Multiple Platelet-Rich Plasma Injections Versus Single Platelet-Rich Plasma Injection in Early Osteoarthritis of the Knee

An Experimental Study in a Guinea Pig Model of Early Knee Osteoarthritis

Devendra K. Chouhan,* MS Ortho, Mandeep S. Dhillon,* MS, FAMS, FRCS,
Sandeep Patel,*† MS Ortho, DNB Ortho, Tungish Bansal,‡ MS Ortho,
Alka Bhatia,§ MD, and Himanshu Kanwat,‖ MS Ortho
*Investigation performed at Department of Orthopaedics, Post Graduate Institute
of Medical Education and Research, Chandigarh, India*

Background: Platelet-rich plasma (PRP) has emerged as the forerunner among disease-modifying treatment options for early osteoarthritis (OA) of the knee. However, no consensus is available regarding optimum dosing schedules.

Purpose: To determine whether multiple injections of PRP (3 injections) provide better short-term and long-term results than a single injection of PRP in a guinea pig model of knee OA.

Study Design: Controlled laboratory study.

Methods: 36 Dunkin-Hartley guinea pigs (weighing ~600-800 g) were chosen for this study. The animals were assigned to group DC (disease control group), group G1 (single-PRP group), and group G2 (multiple-PRP group) containing 10, 10, and 12 animals, respectively. Another 4 animals were used for preparation of allogenic PRP. Groups G1 and G2 received 1 and 3 injections of PRP, respectively, at weekly intervals in the intervention knee while the contralateral knee was injected with normal saline. Group DC received no intervention in either knee. Half of the animals from each group (subgroups DC.3, G1.3, and G2.3) were sacrificed at 3 months, and the remaining half (subgroups DC.6, G1.6, and G2.6) were sacrificed at 6 months after intervention. Both knee joints were harvested for histological assessment of articular cartilage and synovium.

Results: The mean synovial scores for groups G1 and G2 were significantly better than those for group DC at 3 months. No difference was found between groups G1 and G2 at 3 months. At 6 months, group G2 had significantly better mean synovial scores than group G1 and group DC. The mean articular cartilage scores in group G2 were significantly better than those in group DC at 3 months. However, at 6 months, no significant difference was found among any of the groups in terms of mean articular scores.

Conclusion: Both single and multiple injections of PRP exert similar anti-inflammatory effects on the synovium in the short term. However, this effect is sustained in the long term only for multiple injections. Multiple injections of PRP exert a chondroprotective effect, but only in the short term. This effect is not seen with a single injection of PRP.

Clinical Relevance: This study provides insight into the histological basis for the superiority of multiple injections of PRP.

Keywords: platelet-rich plasma; osteoarthritis knee; guinea pig; animal experimental study

Platelet-rich plasma (PRP) has emerged as the forerunner among treatment options for early osteoarthritis (OA) of the knee. Multiple studies have suggested that PRP may play a role in modifying the course of disease through its positive actions on both the synovium[4,35] and the cartilage.[1,31] The safety and efficacy of PRP in knee OA have been proven in various clinical studies.[12,14,32,34] However, the dosage of PRP has varied among studies, ranging from single to multiple injections at varying intervals. The ideal PRP dosage remains a matter of debate and a topic of ongoing research. Patel et al[32] reported in a clinical study that a single injection may be as good as 2 injections. In contrast, studies by Görmeli et al[15] and Kavadar et al[18] have supported the use of multiple injections. To address this issue, we designed an experimental study to evaluate
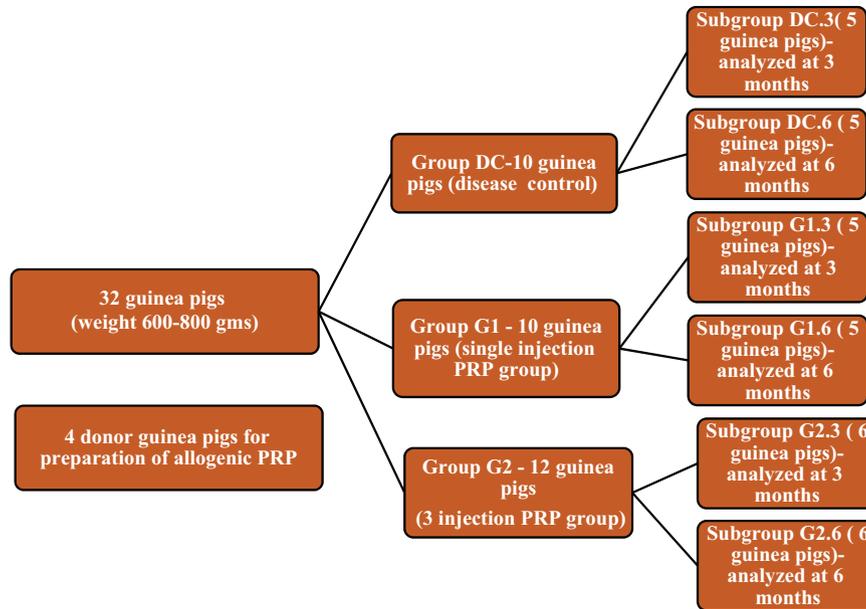
Figure 1. Grouping of animals for the study. PRP, platelet-rich plasma.

the histopathological changes in synovium and cartilage between a single injection and multiple injections of PRP in a guinea pig model of spontaneous OA knee.

METHODS

Selection of Study Participants

This study involved 36 Dunkin-Hartley guinea pigs, 5 months of age and weighing approximately 600 to 800 g. Appropriate clearances were obtained from the institutional animal ethics committee. The animals were divided into a study group (32 animals) and a donor group (4 animals). The 4 animals in the donor group were used for preparation of allogenic PRP. At each time point, 1 donor animal was sacrificed for preparation of PRP. Therefore, 1 animal was used to prepare PRP for the single PRP injection group, and 3 animals (1 at the time of each injection) were used in the multiple-PRP injection group. Study animals were divided into 3 groups, as described below and shown in Figure 1. Both knees of each animal were considered separately, and thus a total of 64 knees were available for study. For each animal, 1 knee was randomly designated the control knee and the contralateral knee was designated the intervention knee.

- Group DC: disease control group (n = 10 animals). This group was further divided into subgroups of 5 animals each (subgroups DC.3 and DC.6), which were sacrificed at 3 and 6 months, respectively. Neither knee received an intervention.
- Group G1: group receiving a single injection of PRP (n = 10 animals), divided into subgroups G1.3 and G1.6 (n = 5 each), sacrificed at 3 and 6 months, respectively. A single PRP injection was given in the intervention knee, and the same amount of saline was injected in the control knee.
- Group G2: group receiving 3 injections of PRP (n = 12 animals), divided into subgroups G2.3 and G2.6 (n = 6 each), sacrificed at 3 and 6 months, respectively. These animals received 3 injections of PRP in the intervention knee and 3 injections of saline in the control knee, at weekly intervals.

We chose Dunkin-Hartley guinea pigs for our study because this animal model provides a spontaneously occurring, weight-induced knee OA model. This model has a temporal progression and histopathological characteristics that are similar to human disease.[5,16,21] Knee OA in guinea pigs is weight induced and progresses with increase in weight.[5] Moreover, the progression and appearance of joint disease in guinea pigs are influenced by well-known risk factors of human disease, such as age, weight gain, sedentary lifestyle, and mechanical loading.[5,16,21]

[†]Address correspondence to Sandeep Patel, MS Ortho, DNB Ortho, Department of Orthopaedics, Post Graduate Institute of Medical Education and Research, Chandigarh, 160012, India (email: sandeepdrpatelortho@gmail.com).
[*]Department of Orthopaedics, Post Graduate Institute of Medical Education and Research, Chandigarh, India.
[‡]Department of Orthopaedics, Maulana Azad Medical College and Lok Nayak Hospital, New Delhi, India.
[§]Department of Experimental Medicine and Biotechnology, Post Graduate Institute of Medical Education and Research, Chandigarh, India.
[‖]Department of Orthopaedics, All India Institute of Medical Sciences, New Delhi, India.

TABLE 1
Mean Synovitis Score and Mean Total Articular
Score of Control Knees of All 6 Subgroups[a]

S. No	Subgroup	Synovitis Score	Total Articular Score
1	DC.3	3.4 ± 0.894	8.6 ± 3.209
2	G1.3	3 ± 2.828	8.8 ± 1.924
3	G2.3	5 ± 3.209	10.17 ± 3.488
4	DC.6	6.2 ± 1.643	12.6 ± 1.140
5	G1.6	5.6 ± 1.817	13 ± 3.000
6	G2.6	6.17 ± 2.683	13.17 ± 1.472

[a]Values are expressed and mean ± SD. There was no significant difference between groups at each time point. DC.3, disease control group, sacrificed at 3 months; DC.6, disease control group, sacrificed at 6 months; G1.3, single injection of platelet-rich plasma, sacrificed at 3 months; G1.6, single injection, sacrificed at 6 months; G2.3, multiple injections, sacrificed at 3 months; G2.6, multiple injections, sacrificed at 6 months; S. No, serial number.

The Osteoarthritis Research Society International (OARSI) is an organization dedicated to research in OA. We used the OARSI recommended scoring systems for synovitis and articular cartilage scoring. The synovitis scoring system includes parameters like synovial hyperplasia (scored 0-2), villous hyperplasia (0-3), and cellular infiltration (0 or 5). A higher score implies more synovitis. OARSI recommends the modified Mankin score for articular cartilage. It consists of 5 components: articular cartilage integrity (scored 0-8), proteoglycan content (0-6), cellularity (0-3), tidemark integrity (0-1), and osteophytes (0-3). A higher score here also implies more articular damage. The mean synovitis scores and mean total articular scores of the control knees of groups DC, G1, and G2 at 3 months and 6 months are shown in Table 1. The control knees were comparable with each other ($P >$ .05 for all comparisons) at 3 months and 6 months. This implies that the disease progression (OA) was similar in all of the groups. Thus, we compared the intervention knees of these groups to look for any differences.

Preparation of PRP

We used allogenic blood obtained from donor animals via cardiac puncture[30] for preparation of PRP. A double-spin technique was used to prepare PRP. Acid citrate dextrose was used as an anticoagulant.[23] The protocol for obtaining PRP was similar to that used in a previous study,[16] and the PRP used was leukocyte-poor PRP. For quality control, a platelet count was performed on the PRP obtained. The normal guinea pig platelet count reference range is 6.2 ± $0.2 \times 10^5/mm^3$.[11] The PRP prepared had a platelet count approximately 3 times the baseline.[10,16]

Instilling PRP in Guinea Pig Knees

Calcium chloride was used for activation of PRP. One part 0.025 M $CaCl_2$ was mixed with 4 parts PRP to obtain activated PRP. PRP and other preparations were instilled in the knee joint (the one chosen for therapy) of the guinea

pig through the inferior patellar tendon with a 26-gauge needle and syringe (100 μL), and same amount of normal saline was injected into the control knee of the same animal.[16,37]

Analysis of Synovium and Articular Cartilage Histology

Subgroups DC.3, G1.3, and G2.3 were sacrificed at 3 months, and joints were harvested. The same procedure was carried out at 6 months for subgroups DC.6, G1.6, and G2.6. The animals were euthanized by an intraperitoneal injection of pentobarbital (100 mg/kg). The harvested tissue was fixed in 10% formaldehyde and sent for histological processing immediately. The collected tissue samples were processed according to the guidelines established by the histopathology initiative.[21] Hematoxylin and eosin stains were used for synovial analysis, and 4-μm sections were obtained. For analysis of articular cartilage, 5-μm sections were stained with toluidine blue stain. Synovial inflammation and articular cartilage degeneration were graded according to semiquantitative scores given and validated for use in guinea pigs by OARSI.[21]

Statistics

The collected data were subjected to statistical analysis with SPSS v 20 software. Descriptive statistics for the 6 subgroups (mean ± SD) were calculated for weight, platelet counts in whole blood and PRP, synovitis scores, and articular cartilage scores. The data were confirmed to be normally distributed. Weight parameters, total synovial scores, and total articular scores were compared among the groups by use of 1-way analysis of variance and post hoc Tukey HSD tests. A P value less than .05 was considered significant.
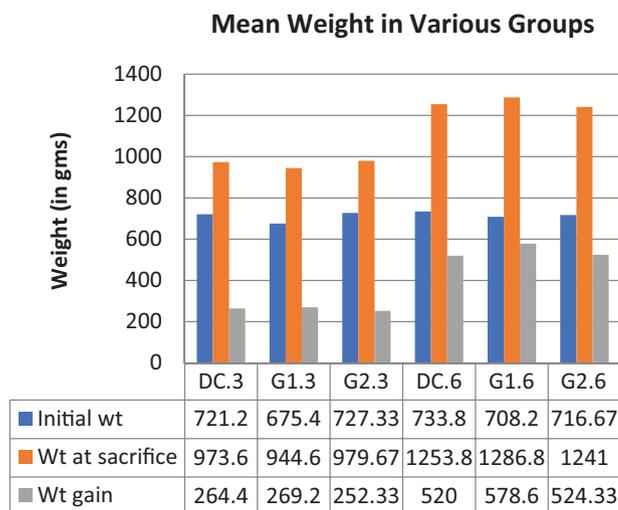
RESULTS

The mean initial weight, weight at sacrifice, and mean weight gain for each subgroup are presented in Figure 2. No statistically significant difference was found in the initial weight of groups DC, G1, and G2. The weight gain and weight at sacrifice were also comparable in subgroups DC.3, G1.3, and G2.3 and in subgroups DC.6, G1.6, and G2.6 ($P >$ .05 for all comparisons) (Table 2). As the initial weight and weight gain correlated with the severity and grading of OA in this model, the groups were homogeneous and thus comparable. The platelet count in whole blood used for PRP preparation was 585,000/μL for group G1 and 565,000/μL for group G2 (mean of 3 injections). The mean platelet count in injected PRP was 1,875,000/μL for group G1 and 1,820,000/μL for group G2 (~3 times the baseline counts).

Mean synovitis scores for the intervention knees of the subgroups are presented in Figure 3. Scores for subgroups G1.3 and G2.3 were significantly better than scores for subgroup DC.3 (P = .011 and .002, respectively) (Table 2). However, no significant difference was found between intervention knees of subgroups G1.3 and G2.3 (P = .991) at 3 months. Subgroup G2.6 had significantly better scores than subgroups DC.6 and G1.6 at 6 months (P = .000 and

TABLE 2
P Values for Comparison Among Intervention Knees of the Subgroups[a]

Subgroup 1 for Comparison	Subgroup 2 for Comparison	*P* Values for Initial Weight	*P* Values for Weight Gain	*P* Values for Mean Synovial Score	*P* Values for Mean Articular Score
DC.3	G1.3	.510	≥.999	.011	.442
DC.3	G2.3	≥.999	.996	.002	.005
G1.3	G2.3	.329	.980	.991	.337
DC.6	G1.6	.947	.461	.562	≥.999
DC.6	G2.6	.989	≥.999	.000	.146
G1.6	G2.6	≥.999	.497	.005	.108

[a]DC.3, disease control group, sacrificed at 3 months; DC.6, disease control group, sacrificed at 6 months; G1.3, single injection of platelet-rich plasma, sacrificed at 3 months; G1.6, single injection, sacrificed at 6 months; G2.3, multiple injections, sacrificed at 3 months; G2.6, multiple injections, sacrificed at 6 months.

Mean Weight in Various Groups

	DC.3	G1.3	G2.3	DC.6	G1.6	G2.6
■ Initial wt	721.2	675.4	727.33	733.8	708.2	716.67
■ Wt at sacrifice	973.6	944.6	979.67	1253.8	1286.8	1241
■ Wt gain	264.4	269.2	252.33	520	578.6	524.33

Figure 2. Graph presenting the mean initial weight, mean weight at sacrifice, and mean weight gain for the subgroups. DC.3, disease control group, sacrificed at 3 months; DC.6, disease control group, sacrificed at 6 months; G1.3, single injection of platelet-rich plasma, sacrificed at 3 months; G1.6, single injection, sacrificed at 6 months; G2.3, multiple injections, sacrificed at 3 months; G2.6, multiple injections, sacrificed at 6 months. SDs for initial weight in the groups were as follows: DC.3, 29.719 g; G1.3, 46.420 g; G2.3, 47.865 g; DC.6, 24.356 g; G1.6, 34.215 g; and G2.6, 34.944 g. SDs for weight gain were DC.3, 22.952 g; G1.3, 32.322 g; G2.3, 71.930 g; DC.6, 63.887 g; G1.6, 39.310 g; and G2.6, 47.731 g.

.005, respectively). However, no significant difference was seen between subgroups DC.6 and G1.6 at 6 months. These results suggest that multiple injections provided better reduction in mean synovial scores at 3 months, which persisted at 6 months, compared with the disease control group. In contrast, a single injection was superior to control only at 3 months, and the effects were not pronounced at 6 months, suggesting decreasing efficacy (Figure 3).

The mean total articular scores of intervention knees for each subgroup are presented in Figure 4. At 3 months, the mean total articular score was significantly better for subgroup G2.3 compared with subgroup DC.3 (*P* = .005) (Table 2). However, no significant difference was found between subgroups DC.3 and G1.3 (*P* = .442) and between subgroups G1.3 and G2.3 (*P* = .337). At 6 months, the mean total articular scores for subgroups DC.6 and G1.6 (*P* ≥ .999), subgroups DC.6 and G2.6 (*P* = .146), and subgroups G1.6 and G2.6 (*P* = .108) were not significantly different. This suggests that multiple injections of PRP might exert a chondroprotective effect in the short term but not in the long term. This effect may not be seen with a single injection of PRP (Figure 4).

DISCUSSION

Although many previous studies have obtained some degree of consensus on the safety and efficacy of PRP in the OA knee, one of the unresolved issues with the use of PRP in early OA seems to be the frequency of administration.[9,12,14,32,34] The dosing schedule of PRP is not clear yet and is often debated. Various experimental studies used either multiple injections[2,24,25] or single injections[19,22] and were able to demonstrate the positive effect of PRP on histological modulation and other objective analyses. Our study was designed to investigate whether 3 injections of PRP are superior to a single injection of PRP and to determine their time-dependent efficacy. To study the effect objectively, we evaluated articular cartilage and synovial histological features, which are the 2 major components of OA. Anti-inflammatory effect[6,29,35] and chondral remodeling[33,36] have been postulated to be the 2 most important mechanisms of PRP action in the OA knee. Thus, we chose to objectively evaluate synovial and articular cartilage histological features for this study. We found no significant differences among groups DC, G1, and G2 in terms of initial weight. Similarly, the mean weight gains for the subgroups were comparable at 3 and 6 months. This implies that these groups were also comparable in terms of OA. To date, no in vivo experimental study has compared a single injection versus multiple injections of PRP. A few clinical studies compared PRP dosages. Patel et al,[32] in a randomized controlled trial, noted

Mean Total Synovial Score

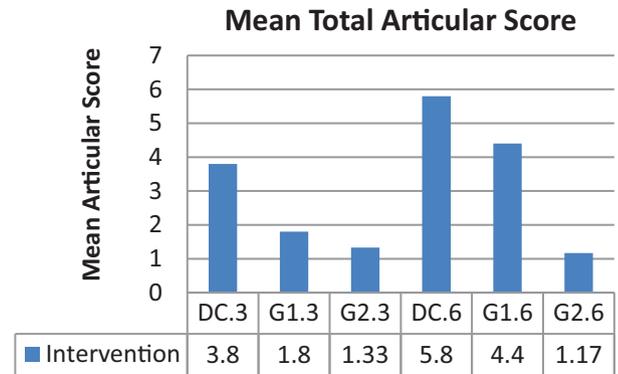|	DC.3	G1.3	G2.3	DC.6	G1.6	G2.6
■ Intervention	3.8	1.8	1.33	5.8	4.4	1.17
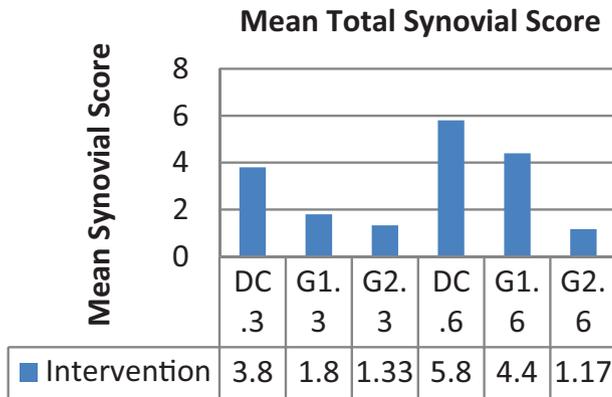
Figure 3. Graph presenting the mean total synovial scores in the intervention knees of subgroups of the study. DC.3, disease control group, sacrificed at 3 months; DC.6, disease control group, sacrificed at 6 months; G1.3, single injection of platelet-rich plasma, sacrificed at 3 months; G1.6, single injection, sacrificed at 6 months; G2.3, multiple injections, sacrificed at 3 months; G2.6, multiple injections, sacrificed at 6 months. SDs in the score for the intervention knee were as follows: DC.3, 0.447; G1.3, 1.304; G2.3, 1.033; DC.6, 2.168; G1.6, 1.517; and G2.6, 0.894.

Mean Total Articular Score

	DC.3	G1.3	G2.3	DC.6	G1.6	G2.6
■ Intervention	3.8	1.8	1.33	5.8	4.4	1.17

Figure 4. Graph presenting the mean total articular scores in the intervention knees in the subgroups of the study. DC.3, disease control group, sacrificed at 3 months; DC.6, disease control group, sacrificed at 6 months; G1.3, single injection of platelet-rich plasma, sacrificed at 3 months; G1.6, single injection, sacrificed at 6 months; G2.3, multiple injections, sacrificed at 3 months; G2.6, multiple injections, sacrificed at 6 months. SDs in the score for the intervention knee were as follows: DC.3, 3.050; G1.3, 1.304; G2.3, 1.366; DC.6, 2.864; G1.6, 2.000; and G2.6, 1.643.

no significant difference between the Western Ontario and McMaster Universities Osteoarthritis Index scores of groups treated with 1 or 2 injections of PRP at 6 months after injection. In another randomized controlled trial, Görmeli et al[15] noted significantly better pain and functional scores in knees treated with 3 injections of PRP compared with a single injection of PRP or hyaluronic acid. In a randomized controlled trial comparing 1, 2, and 3 injections of PRP, Kavadar et al[18] concluded that 2 and 3 injections of PRP were significantly better than a single injection in terms of pain and functional scores at 6 months. Based on the above clinical studies, it appears that multiple injections are either equal to or superior to a single injection of PRP. A preclinical in vitro study by Moussa et al[27] also supported the concept of dose-dependent improvement in chondrogenesis with PRP. However, it is still not clear why multiple injections of PRP are more effective than a single injection. Moreover, in clinical practice, multiple injections increase patient morbidity, laboratory burden, and treatment cost.

We used allogenic PRP in our study. Multiple studies have supported the use of allogenic PRP in animals and have shown that it is safe and reliable and provides more consistent results with respect to concentration of platelets.[17,38] Allogenic PRP also has been shown to be nonimmunogenic and efficacious.[17] We were able to eliminate inter- and intra-animal variations by using a uniform and consistent product. The platelet count in PRP injections used was approximately 3 times the baseline and hence within desirable limits.[28]

In OA, the most common symptom for which medical attention is sought is pain. Pain in OA has been attributed to such factors as changes in the articular cartilage,

synovial inflammation, joint effusion, subchondral cysts, and osteophyte formation.[13] Synovitis is an important cause of pain in OA, and the severity of synovitis relates to the severity of pain.[7,8] The positive effect of PRP on synovium has been noted by a number of in vitro studies.[6,29,35] PRP has been known to decrease synovial inflammation by decreasing an interleukin-1–mediated increase in matrix metalloproteinase (MMP) 1, 3, and 13, which are inflammatory and catabolic.[3,4] PRP also increases the secretion of hyaluronic acid and positively influences angiogenesis.[4,35]

Liu et al[25] studied the effect of PRP application in a rabbit knee OA model induced with intra-articular papain injection. The investigators administered 10 injections of autologous PRP at weekly intervals; the controls were untreated individual animals. Liu et al showed a significantly increased erythrocyte sedimentation rate and interleukin β1 levels in the serum of control samples compared with intervention samples. Histological analysis showed that the Mankin synovitis score was significantly increased in control samples compared with intervention samples at 2, 4, 6, and 10 weeks after PRP injection. Using a rat model, Khatab et al[20] demonstrated less pain, less synovial thickness, and higher concentrations of anti-inflammatory markers (CD163 and CD 206) at 3 weeks after 3 PRP injections. Thus, these studies show the reduction in knee joint inflammation with PRP application. However, none of these studies had an evaluation period beyond 3 months, and thus the long-term effects of PRP on the inflammatory milieu of the joint are not clear. In our study, synovitis was analyzed histologically (Figure 5) using the Pelletier score, which has been validated for use in guinea pigs by OARSI.[21] At 3 months, the mean synovitis scores were significantly better in both the single-PRP and multiple-PRP groups compared with the disease control group ($P < .05$). However, no
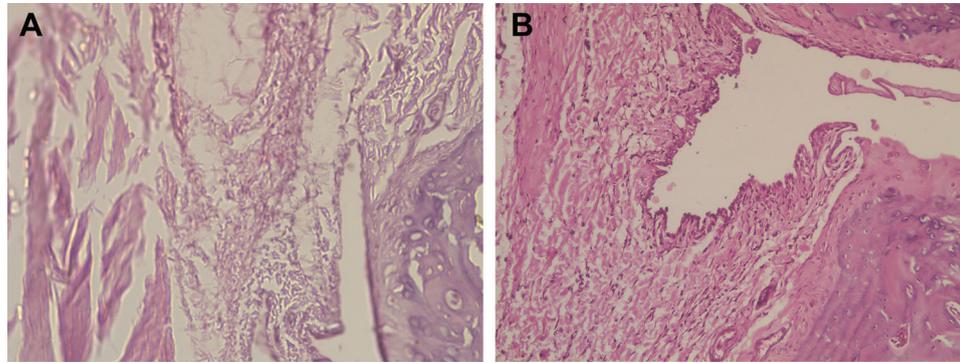
Figure 5. Hematoxylin and eosin staining. (A) Synovial morphological characteristics of a knee treated with multiple injections of platelet-rich plasma with minimal cellular infiltration and no synovial hyperplasia at 6 months. (B) Synovial morphological characteristics of a knee from the single-injection group showing notable cellular infiltration and synovial hyperplasia at 6 months.
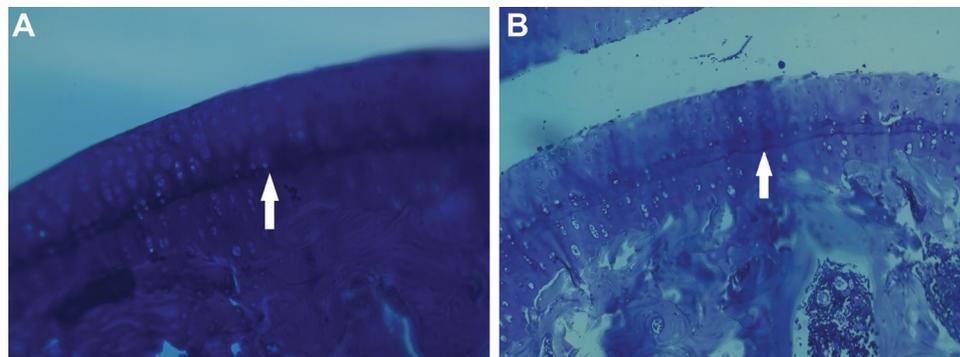
Figure 6. Toluidine blue stain for cartilage. (A) Knee treated with multiple injections of platelet-rich plasma at 3 months showing no loss of proteoglycan, no fissures, and no duplication of tidemark (white arrow) and with normal cellularity. (B) Knee from the disease control group, showing patchy loss of proteoglycan, superficial fissures, and duplication of tidemark (white arrow) with regions of hypercellularity at 3 months.

significant difference was found between single-PRP and multiple-PRP subgroups at 3 months. This means that both single and multiple injections of PRP are effective in decreasing synovial inflammation in the short term, and their effects are comparable in the short term. These results corroborated those of proponents of a single injection of the PRP in the OA knee.[26,32] However, at 6 months (long term), histological synovitis grades of the single-PRP group and the disease control group were comparable. The multiple-PRP group showed an extended anti-inflammatory effect compared with the single-PRP group and disease control group.

Our findings are in line with those of Liu et al,[25] who used 10 injections of PRP and noted a decrease in synovial inflammation in the short term. Kanwat et al[16] demonstrated similar findings: 3 injections of PRP were effective in decreasing synovial inflammation in both the short term (3 months) and the long term (6 months). These results may provide a histological basis for the reduction of pain and improvement in quality of life after intra-articular PRP injections, as observed in various clinical studies. The findings also correlate with the randomized controlled

trial by Patel et al,[32] where a single injection of PRP provided pain relief and improved functional score over short periods of time, with the best scores observed at 3 months.

Several in vitro studies have shown that PRP has a positive effect on cartilage through stimulation of lubrication, synthesis of superficial zone protein,[33] and inhibition of inflammatory process in chondrocytes.[36] Moussa et al,[27] in an in vitro study, highlighted the increased chondrogenesis with increasing doses of PRP. In our study, the mean articular cartilage score was significantly lower in the multiple-PRP group compared with the disease control group at 3 months (Figure 6). However, no significant difference was found between the single-PRP group and disease control groups at 3 months. The scores in the intervention subgroups were comparable among all subgroups at 6 months. This shows that multiple injections of PRP might exert a chondroprotective effect in the short term, which may not persist in the long term. The short-term beneficial effects of multiple injections of PRP on the cartilage have been shown by other authors[24] in rabbit and rat models[2] of OA. Using a rabbit model, Kwon et al[22] showed the chondroprotective effects of a single injection of

PRP, evidenced by better histological scores at 4 weeks. However, it is not clear whether these effects were sustainable for longer periods. Kazemi and Fakhrjou[19] used a single application of activated PRP gel and PRP fibrin clot for full-thickness articular defects in a canine model. They noted significantly better results at short term (12 and 16 weeks) but not in the long term (24 weeks). Of note, those investigators had the advantage of direct local application of PRP in the defects. Thus, the literature supports our findings that multiple injections of PRP may provide a chondroprotective effect for cartilage in the short term. This effect tends to decrease in the long term. This benefit is not seen with a single injection of PRP, which may principally act by reducing inflammation in the joint.

CONCLUSION

Long-term reduction in inflammation is better with multiple injections of PRP; moreover, a chondroprotective effect in the OA knee at short term is seen only with multiple injections of PRP.

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