


# Steroids and Platelet-Rich Plasma as Coadjuvants to Microfracture for the Treatment of Chondral Lesions in an Animal Model: Can the Healing Be Enhanced?

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## Abstract

**Objective:** The aim of this study was to evaluate the contribution to hyaline cartilage regeneration of the microfracture (MFx) technique plus intraarticular betamethasone (BMS) or platelet-rich plasma (PRP). **Design:** Full-thickness chondral defects of  $3 \times 6 \text{ mm}^2$  were surgically performed in both femoral condyles of each knee in 13 New Zealand rabbits and then treated with MFx associated with intraarticular BMS or PRP. At 12 weeks postimplantation, the animals were killed and the condyles were characterized macroscopically, molecularly according to collagen type II and I gene expression (quantitative reverse transcriptase–polymerase chain reaction), and histologically (hematoxylin–eosin staining). For the latter, samples were scored using the International Cartilage Repair Society visual histological scale. Data of MFx/BMS-treated and MFx/PRP-treated condyles were compared against untreated, MFx-treated, or normal condyles without lesions. **Results:** Our macroscopic findings showed that in MFx/BMS-treated and MFx/PRP-treated groups, the defects were filled with an irregular, partially rough tissue similar to the MFx-treated group. No differences in the ratio between collagen type II versus collagen type I expression were observed among groups. Histological changes were observed between MFx/BMS-treated and MFx/PRP-treated groups versus untreated defects mainly in surface regularity and cell distribution. However, International Cartilage Repair Society score analysis did not support statistical differences between MFx/BMS-treated and MFx/PRP-treated groups versus MFx-treated group. **Conclusions:** These results provide evidence that the use of intraarticular BMS or PRP as coadjuvants to the microfracture technique in the treatment of acute chondral lesions is not associated with a significant improvement of hyaline cartilage regeneration.

## Keywords

microfracture, cartilage, betamethasone, platelet-rich plasma, tissue engineering

## Introduction

Chondral injuries are common and disabling lesions among athletes, especially during the practice of contact sports.<sup>1</sup> Because of its poor intrinsic potential for healing, mild cartilage injuries can lead to progressive lesions deteriorating the joint's capacity to absorb and to distribute mechanical loads, causing limitation of joint mobility and articular pain, and leading to high levels of disability, especially in athletes.<sup>2,3</sup>

Multiple strategies to prevent progression of these lesions and to treat the defects have been advocated, such as subchondral drilling, osteochondral grafting, and autologous chondrocyte implants. However, none of these strategies have been able to regenerate hyaline cartilage.<sup>4–6</sup>

The microfracture technique has been proposed as a first-line treatment option for smaller lesions (less than  $4 \text{ cm}^2$ ) because of its minimal invasiveness, technical ease, and high cost-effectiveness.<sup>7</sup> Several studies have described the use of this technique for the treatment of chondral injuries.<sup>1,8,9</sup> However, many of these studies have shown that

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the repair tissue is at best a fibrocartilage and that the clinical outcomes diminish after 2 years of follow-up.<sup>9</sup>

Since the principle of microfracture is to allow for mesenchymal stem cells (MSC) to migrate from the medullary cavity to the chondral defect, where they differentiate and generate a fibrocartilage tissue, the *in vivo* use of adjuvants that induce MSC chondrogenic differentiation could enhance the biology of the microfracture technique, leading to better and longer-term results and, it is hoped, to regenerate hyaline cartilage.

Among the potential adjuvants, steroids have already proven to induce chondrogenic differentiation<sup>10-12</sup> and to stimulate aggrecan gene expression in MSC.<sup>10,13</sup> Intraarticular betamethasone (BMS), a commonly used steroid in the clinical practice, although not routinely used in association with microfracture, could by these means improve the cartilage repair. It also has the advantages of a low cost, ease of availability, and relatively safety for using *in vivo*.

On the other hand, platelet-rich plasma (PRP) is a product derived from fresh whole blood that contains high concentrations of platelet and growth factors like platelet-derived growth factor and transforming growth factor- $\beta$ .<sup>14-16</sup> The advantages of the use of PRP as an adjuvant to microfracture are the sealant properties for the implant, the capacity to promote cellular implantation, and the presence of growth factors that are involved in MSC chondrogenic differentiation.<sup>14,15</sup>

The aim of this study was to evaluate, in the rabbit model, the contribution to hyaline cartilage regeneration in the treatment of full-thickness chondral defects with microfracture associated with intraarticular BMS or PRP.

## Methods

**Study design:** See **Figure 1**.

**Animals.** Femoral condyles ( $n = 52$ ) from 13 New Zealand male rabbits (3 months old, 2.5–3.5 kg) were used in this study. The animals were housed by themselves, at constant temperature and humidity, with a 12:12 hour light-dark cycle and with unrestricted access to a standard diet and water, in individual 40 cm  $\times$  40 cm  $\times$  60 cm cages. The research protocol was reviewed and approved by the Ethics Committee of the Faculty of Medicine of Clínica Alemana–Universidad del Desarrollo. All the procedures were carried out under aseptic conditions, using intramuscular anesthesia with ketamine (35 mg/kg), xylazine (5 mg/kg), and acepromazine (1 mg/kg). Enrofloxacin (10 mg/kg) and tramadol (4 mg/kg) were administered to all the animals preoperatively and up to 2 days after surgery.

**Chondral defect interventions.** Full-thickness chondral defects of 3 mm  $\times$  6 mm, as described by Hui *et al.*, were created in the weight-bearing area of both femoral condyles of each knee by making a lateral longitudinal parapatellar arthrotomy.<sup>17</sup> The animals were divided into five study

groups: condyle with lesion untreated group ( $n = 8$ ), condyle with lesion MFx-treated group ( $n = 8$ ), condyle with lesion MFx/BMS-treated group ( $n = 12$ ), and condyle with lesion MFx/PRP-treated group ( $n = 12$ ). An unlesioned, untreated group of condyles was used as a control group (normal group,  $n = 12$ ).

Less than 1-mm-diameter microfracture holes (five) penetrating the subchondral bone plate were performed into the periphery of the defect first and then into the center of the defect as previously described, leaving 1- to 2-mm bone bridges between the holes.<sup>7</sup> The arthrotomies were then closed by layers with a 4,0 Vycril suture in the deep layer and 3,0 Nylon to the skin.

After surgery, the animals were kept in separate cages and allowed to walk freely with full weight bearing and without immobilization. No complications were observed and no animal had to be killed before the end of the study. Twelve weeks postintervention, the rabbits were killed using an intravenous overdose of pentobarbital and the condyles were dissected and analyzed.

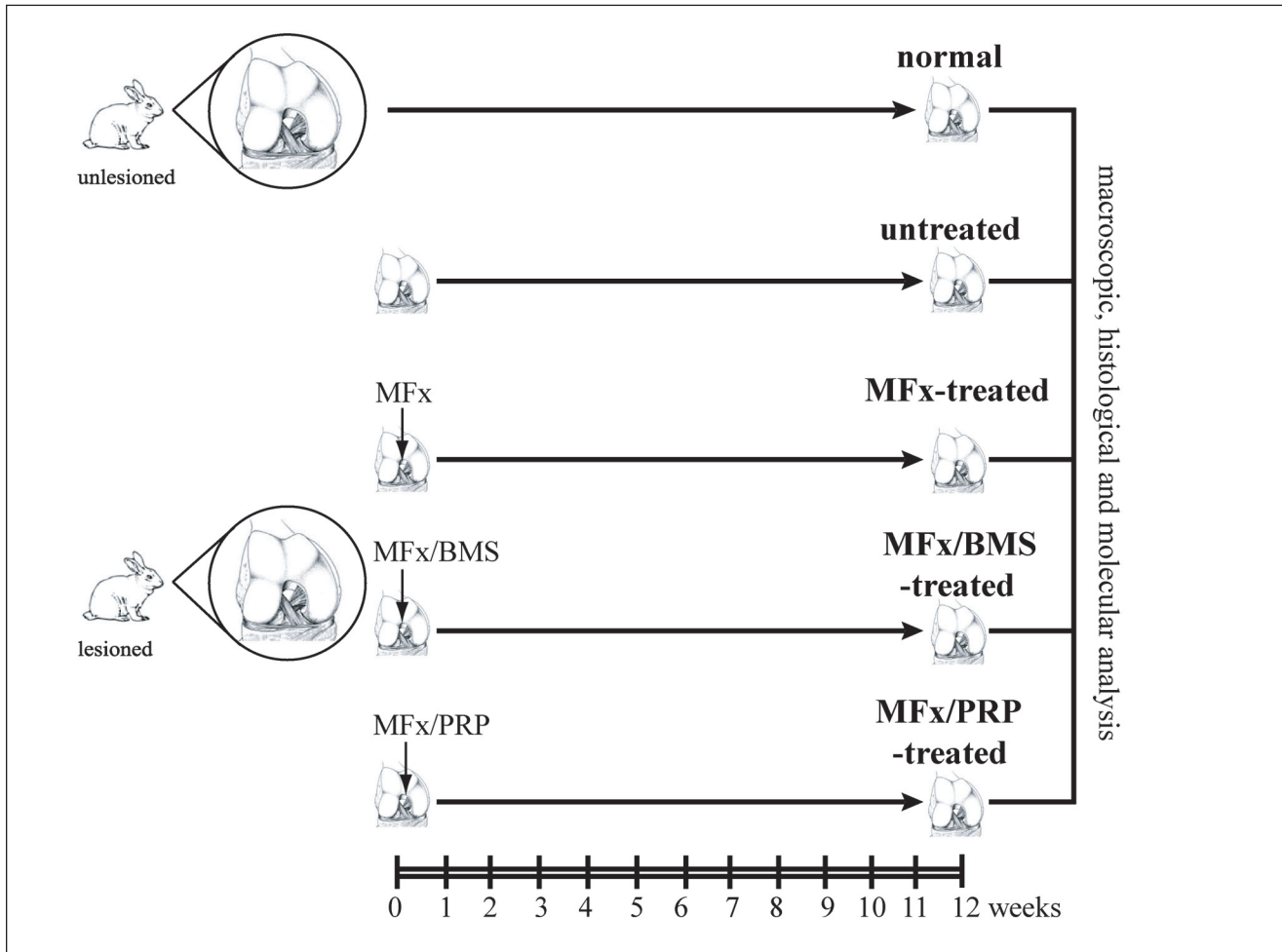
**Preparation of PRP.** To obtain PRP, 10 mL of venous blood was drawn from each cell donor rabbit by using a 20-mL sterilized syringe. The blood was subjected to centrifugation for 10 minutes at 1,800 rpm and the obtained supernatant was transferred to another tube. The supernatant was subjected to centrifugation for 10 minutes at 3,600 rpm to obtain platelet-poor plasma (PPP) and PRP. The top layer was discarded.

PRP aliquots were analyzed by flow cytometry. Platelets were identified according to their size (forward scatter) and granularity (side scatter). The mean platelet count of rabbit PRP was  $1.5 \times 10^6$  platelets/ $\mu$ L. Thus, rabbit PRP was comparable to a standard active human PRP ( $1 \times 10^6$  platelets/ $\mu$ L).<sup>16</sup>

Approximately 1 mL of PRP was aspirated and put into another tube. Subsequently, 0.15 mL of 10% CaCl<sub>2</sub> was combined with the PRP 5 minutes before injection, for activation purposes. In addition, 0.5 mL of the activated PRP was instilled in each knee directly on top of the treated chondral defect, allowing it to coagulate for 1 minute.

**Steroid injection.** In the condyle with lesion MFx/BMS-treated group, 0.4 mg of betamethasone sodium phosphate (Laboratorio Chile, Chile) was injected intraarticularly once, immediately after closure of the arthrotomy. The usual dose recommended for a 70 kg human adult is between 6 and 9 mg of intraarticular betamethasone.<sup>18</sup> The rabbits weighed approximately 1/20 of the human body weight equivalent, so the dose was adjusted accordingly to the weight, as previously reported by Albano *et al.*<sup>19</sup>

**Macroscopic evaluation.** All of the condyles were observed directly, immediately after sacrificing the animals, and the characteristics of the lesion were recorded describing color, surface texture, and the presence or absence of exposed subchondral bone.



**Figure 1.** Study design. Surgical full-thickness chondral defects were established in both femoral condyles of each knee of adult New Zealand male rabbits. Defects were not treated (untreated group,  $n = 8$ ) or treated with microfracture alone (condyle with lesion MFx-treated group,  $n = 8$ ), microfracture plus betamethasone (condyle with lesion MFx/BMS-treated group,  $n = 12$ ), or microfracture plus platelet-rich plasma (condyle with lesion MFx/PRP-treated group,  $n = 12$ ). Twelve weeks after the initiation of the interventions, condyles were analyzed macroscopically, molecularly, and histologically. Untreated condyles, without lesions, were used as controls (normal group,  $n = 12$ ).

**Collagen type II and collagen type I mRNA quantification.** Twenty-six condyles, representing the different study groups were frozen and pulverized with a mortar. The powder thus obtained was dissolved in 1 mL of Trizol reagent (Invitrogen, Carlsbad, CA) and incubated for 30 minutes at room temperature. The total RNA was extracted with 300  $\mu\text{L}$  of chloroform and precipitated with 500  $\mu\text{L}$  of isopropanol for 24 hours at  $-20^\circ\text{C}$ . After washing with 75% ethanol, the RNA pellet was dried and dissolved in 11  $\mu\text{L}$  of DMPC-water. RNA concentration was determined spectrophotometrically at 260 nm. First-strand cDNA was reverse transcribed from 1  $\mu\text{g}$  of RNA, incubating with 1  $\mu\text{g}$  oligodT (Tib Molbiol, Berlin, Germany) for 10 minutes at  $70^\circ\text{C}$  at a final volume of 12  $\mu\text{L}$ . A mix (8  $\mu\text{L}$ ) containing 1  $\mu\text{L}$  of dNTPs 10 nM (Fermentas Life Sciences, Hanover, MD),

buffer M-MLV 1x (Tris-HCl 50 nM [pH 8.3], KCl 7 mM,  $\text{MgCl}_2$  3 mM, DTT 10 mM), 1  $\mu\text{L}$  of M-MLV reverse transcriptase 200 U/ $\mu\text{L}$  (Promega, Madison, WI), RNasin inhibitor 1x (Promega) and DMPC-water was added to a final volume of 20  $\mu\text{L}$ . Polymerase chain reaction (PCR) amplification was carried out in a reaction volume of 10  $\mu\text{L}$  containing 0.15  $\mu\text{g}$  of cDNA, 0.8  $\mu\text{L}$   $\text{MgCl}_2$  25 mM, each of the sense and antisense primers at 0.5  $\mu\text{M}$  (Genética y Tecnología Ltda, Santiago, Chile) and 1  $\mu\text{L}$  FastStart DNA Master SYBR Green I 10x (FastStart Taq DNA polymerase, dNTPs, SYBR Green I,  $\text{MgCl}_2$  10 nM) (Roche). Rabbit collagen type II (col2a1), collagen type I (col1a1), and gapdh primers used are shown in **Table 1A**. PCR conditions are described in **Table 1B**. For each sample, the threshold cycle at which there was the first detectable increase in

**Table 1.** Primers, Amplicons (A), and PCR Conditions (B)

A						
Gene	GenBank Access no.	Sense (5'→3')	Antisense (5'→3')	Amplicon		Reference
				Size (bp)	Temperature (°C)	
Collagen II	D83228	ACACTGCCAA CGTCCAGATG	GTGATGTTCT GGGAGCCCTC	64	85	16
Collagen I	S61950	TCCCTTCCTTG ATATTGCACCT	GGCCAACGTCC ACATAGAATTC	68	82	16
gapdh	L23961	GGTGAAGGTC GGAGTGAACG	AGTTAAAAGCA GCCCTGGTGAC	65	83	16

B			
Gene	Annealing (temperature; time)	Extension (temperature; time)	Cycle no.
Collagen II	62 °C; 5 s	72 °C; 5 s	45
Collagen I	56 °C; 5 s	72 °C; 5 s	45
gapdh	57 °C; 10 s	72 °C; 5 s	45

Note: PCR = polymerase chain reaction.

fluorescence (Ct value) was determined as the cycle number at which all samples were in the exponential phase of amplification. The ratio between collagen type II and collagen type I was used as an indicator of hyaline versus fibrous cartilage.<sup>20,21</sup>

**Histological analysis.** Condyles ( $n = 26$ ) were fixed in formalin, decalcified in nitric acid, and embedded in paraffin. Sagittal cross sections 10  $\mu$ m thick were cut through the tissue.

Sections were stained with hematoxylin/eosin. The samples were blindly evaluated by a trained pathologist and graded according to the International Cartilage Repair Society (ICRS) histological assessment of cartilage repair.<sup>22</sup> The scale was composed of six categories assigning a score to the most prominent feature on each sample.

**Statistical analysis.** The unit of analysis used in this study was each femoral condyle ( $n = 52$ ). The estimated number of femoral condyles required per group based on an average difference between groups of 25% and a power of 90% was a minimum of 7.

PCR results (means  $\pm$  standard deviations) were analyzed by ANOVA and Tukey posttest to prove statistical differences among groups. ICRS scores (medians) were analyzed by Mann-Whitney  $U$  test to prove statistical differences. Values of  $P < 0.05$  were considered statistically significant.

## Results

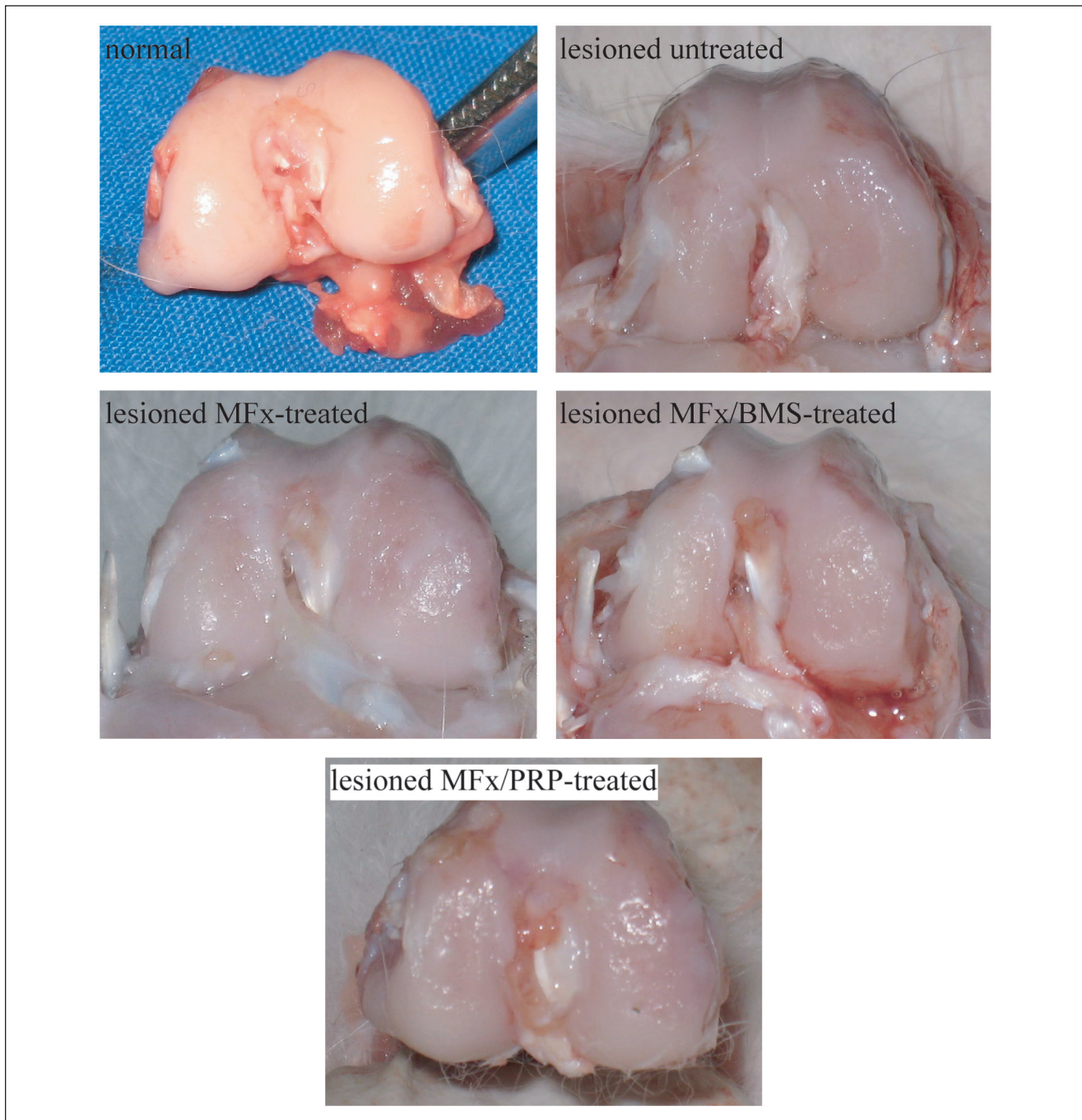
### Macroscopic Observations

As expected, the normal group presented a smooth, shiny, semitransparent tissue. At 12 weeks after intervention, in

the untreated group the newly formed cartilage tissue was thin, irregular, and rough-looking with zones of bone exposure. In the condyle with lesion MFX-treated group, the tissue was irregular and opaque. The condyle with lesion MFX/BMS-treated group showed differences in gross observation, ranging from samples with tissue slightly irregular, soft, and with similar color to the surrounding cartilage to others with irregular tissue and bone exposure. Finally, in the condyle with lesion MFX/PRP-treated group, the same variability observed in the condyle with lesion MFX/BMS-treated group was present, with a high frequency of bone exposure (**Fig. 2**).

**Histological analysis.** As seen in **Figure 3**, normal condyles presented a regular surface with a hyaline matrix and chondrocytes organized in columns. Condyle with lesion-untreated group showed a mostly irregular surface with a hyaline matrix mixed with fibrocartilage and disorganized cell distribution. In the condyle with lesion MFX-treated group, 50% of the samples presented an irregular cartilage surface, with a predominantly disorganized cell distribution. The condyle with lesion MFX/BMS-treated group showed mainly a regular surface with differences in cell distribution criteria, ranging from samples with a disorganized cell distribution to others with mixed columnar to cluster distribution. In the condyle with lesion MFX/PRP-treated group, the surface of the tissue was mostly irregular, with a hyaline matrix mixed with fibrocartilage in some areas, but with a more organized tissue in cell distribution criteria than control.

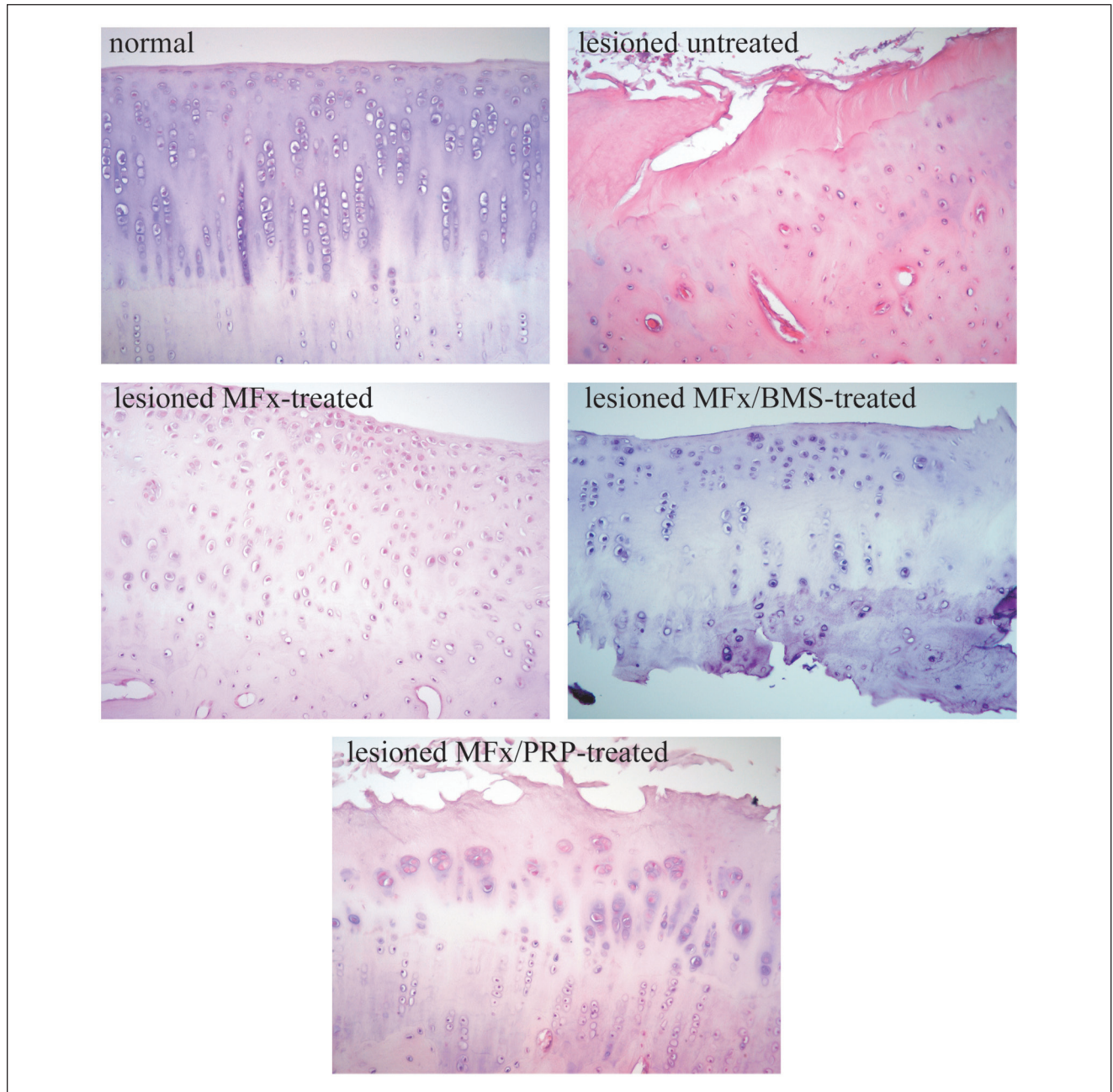
Quantitative analysis of histological data showed that cell population viability and cartilage mineralization were unaltered in all groups. Nonetheless, surface, matrix, cell



**Figure 2.** Macroscopic observations after chondral defects intervention. Macroscopical characterization of the tissue formed 12 weeks after intervention of full-thickness chondral defect. Data shown are representative of each group ( $n = 8-12$ ).

distribution and subchondral bone varied among them (**Fig. 4**). In these four categories, the normal group presented an ICRS score of 3. When compared with the normal group, the condyle with lesion-untreated group showed a significant reduction in scores in all categories (3 vs. 0, 3 vs. 2, 3 vs. 0, and 3 vs. 2, respectively;  $P < 0.05$ ). The condyle with

lesion MFx/BMS-treated group showed a median score for surface category higher than that of the untreated group (3 vs. 0, respectively) and equal to that of the normal group (3 vs. 3, respectively). In the former case, no statistical significance was proven. The condyle with lesion MFx-treated group showed no significant improvement in all the criteria

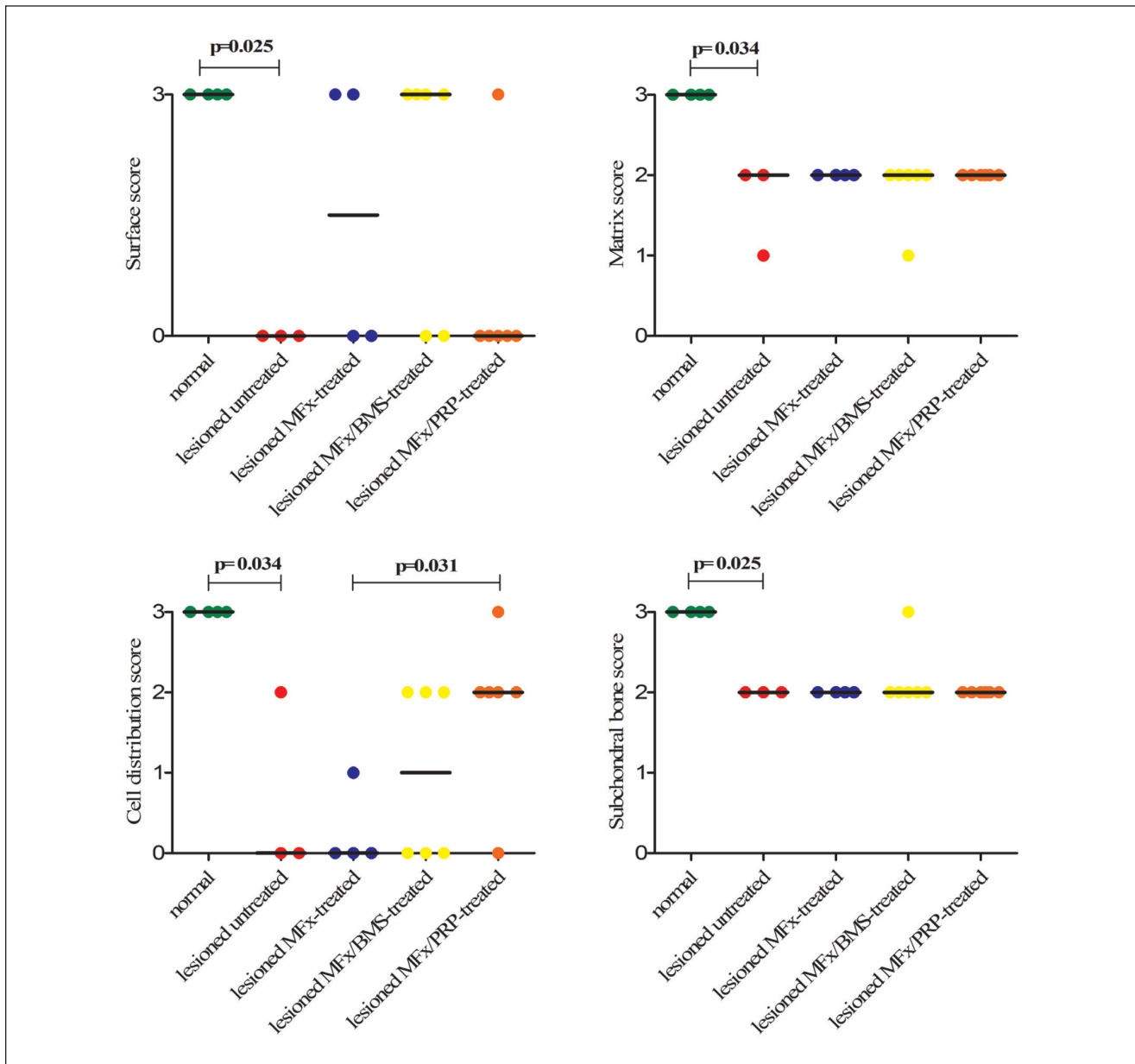


**Figure 3.** Qualitative histological analysis after chondral defects intervention. Cartilage histological analysis six weeks after the initiation of the interventions. Data shown are representative of 4 sections (20x) per animal, for at least 3 animals.

evaluated when compared against the untreated group (1.5 vs. 0, 2 vs. 2, 0 vs. 0, and 2 vs. 2, respectively). However, in the condyle with lesion MFx/PRP-treated group, a significant improvement in cell distribution score was observed compared with the condyle with lesion MFx-treated group (2 vs. 0, respectively;  $P < 0.05$ ).

*Hyaline versus fibrous cartilage markers expression.* The ratio between collagen type II expression versus collagen type I expression is used as an indicator to distinguish

between hyaline versus fibrous cartilage.<sup>21</sup> Accordingly, this ratio was near to 1.5 in the normal group ( $1.47 \pm 0.16$ ) and decreased near to 1 in the condyle with lesion untreated group ( $1.12 \pm 0.12$ ,  $P < 0.05$ ) (**Fig. 5**). In the case of condyle with lesion MFx/BMS-treated group, the mean value was  $1.04 \pm 0.15$ . In the condyle with lesion MFx/PRP-treated group, the mean was  $1.13 \pm 0.11$ . No statistically significant difference was observed between these groups and the condyle with lesion MFx-treated group.

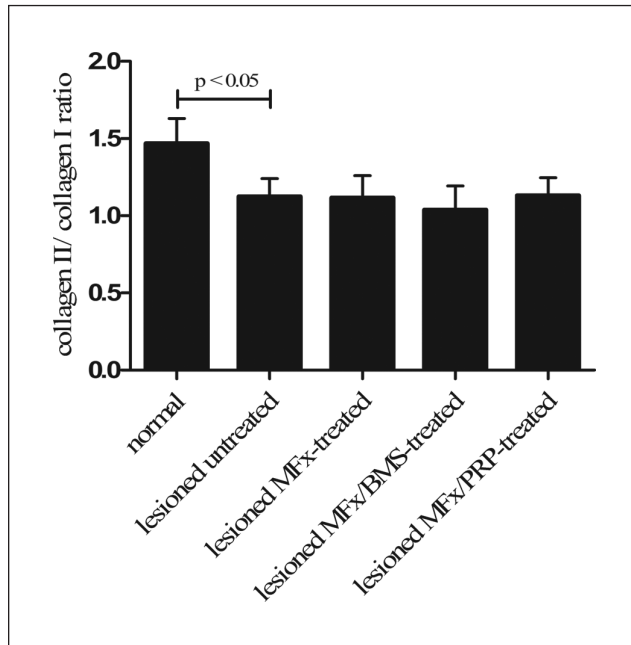


**Figure 4.** Quantitative histological analysis after chondral defects intervention. ICRS histological assessment of cartilage repair score was determined 12 weeks after the initiation of the interventions. For cell population viability and cartilage mineralization categories, the score was 3, in all groups (not shown). For the shown categories, circles correspond to individual scores and the horizontal line, to group median score. Normal ( $n = 4$ ), condyle with lesion untreated ( $n = 4$ ), condyle with lesion MFx-treated ( $n = 4$ ), condyle with lesion MFx/BMS-treated ( $n = 6$ ), and condyle with lesion MFx/PRP-treated ( $n = 6$ ). Only significant  $P$  values are shown.

## Discussion

In the present study, we evaluated the treatment of acute full-thickness chondral defects with microfracture associated with intraarticular betamethasone or platelet-rich plasma. The use of these adjuvants, however, did not contribute significantly to cartilage repair compared to microfracture treatment alone after 12 weeks.

Although an improvement in the macroscopic observation was observed in the groups that received BMS or PRP, the more sensible analysis of histology and molecular biology did not support this. In fact, within the criteria evaluated according to the histological ICRS score, only the cell distribution item showed a significant improvement with the use of PRP associated with microfracture compared with the microfracture treatment alone. However,



**Figure 5.** Hyaline versus fibrous cartilage markers expression after chondral defects intervention. Cartilage relative gene expression of collagen II versus collagen I at 12 weeks after the initiation of the interventions. Data correspond to group mean  $\pm$  standard deviation. Normal untreated condyle, without lesion ( $n = 8$ ), condyle with lesion untreated ( $n = 4$ ), condyle with lesion MFX-treated ( $n = 4$ ), condyle with lesion MFX/BMS-treated ( $n = 6$ ), and condyle with lesion MFX/PRP-treated ( $n = 6$ ). Only significant  $P$  values are shown.

this is only one item among many parameters evaluated by this score.<sup>22</sup>

The contribution to hyaline cartilage regeneration of the microfracture technique has been evaluated in several studies.<sup>8,9,23,24</sup> Although good results have been reported mainly in young patients with small defects, the initial improvement tends to deteriorate over 18–24 months.<sup>25</sup> Mithoefer *et al.* reported a deterioration of clinical outcomes over time in 47% of athletes after an initial improvement.<sup>26</sup> This could be explained because of the less durable fibrocartilage produced after microfracture and the poor integration of this tissue with the native cartilage. This fact is also consistent with our results, since in the condyle with lesion MFX-treated group the newly formed tissue that filled the defect was fibrocartilage. In search of a “more” hyaline-like cartilage, we evaluated the contribution of BMS or PRP to cartilage regeneration. Although good clinical results have been reported with the use of PRP in degenerative chondral defects,<sup>27</sup> we could not demonstrate, at the molecular and histological levels, any benefits in association with microfracture. In fact, the available studies on the efficacy of PRP in the treatment of chondral injuries are insufficient to predict the real clinical benefit of this therapy.<sup>28</sup>

At the present time, the only study evaluating the effect of PRP on microfracture for the treatment of chronic full-thickness chondral lesions in animals, performed in sheep by Milano *et al.*,<sup>29</sup> concluded that this treatment produced, at 6 months, a repair tissue showing better macroscopic, mechanical, and histological results than those observed after isolated microfractures and that a PRP gel was more effective than a liquid injectable PRP. Milano *et al.* used larger animals (sheep) and chronic 8-mm defects, as opposed to our study, where we used smaller animals and acute defects and our PRP had a higher comparative platelet count, so both studies are not totally comparable. Nevertheless, none of the experimental treatments produced hyaline cartilage. One pitfall of the study by Milano *et al.* is that they did not perform a molecular analysis of the generated cartilage. In our study, the molecular analysis was consistent with the histology, reaffirming that the repair tissue in the different treatment groups was not significantly different when using betamethasone or PRP as coadjuvants to microfracture.

This suggests that more thorough studies, assessing the concentration, method of activation, and administration of PRP components are necessary before the widespread use of this therapy in patients with chondral lesions.

Regarding intraarticular steroids, although frequently used to treat various joint diseases, their long-term effects on cartilage remain controversial both at the experimental and clinical levels.<sup>19,30,31</sup> In the condyle with lesion MFX/BMS-treated group, no improvements on the histological or molecular parameters were observed when compared to the condyle with lesion MFX-treated group, suggesting that betamethasone in association with microfracture has no effect on the regeneration of hyaline cartilage. This conclusion is only valid for the dosage used in this study (one intraarticular dose of 0.4 mg betamethasone) and not for other schemes, doses, or steroids that might effectively modify the joint microenvironment in order to facilitate cartilage regeneration.

In addition to lack of benefits from betamethasone or PRP on microfracture, we also did not find any deleterious effects in the parameters evaluated in this study. This suggests that it is probably safe to use these therapies intraarticularly in living animals.

A limitation of our study could be attributed to the fact that no significant differences were found, in general, among treatment groups. This could be due to a limited number of animals or because the betamethasone dose or PRP concentration was not adequate to promote chondral healing. However, in our study, the preparation of PRP was performed by using the protocol described by Wu *et al.*<sup>32</sup> In their research, Wu *et al.* successfully reconstructed an injectable cartilage graft by using autologous PRP and cultured chondrocytes, without performing a platelet count, so there is evidence that PRP prepared with this protocol

should at least be effective to allow for chondrocyte and chondral matrix growth. Moreover, the platelet count in our PRP was comparable to a standard human PRP.<sup>16</sup>

Another limitation of this study is that we only evaluated morphological parameters and no functional data were analyzed. However, our aim was to describe the effect of BMS and PRP in the biological quality of the tissue generated after microfracture and not their impact in joint function.

The strong points of this study include the use of an easy and reproducible method, and using the same anesthetic procedure, operative technique, and perioperative management protocol. The fact that the histological and molecular results were similar also suggests that our analysis was accurate and represents a “double check” of the generated tissue.

In the present work, we were unable to demonstrate a beneficial effect of intraarticular administration of betamethasone or platelet-rich plasma as coadjuvants of microfracture in order to regenerate hyaline cartilage in acute full-thickness chondral lesions in a rabbit model. Further investigation is necessary to prove if this will also be valid in the clinical setting.

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### Declaration of Conflicting Interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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