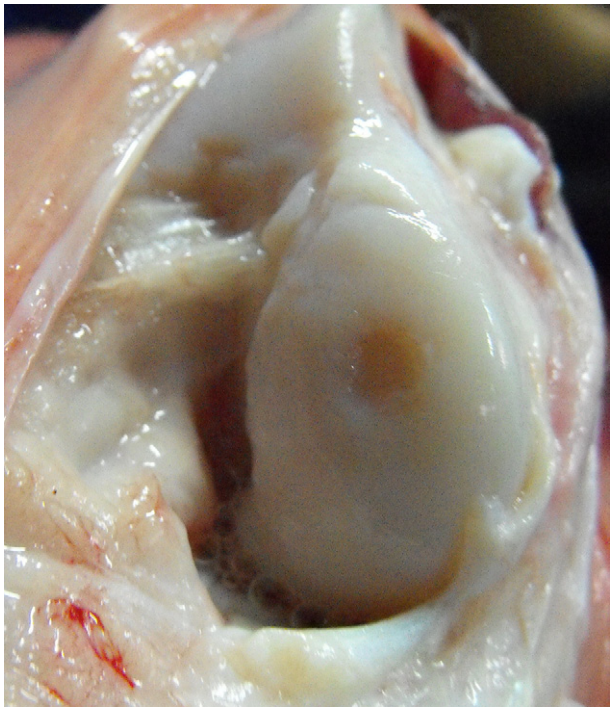

Fig. 1. Macroscopic appearance of an 8-mm full-thickness chondral defect on the weight-bearing area of the medial femoral condyle 12 months after surgery. A mild reparative response from surrounding cartilage can be observed along the margins of the defect.

PRP preparation

PRP was prepared by harvesting 60 ml of autologous blood from each animal before the induction of anaesthesia. Blood was centrifuged at 2400 rpm for 3 min. The precipitate was separated and supernatant was centrifuged at 3000 rpm for 12 min. The precipitate was collected and the final product was 6–8 ml of liquid PRP, which was used for intra-articular injection in group 3. PRP gel was prepared by adding 2 ml of a Ca-gluconate solution (1 g/10 ml) and 2 ml of fibrin glue, consisting of a combination of fibrinogen and thrombin, to 6 ml of liquid PRP.

In a previous pilot study, centrifugation procedure of sheep blood was optimized in order to obtain the greatest platelet concentration[18]. Based on this study, we chose the setting with the best PRP quantity/platelet concentration ratio. To confirm this result, on five animals, 1 week before surgery, 60 ml of autologous blood were harvested for PRP preparation, according to the previously described method, 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 to those showed by PRP samples. Each sample was tested using a digital haematology analyzer (Advia 120 Hematology Analyzer; GMI Inc., Ramsey, MN) and mean platelet concentration ($\pm$standard deviation (SD)) was assessed in PRP and whole blood. A non-parametric test (Mann–Whitney *U*-test) was used to compare mean platelet concentration between PRP and whole blood. Data analysis showed a fourfold greater platelet concentration in PRP ($1415 \pm 164 \times 10^3$/ml) than in whole blood ($351 \pm 64 \times 10^3$/ml) with a highly significant difference between them ($P < 0.0001$).

Randomization

Five animals were randomly assigned to each group after first treatment. Randomization was performed using a random number generator and assigning the first five numbers of the random list to the animals of group 1, the following five numbers to those of group 2, and the last five numbers to group 3. The randomization list was then put in increasing order from 1 to 15 and kept by an independent researcher, and the assignment code of each animal was revealed to the surgeon only at the time of the second surgery.

Post-operative treatment

After second surgery, the animals have been 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 veterinary.

Outcome measurements

Animals were euthanized by intravenous injection of 1 ml/kg pentobarbital sodium 6 months after treatment (second surgery). Distal femurs of the operated and contralateral limb were harvested after removing all peri-articular soft tissues.

On each operated sample, a macroscopic evaluation of the treated defect was performed to evaluate repaired tissue. Moreover, a biomechanical test and a histological evaluation were performed. Histological assessment was considered the primary outcome of the study.

Macroscopic evaluation of cartilage repair was assessed using the International Cartilage Repair Society (ICRS) evaluation score[25,26] (Table I).

Biomechanical evaluation was performed by measurement of articular cartilage stiffness using the Artscan 200 (Artscan Oy, Helsinki, Finland). The Artscan 200 is an electromechanical indentation probe which consists of a measurement rod (length = 150 mm and diameter = 5 mm) attached to a handle. The distal end of the rod consists of an inclined reference plate and a protruding cylindrical indenter (height = 0.3 mm and diameter = 1.0 mm). When the reference plate is pressed against cartilage surface, the indenter induces a constant deformation on the cartilage. The force by which the cartilage resists the deformation is detected as indenter force (N). Consistent measurements were taken by applying manually a constant 10 N force to the cartilage surface to be tested for 1 s intervals over 60 s under Artscan 200 software's control, and recording the mean indenter force, which is a measure of stiffness. An average of three mean stiffness values was taken and calculated. 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.

Table I
ICRS 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
3/4 of graft integrated, 1/4 with a notable border >1 mm width	2
1/2 of graft integrated with surrounding cartilage, 1/2 with a notable border >1 mm	1
From no contact to 1/4 of graft integrated with surrounding cartilage	0
Macroscopic appearance	
Intact smooth surface	4
Fibrillated surface	3
Small, scattered fissures or cracs	2
Several, small or few but large fissures	1
Total degeneration of grafted area	0
Overall repair assessment	
Grade I: normal	12
Grade II: nearly normal	11–8
Grade III: abnormal	7–4
Grade IV: severely abnormal	3–1

After biomechanical evaluation, each operated sample was fixed in 10% buffered neutral formalin for 7 days, decalcified in ethylene diamine tetra-acetate (EDTA) buffered saline (pH 7.4) (0.25 mol/l), dehydrated by serial ethanol washing and embedded in paraffin wax. Samples were cut under visual control at three levels. Five sagittal cuts (6 μm thick) from the central third of the defect were obtained, using a motorized microtome. Slices were stained with haematoxylin–eosin (H–E) and Safranin-O/fast green, and examined under the light microscope.

Each slice was evaluated by three different authors and scored according to a modified version of the grading system developed by O'Driscoll *et al.*[27,28] (Table II). On each sample, the mean score from observation of all histological sections was obtained from the three observers, and the mean of the three mean observations was then calculated.

Investigators that assessed outcomes were well-experienced in managing outcome measurement tools and were blind to treatment.

Data analysis

Data were analyzed using statistical software SPSS 10.1 (SPSS Inc.). Outcome variables were: macroscopic evaluation score, cartilage stiffness, and histological score. Each variable was expressed as mean value (±SD) for each group. Comparison between groups for each outcome was performed with the Kruskal–Wallis test. A *post-hoc* analysis was then performed with the Tukey's test for multiple pairwise comparisons. 95% confidence intervals (CI) of estimated differences between means were calculated for each comparison. Significance was set at $P < 0.05$.

A *post-hoc* power analysis was based on the primary outcome (histological score), and according to the primary hypothesis of the study.

Results

Macroscopic evaluation

Macroscopic evaluation showed that in group 1 (microfractures), repaired tissue partially covered the defect. Healing tissue was much thinner than normal surrounding cartilage and was more evident in the areas over and surrounding the microfractures. In some areas, subchondral bone was still exposed [Fig. 2(A)].

In group 2 (microfractures + PRP gel), the defect area was completely covered by repair tissue which showed a good integration with healthy surrounding cartilage. Repaired tissue showed similar thickness, appearance and colour to that of normal cartilage. Macroscopic evaluation did not show fibrillation and/or fissuration of the repaired tissue [Fig. 2(B)].

In group 3 (microfractures + liquid PRP), repaired tissue covered almost completely the defect. Microfractures holes were not

Table II
Modified O'Driscoll histological score

Variable	Comment	Score
Tissue Morphology (Ti)	Mostly hyaline cartilage	4
	Mostly fibrocartilage	3
	Mostly non-cartilage	2
	Exclusively non-cartilage	1
Matrix staining (Matx)	None	1
	Slight	2
	Moderate	3
	Strong	4
Structural integrity (Stru)	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 (Clus)	25–100% of the cells clustered	1
	<25% of the cells clustered	2
	No clusters	3
Intactness of the calcified layer, formation of tidemark (Tide)	<25% of the calcified layer intact	1
	25–49% of the calcified layer intact	2
	50–75% of the calcified layer intact	3
	76–90% of the calcified layer intact	4
	Complete intactness of the calcified cartilage layer	5
Subchondral bone formation (Bform)	No formation	1
	Slight	2
	Strong	3
Histological appraisal of surface architecture (SurfH)	Severe fibrillation of disruption	1
	Moderate fibrillation or irregularity	2
	Slight fibrillation or irregularity	3
	Normal	4
Histological appraisal defect filling (FilH)	<25%	1
	26–50%	2
	51–75%	3
	76–90%	4
	91–110%	5
Lateral integration of implanted material (Latl)	Not bonded	1
	Bonded at one hand/partially both ends	2
	Bonded at both sides	3
Basal integration of implanted material (Basl)	<50%	1
	50–70%	2
	70–90%	3
	91–100%	4
Inflammation (InfH)	No inflammation	1
	Slight inflammation	3
	Strong inflammation	5
Maximum total score		45

evident; however, repair tissue was thin, irregular and inconsistent in the central aspect of the defect. A good integration with the healthy surrounding cartilage was evident [Fig. 2(C)].

Macroscopic scoring analysis revealed a significant difference between groups ($P = 0.0001$). *Post-hoc* analysis showed that ICRS score of group 2 was significantly greater than those of the other groups, and score of group 1 was significantly lower than those of the other groups. ICRS scores of group 1 and 3 were significantly lower than that of normal cartilage (Table III).

Biomechanical evaluation

Biomechanical evaluation showed a significant difference between groups ($P = 0.007$). *Post-hoc* analysis showed that mean stiffness of group 2 was greater than those of the other two groups, although differences between the three groups were not significant. Only mean cartilage stiffness of group 2 approximated that of controls. On the contrary, groups 1 and 3 showed a significantly lower mean stiffness than normal cartilage (Table IV).

Histological evaluation

Histological evaluation showed that in group 1, repair tissue consisted of a thin layer of non-cartilaginous tissue. In some areas, where microfractures holes had been performed, we found a great amount of rounded cells resembling chondrocytes embedded in a fibrous and poorly organized ECM. In those areas, we observed an intense Safranin-O staining. Tidemark was almost completely absent in all the area of the defect. Healthy cartilage surrounding the defect showed signs of suffering with a poor Safranin-O staining, an intense cell proliferation and clusters of chondrocyte-like cells in the transitional and radial zones (Fig. 3).

In group 2, repair tissue covered almost completely the defect. Its thickness was similar to that of healthy surrounding cartilage, and contained a great amount of small rounded cells. Tissue showed an intense Safranin-O staining of ECM, and a partial integration with the surrounding healthy cartilage in the radial zone, although a clear transition between them was evident in the transitional and superficial zones. Moreover, we observed that surrounding cartilage flowed over the defect. In many areas, we observed numerous clusters of chondrocyte-like cells in the transitional and radial zones and some clefts that deepened to the radial zone. In these areas, Safranin-O staining was poor. Tidemark was observed in some zones, but it did not appear completely restored. Healthy surrounding cartilage showed an intense cell proliferation with formation of cell clusters (Fig. 4).

In group 3, we observed a thin layer of repair tissue that covered almost completely the defect, although it was thinner than normal

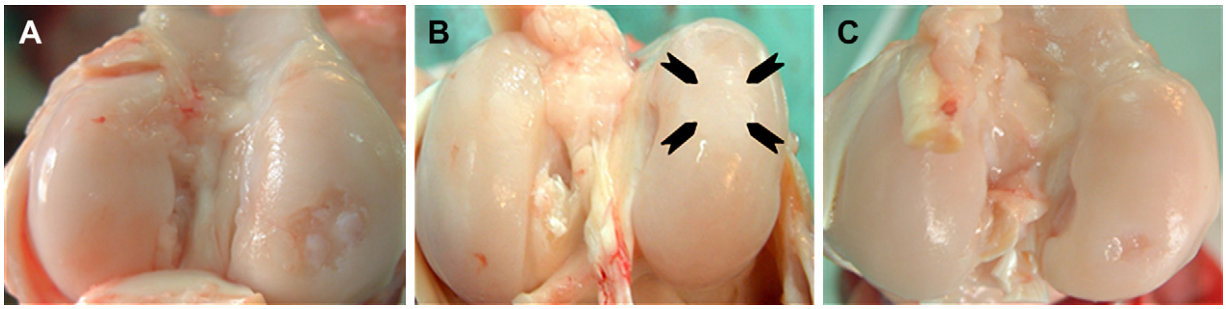

Fig. 2. Macroscopic appearance of three samples from experimental groups: A. Group 1 (microfractures): healing tissue is much thinner than normal surrounding cartilage and more evident in the areas over and surrounding the microfractures; B. Group 2 (microfractures + PRP gel): repaired tissue (arrows) shows similar thickness, appearance and colour to that of normal cartilage, and a good integration with healthy surrounding cartilage; C. Group 3 (microfractures + liquid PRP): repaired tissue covers almost completely the defect, but is thin, poorly regular and consistent, especially in the inner part of the defect.

Table III
Results of macroscopic evaluation

ICRS score (Mean ± SD)				Differences between means		*P* value	95% CI	
Group 1 ($N=5$)	Group 2 ($N=5$)	Group 3 ($N=5$)	Group 4 (controls) ($N=15$)	Groups	Value		Lower limit	Upper limit
3.6 ± 1.34	11 ± 1	5.8 ± 1.09	12	1–2	−7.4*	0.000	−9.2095	−5.5905
				1–3	−2.2*	0.015	−4.0095	−0.3905
				1–4	−8.4*	0.000	−10.2095	−6.5905
				2–3	5.2*	0.000	3.3905	7.0095
				2–4	−1.0	0.416	−2.8095	0.8095
				3–4	−6.2*	0.000	−8.0095	−4.3905

* Difference between means is statistically significant for $P < 0.05$.

surrounding cartilage with a clear demarcation respect to healthy tissue. Tissue showed a good Safranin-O staining, a great amount of chondrocyte-like cells well-organized in columns, and rare clusters in the in the transitional and radial zone. However, in some areas we found a fibrocartilaginous tissue with poor Safranin-O staining. Tidemark was almost completely absent in the defect. Normal surrounding cartilage showed an intense cell proliferation with cluster formation (Fig. 5).

Histological scoring assessment showed a significant difference between groups for the following variables: matrix staining (Matx) ($P = 0.001$), structural integrity (Stru) ($P = 0.001$), surface architecture (SurfH) ($P = 0.006$), defect filling (FillH) ($P = 0.0001$), basal integration (Basl) ($P = 0.0001$) and total score ($P = 0.0001$). *Post-hoc* analysis revealed that PRP groups (group 2 and 3) had a significantly greater mean score than that of microfracture group (group 1) for all the above mentioned variables. For FillH and Basl variables, mean scores of group 2 were also significantly greater than those of group 3 (Table V).

Power analysis

Post-hoc power analysis based on the primary outcome (histological score) showed an effect size of 1.98 and a power ($1 - \beta$) of 0.78.

Discussion

Although treatment algorithm for chondral injuries is not well-defined, microfracture technique can be actually considered as a first-line option and is frequently used as standard treatment against which other cartilage repair or reconstruction procedures, such as autologous osteochondral graft or chondrocyte implantation, are compared[3,5,29–32].

A recent systematic literature review on clinical efficacy of microfractures showed a high consensus between studies for the rate of clinical improvement, while high variability was observed for repair cartilage fill grade[32]. Although almost all reviewed studies reported a short-term clinical improvement, longer follow-up showed consistent functional decline and increased failure rate[32].

Several experimental *in-vitro* studies have demonstrated that BMSCs have a high chondrogenic differentiation potential[33–36], and that cells originating from subchondral plate are similar to human adult mesenchymal stem cells derived from bone-marrow aspirates[17]. However, *in-vivo* studies showed that BMSCs, both suspended or implanted over scaffolds, repaired cartilage defects by formation of fibrocartilaginous tissue[37–43]. Similarly, histological studies on human biopsies or animal models confirmed that microfracture procedure cannot restore normal hyaline cartilage, but usually leads to the formation of fibrocartilage or hybrid repaired tissue with a variable amount of type II collagen[1–6]. For this reason, many researchers made efforts to improve mechanical and biochemical quality of repaired tissue obtained after microfractures.

Some authors showed that biologic or synthetic biocompatible scaffolds could improve chondrogenic differentiation of BMSCs derived from microfractures[44–48]. *In-vitro* studies showed that chondrogenesis can be also stimulated by some GFs, such as TGF-beta superfamily[8,9,13,16,17], BMP 2, 4, 6, and 7[12,14,15], and FGF 2 and 18[10,11]; other authors combined the use of scaffolds and GFs to improve cartilage repair after microfractures[49–53].

Potential efficacy of PRP to repair chondral defects was initially supposed for its high content in chondrogenic GFs[54]. Gaissmaier *et al.*[20] showed that addition of human platelet supernatant to monolayer cultures of chondrocytes induced cell proliferation but de-differentiation towards a fibroblast-like phenotype. Subsequently, Akeda *et al.*[21], in an experimental study on porcine model, showed that chondrocytes cultured in a medium containing 10% of autologous PRP underwent a small but significant increase in proliferation and anabolic activity. Furthermore, recent *in-vitro* studies showed that PRP increase proliferation and chondrogenic differentiation of BMSCs[22,24]. Drengk *et al.*[23], in an experimental model similar to that used in the present study, showed that PRP had a primary effect on cultured chondrocytes, that maintained stable phenotype; however it revealed a smaller but consistent promotive effect on proliferation and chondrogenic differentiation of BMSCs. According to these *in-vitro* studies, PRP could effectively

Table IV
Results of biomechanical evaluation

Stiffness (indenter force: N) (Mean ± SD)				Differences between means		*P* value	95% CI	
Group 1 ($N=5$)	Group 2 ($N=5$)	Group 3 ($N=5$)	Group 4 (controls) ($N=15$)	Groups	Value		Lower limit	Upper limit
2.92 ± 0.81	4.98 ± 1.54	3.60 ± 0.85	5.81 ± 1.44	1–2	−2.06	0.068	−4.2429	0.1229
				1–3	−0.674	0.813	−2.8569	1.5089
				1–4	−2.89*	0.008	−5.0729	−0.7071
				2–3	1.386	0.302	−0.7969	3.5689
				2–4	−0.83	0.702	−3.0129	1.3529
				3–4	−2.216*	0.046	−4.3989	−0.0331

* Difference between means is statistically significant for $P < 0.05$.

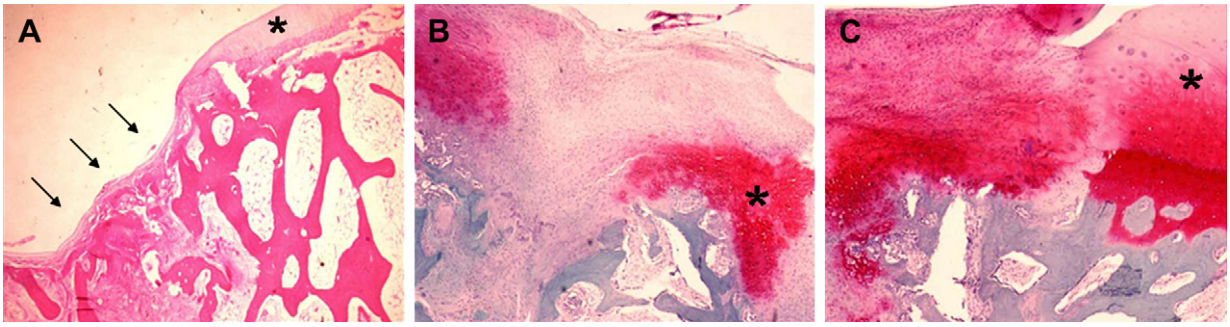

Fig. 3. Histological sections of group 1 (microfractures): A. Repaired tissue (arrows) was much thinner than normal cartilage (asterisk) (H–E staining. Original magnification 12.5×); B. In some areas, corresponding to microfracture holes, a great amount of rounded cells were embedded in a fibrous and poorly organized ECM. In those areas, some spots of intense Safranin-O staining could be seen (asterisk). (Safranin-O staining. Original magnification 40×); C. Repaired tissue (left) was partially integrated with the surrounding healthy cartilage (asterisk), which showed signs of suffering with a poor Safranin-O staining, and cluster formation (Safranin-O staining. Original magnification 100×).

promote cartilage repair; however, the only *in-vivo* study on the chondrogenic effect of PRP was performed in an extra-articular environment[55] Authors implanted a PRP gel used as carrier for cultured autologous chondrocytes in the subcutaneous tissue of rabbits and observed cartilage formation into the PRP scaffold[55].

The primary hypothesis of the present study was that intra-articular use of PRP after microfractures can improve formation rate and quality of repaired tissue in comparison with microfracture procedure alone. The use of PRP to improve the outcome of microfractures has several potential advantages. First, PRP preparation is simple, rapid and not expensive, and can represent a valid therapeutic option to employ, as indicated, without any thorough preoperative planning. Further, it can be used as one-stage treatment as does not necessitate cultured cells, but acts on cells drawn into the defect from microfractures. Finally, it does not need any supplementary scaffold that, besides costs, implies a potential risk of inflammatory reaction, and a more complex surgical technique that is not always suitable for an all-arthroscopic procedure.

Nevertheless, subchondral perforations we performed, partially differed from standard microfracture procedure described by Steadman[56]. First, we used a 1.5-mm K-wire instead of dedicated arthroscopic awls, as, in our opinion, the tip of the commercially available awls was too large to perform multiple perforations in an about 0.5-cm^2 lesion. In fact, using a 1.5-mm K-wire, and leaving a distance of at least 1 mm between each hole and the margins of the defect, microfractures were separated by a 3-mm bone bridge, according to suggestions by Steadman[56]. Second, as subchondral bone of the distal medial condyle of sheep stifle is thinner than that of human knee[57], extending perforations to 5 mm in depth probably caused a deep penetration beyond the subchondral plate and into the marrow cavity, which can affect reparative response[58].

Results of the present study showed that, compared with isolated microfracture procedure, adjunctive use of PRP produced repair tissue that was more mechanically competent and histologically differentiated. Moreover, on comparing two different PRP preparations, we observed that solid gel applied over the chondral defect was more effective than liquid solution injected into the joint, determining better mechanical and histological results, although without a significant difference, except for defect filling and basal integration of repair tissue.

The use of fibrin glue was previously reported as carrier for chondrocytes and BMSCs[40,59–61]. *In-vitro* studies showed that chondrocytes seeded in fibrin glue produced high-quality cartilage[59–61]; however, Shao *et al.*[40] in an *in-vivo* study in rabbits, observed that BMSCs seeded in fibrin glue matrix leaded to formation of a poor-quality cartilage-like tissue. In the present study, we did not use fibrin glue as scaffold for cell seeding, but it was mixed to PRP to enhance adhesive capacity and consistence of gel, and reduce the risk of PRP dispersal into the joint. However, we did not test separately the effect of PRP gel and fibrin glue combined with microfractures, so that we cannot establish whether the improvement observed was caused by PRP or fibrin glue, or the combination thereof.

We did not observe macroscopic signs of inflammation within the joint, nor histological findings of cell reaction typical of immune response in the repair tissue associated with the use of xenogenic fibrin glue, as reported in other studies with different animal models[28].

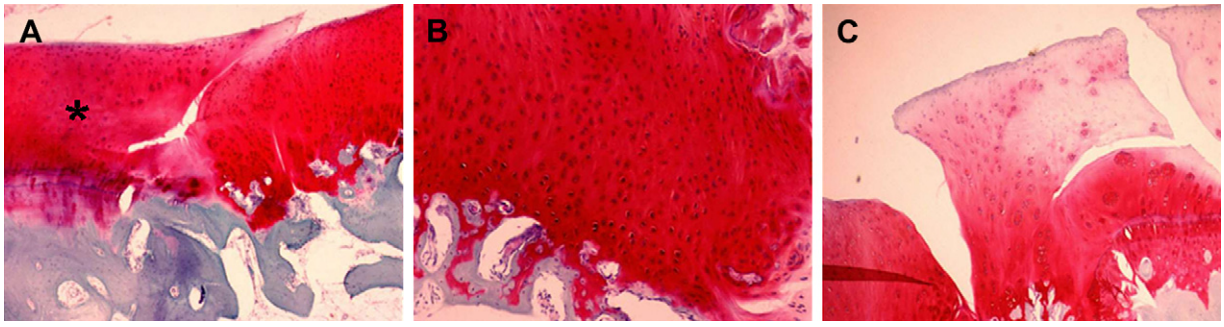

Fig. 4. Histologic sections of group 2 (microfractures + PRP gel): A. Thickness of repaired tissue (right) was similar to that of healthy surrounding cartilage (asterisk) with a partial lateral integration with the surrounding cartilage in the radial layer, although a clear transition between the two zones was still evident in the transitional and superficial layers. (Safranin-O staining. Original magnification 100×); B. Repaired tissue showed a great amount of small rounded cells and an intense Safranin-O staining of ECM. A calcified layer could be observed in some areas. (Safranin-O staining. Original magnification 200×); C. Numerous clusters of chondrocyte-like cells in the transitional and radial zones and some clefts that deepened to the radial zone, with a poor Safranin-O staining were observed in some areas. (Safranin-O staining. Original magnification 100×).

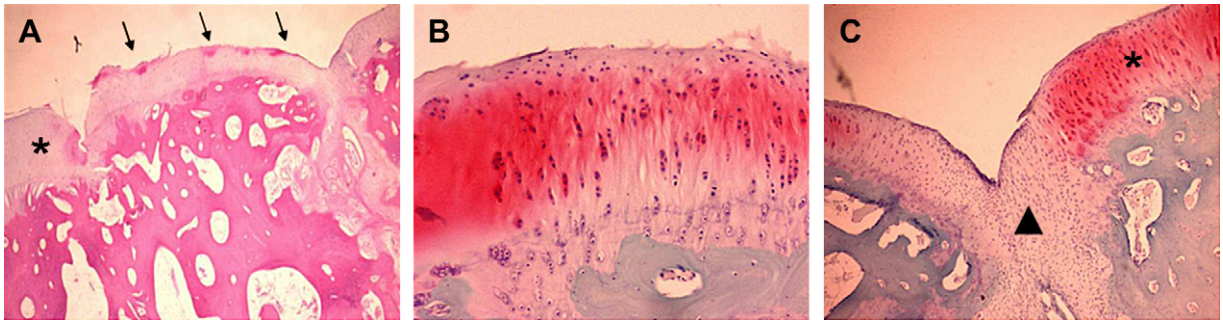

Fig. 5. Histologic sections of group 3 (microfractures + liquid PRP): A. A thin layer of repaired tissue (arrows) covered almost completely the defect, with a clear demarcation respect to healthy tissue (asterisk) (H–E staining. Original magnification 12.5×); B. A great amount of chondrocyte-like cells well-organized in columns, and rare clusters in the transitional and radial zone could be seen. ECM showed a good Safranin-O staining. (Safranin-O staining. Original magnification 100×); C. In some areas, a fibrocartilaginous tissue with poor Safranin-O staining could be observed (arrow head). Normal surrounding cartilage (asterisk) showed an intense cell proliferation with cluster formation. (Safranin-O staining. Original magnification 40×).

Although biomechanical results showed that repaired tissue obtained in the PRP-gel group was not significantly different from normal cartilage and stiffer than that observed in the other experimental groups, histological analysis revealed that none of experimental treatments produced hyaline cartilage. Repaired tissue observed in the PRP-gel group was better than that observed in the liquid-PRP group; however, there was a great amount of cell clusters and small but deep clefts, and lateral integration with

Table V
Results of histological evaluation (15 observations per sample)

Variables	Mean score ± SD			Differences between means		*P* value	95% CI	
	Group 1 ($N=5$)	Group 2 ($N=5$)	Group 3 ($N=5$)	Groups	Value		Lower limit	Upper limit
TI	2.3 ± 0.67	2.8 ± 0.71	2.4 ± 0.90	1–2	−0.5333	0.278	−1.3701	0.3034
				1–3	−0.1211	0.921	−0.8845	0.6424
				2–3	0.4123	0.353	−0.3083	1.1329
MATX	1.6 ± 0.84	2.9 ± 0.90	2.5 ± 0.69	1–2	−1.3167*	0.001	−2.1475	−0.4859
				1–3	−0.9263*	0.014	−1.6844	−0.1683
				2–3	0.3904	0.387	−0.3251	1.1058
STRU	1.6 ± 0.51	2.7 ± 0.62	2.8 ± 0.95	1–2	−1.1500*	0.004	−1.9655	−0.3345
				1–3	−1.2421*	0.001	−1.9862	−0.4980
				2–3	−0.0921	0.945	−0.7944	0.6102
CLUS	1.1 ± 0.31	1.2 ± 0.45	1.2 ± 0.45	1–2	−0.1500	0.689	−0.5928	0.2928
				1–3	−0.1632	0.591	−0.5672	0.2409
				2–3	−0.0132	0.996	−0.3945	0.3682
TIDE	1.5 ± 0.84	2 ± 0.95	1.5 ± 0.77	1–2	−0.5000	0.362	−1.3841	0.3841
				1–3	−0.0263	0.997	−0.8330	0.7803
				2–3	0.4737	0.294	−0.2877	1.2350
BFORM	2 ± 0.66	2.1 ± 0.83	1.6 ± 0.88	1–2	−0.1667	0.885	−1.0266	0.6933
				1–3	0.3158	0.593	−0.4689	1.1005
				2–3	0.4825	0.263	−0.2581	1.2230
SURFH	1.1 ± 0.31	1.9 ± 0.9	1.8 ± 0.56	1–2	−0.8167*	0.014	−1.4859	−0.1475
				1–3	−0.7947*	0.008	−1.4053	−0.1841
				2–3	0.0219	0.995	−0.5544	0.5982
FILH	1.6 ± 0.51	4 ± 0.85	2.6 ± 0.76	1–2	−2.4000*	0.000	−3.1730	−1.6270
				1–3	−1.0316*	0.003	−1.7369	−0.3263
				2–3	1.3684*	0.000	0.7028	2.0341
LATL	1.2 ± 0.42	1.5 ± 0.51	1.7 ± 0.87	1–2	−0.3833	0.407	−1.1060	0.3394
				1–3	−0.5368	0.130	−1.1963	0.1226
				2–3	−0.1535	0.820	−0.7759	0.4689
BASL	1.6 ± 0.51	3.2 ± 0.62	2.4 ± 0.90	1–2	−1.6500*	0.000	−2.4334	−0.8666
				1–3	−0.8737*	0.014	−1.5885	−0.1589
				2–3	0.7763*	0.021	0.1017	1.4510
INFH	1.8 ± 1.03	1.1 ± 0.57	1.6 ± 0.95	1–2	0.6333	0.228	−0.2897	1.5563
				1–3	0.1684	0.878	−0.6737	1.0106
				2–3	−0.4649	0.338	−1.2598	0.3299
Total	17.4 ± 3.23	25.8 ± 5.07	22.6 ± 4.64	1–2	−8.4333*	0.000	−13.1232	−3.7434
				1–3	−5.2316*	0.014	−9.5108	−0.9524
				2–3	3.2018	0.143	−0.8371	7.2406

* Difference between means is statistically significant for $P < 0.05$.

surrounding cartilage reported at macroscopic evaluation was not confirmed at histology. These findings can represent negative prognostic factors for long-term survival. Furthermore, presence of poorly stained areas at Safranin-O staining was indicative of limited metabolic cell activity and poor-quality extra-cellular matrix. Reparative response observed in the liquid-PRP group was better than that observed in the microfracture group at the histological evaluation; however, differences between the two groups were not significant at mechanical evaluation. It would be interesting to investigate if prolonged treatment with liquid PRP, by repeating intra-articular injections during first period after surgery, can improve the effects of platelet-derived GFs on reparative process.

One of the limits of PRP preparation is that method is not standardized. Several studies on the use of PRP for connective tissue repair reported discrepancies in centrifugation setting, quantity of blood harvested, and quantity of PRP obtained; moreover, a wide variability of GF concentration was observed[62–64]. We did not analyze GF content of PRP, but performed a preliminary study to optimize centrifugation setting that provided platelet concentration approximating fourfold that of entire blood. This concentration has been suggested as optimal for best efficacy of PRP[65]; however, it is still unclear if effects of PRP on cell proliferation and differentiation are dose-dependent, and related to platelet or GF concentration.

In the present study, we realized an experimental model of chronic isolated chondral defect, without joint instability. Although at the time of reparative treatment we refreshed the lesion, as usually performed during arthroscopic microfracture procedure, we supposed that chronic model would resemble intra-articular environment usually observed in clinical practice during treatment of this type of lesions. However, we did not quantified markers of cartilage degradation and inflammatory response associated to chronic chondral damage, and we do not know if reparative response can be influenced by timing of lesion.

We recognized some other shortcomings of the study. First, we did not perform a histological evaluation at time zero (before second surgery) to evaluate reparative response to induced full-thickness chondral injury. In fact, hand curettage of articular cartilage frequently removes the calcified cartilage layer, thus promoting tissue repair; moreover, it is difficult to distinguish macroscopically between noncalcified and calcified cartilage, and we did use any validated method, as suggested by Frisbie *et al.*[66], to assess retention or removal of calcified cartilage during first surgery. However, macroscopic appearance of the defects at 12 months after first surgery did not show a consistent basal repair. Second, follow-up was too short to evaluate the outcome of a treatment that has shown a consistent deterioration over time[32]. Third, we did not provide a direct proof of the chondrogenic effect of PRP, as the purpose of the study was limited to the evaluation of reparative response after microfractures with and without PRP. Finally, we did not verify retention of PRP gel into the defect; therefore, we do not know if an eventual loosening of PRP gel played a role in limiting the efficacy of this treatment.

In conclusion, the use of PRP combined with microfractures for the treatment of chronic full-thickness focal chondral defects of femoral condyle produced, at 6 months, a repair tissue showing better macroscopic, mechanical, and histological results than those observed after isolated microfractures. PRP gel was more effective than liquid injectable PRP. However, none of the experimental treatments produced hyaline cartilage.

Conflict of interest

No conflict of interest for all authors.

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