

Original Article

Comparative evaluation of leukocyte- and platelet-rich plasma and pure platelet-rich plasma for cartilage regeneration in a cartilage defects model in rabbits

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Abstract: The increased level of leukocytes in leukocyte- and platelet-rich plasma (L-PRP) may activate NF- κ B pathway via interleukin 1 β (IL-1 β) and tumor necrosis factor α (TNF- α) delivery to counter the beneficial effects of growth factors on cartilage regeneration. The objective of this study is to substantiated that *in vivo*. In this study, L-PRP and pure platelet-rich plasma (P-PRP) were prepared for the measurement of concentrations of leukocytes and platelets by whole blood analysis and growth factors and pro-inflammatory cytokines delivery by enzyme-linked immunosorbent assay (ELISA), and the treatment of rabbit cartilage defects models. The outcomes were evaluated by the quantification of the concentrations of IL-1 β and prostaglandin E2 (PGE2) in the synovial fluid by ELISA, macroscopic observation according to International Cartilage Regeneration Society (ICRS) macroscopic scores, micro-computed tomography scanning, and histological observation according to ICRS histological scores. Our results demonstrated that L-PRP and P-PRP used were similar in platelet concentration and growth factors delivery, but L-PRP had significantly higher leukocyte concentration and released significantly more pro-inflammatory cytokines than P-PRP. Additional analysis demonstrated that pro-inflammatory cytokines delivery of platelet-rich plasma (PRP) formulations had significantly positive correlations with leukocyte concentration in them. Besides that, the results showed that L-PRP increased IL-1 β and PGE2 concentrations in the synovial fluid *in vivo*, which were significantly reversed by inhibition of NF- κ B activation using caffeic acid phenethyl ester (CAPE) intraperitoneal injections. With respect to cartilage regeneration in the rabbit cartilage defects, P-PRP scaffolds induced better effects than L-PRP scaffolds. Moreover, CAPE enhanced the effects of L-PRP scaffolds on cartilage regeneration to a level similar to that of P-PRP scaffolds, while inhibition of NF- κ B activation had no influence on P-PRP scaffolds treatment. These findings suggested that leukocytes in L-PRP might activate NF- κ B pathway via the delivery of IL-1 β and TNF- α to induce harmful effects on cartilage regeneration and result in the inferior effects of L-PRP compared with P-PRP. Hence, P-PRP may be more suitable than L-PRP for the treatment of cartilage defects.

Keywords: Leukocyte- and platelet-rich plasma, pure platelet-rich plasma, cytokines, rabbits, cartilage, NF- κ B

Introduction

Articular cartilage defects generally do not heal spontaneously because of the poor regenerative capability of this tissue. Numerous attempts have been made to induce cartilage regeneration, including autografts, allografts, and xenografts, in an order to prevent progression of cartilage defects to osteoarthritis [1]. However, these techniques suffer from either the inadequate tissue availability and associated donor site morbidity, or the risks of disease transmission and rejection [2]. Moreover, long-term outcomes of them remain variable [3]. Consequently, cartilage repair through tis-

sue engineering has become a promising alternative in the last decade.

Platelet-rich plasma (PRP) is an autologous derivative of whole blood that contains concentrated platelets. PRP is often considered as a safe, simple and cost-efficient delivery medium of growth factors that have beneficial effects on cartilage regeneration, such as platelet-derived growth factor (PDGF), transforming growth factor (TGF), insulin-like growth factor (IGF), fibroblast growth factor (FGF) and epidermal growth factor (EGF) [4-6]. Besides that, PRP can also be activated to form scaffolds capable of encapsulating chondrogenic cells for cartilage

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tissue engineering [7]. Therefore, the use of autologous PRP has gained popularity in the field of cartilage tissue engineering.

Despite the increasing use of PRP, there is no standardized protocol for PRP preparation, and different protocols may result in formulations that differ in leukocyte concentration [8]. It has been reported that leukocyte- and platelet-rich plasma (L-PRP) that has high leukocyte concentration contains increased levels of interleukin 1 β (IL-1 β) and tumor necrosis factor α (TNF- α) compared with pure platelet-rich plasma (P-PRP), which has low or undetectable leukocyte concentration [9]. IL-1 β and TNF- α have been described to inhibit the formation and enhance the degradation of extracellular matrix in chondrocytes [10, 11] and impair the chondrogenic capability of chondrogenic cells [12]. Besides acting independently, IL-1 β and TNF- α also act in synergy to induce more articular cartilage destruction *in vivo* than either cytokine alone [13]. The *in vitro* study by Cavallo and colleagues proved that L-PRP and P-PRP, which differed in leukocyte and pro-inflammatory cytokines concentrations, induced distinct effects on human articular chondrocytes with regard to cell proliferation, extracellular matrix deposition and catabolic genes expression [14]. Thus, the concentrated levels of IL-1 β and TNF- α in L-PRP may induce harmful effects on cartilage regeneration to counter the beneficial effects of growth factors. However, these findings have not yet been substantiated *in vivo*.

NF- κ B pathway is one of the major pathways involved in the harmful effects of IL-1 β and TNF- α on cartilage. IL-1 β and TNF- α activate NF- κ B pathway via the canonical pathway, which involves the phosphorylation and degradation of inhibitory κ B, and the release, phosphorylation, and nuclear translocation of NF- κ B heterodimers, which then activate expression of a wide range of catabolic genes, including IL-1 β , inducible nitric oxide synthase, cyclooxygenase-2 and matrix metalloproteinases [15]. Hence, L-PRP may activate NF- κ B pathway via increased concentrations of IL-1 β and TNF- α to induce inferior effects of it on cartilage regeneration compared with P-PRP. However, no relevant study has investigated that to date.

The objective of the current study is to evaluate the effects of L-PRP and P-PRP on cartilage regeneration and NF- κ B pathway in a rabbit

model, in order to develop an alternative method for the treatment of cartilage defects in clinical practice.

Materials and methods

The Animal Care and Use Committee of Shanghai East Hospital Affiliated to Tongji University approved the protocols of this study.

Preparation of L-PRP and P-PRP

Whole blood used for L-PRP and P-PRP preparation was collected from male mature New Zealand white rabbits (weighing 2.5-3.0 kg) through the central auricular artery and anticoagulated with acid-citrate dextrose solution A (ACD-A) at a ratio of 9:1 (v/v).

L-PRP was prepared using a double-spin centrifugation process as described elsewhere [16]. In brief, 10 ml of anticoagulated whole blood was added to a 15-ml centrifuge tube and spun at 250 \times g for 10 min. After the first-spin, the blood was separated into three components: erythrocytes at the bottom, buffy coat in the middle, and platelet-containing plasma at the top. The top and middle layers were transferred to a new tube and spun again at 1000 \times g for 10 min. After the second-spin, the supernatant platelet-poor plasma was discarded and the remaining 1 ml of plasma containing precipitated platelets was blended evenly and designated as L-PRP.

For P-PRP preparation, the first-spin at 160 \times g for 10 min was employed to separate 15 ml of anticoagulated whole blood into three components as above. Then, platelet-containing plasma was collected and transferred to a new tube while buffy coat and erythrocytes were discarded to remove leukocytes and erythrocytes. The collected platelet-containing plasma was spun again at 1000 \times g for 10 min. After discarding the supernatant platelet-poor plasma, the precipitated platelets were resuspended in the residual plasma to obtain 1 ml of P-PRP.

Quantification of leukocyte and platelet concentrations in whole blood and PRP formulations

Whole blood analysis was performed using an automatic hematology analyzer (XS-800i, Sysmex, Kobe, Japan) in the clinical laboratory

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of the hospital to determine leukocyte and platelet concentrations in the anticoagulated whole blood, L-PRP and P-PRP.

Preparation of PRP scaffolds and transplanted constructs

PRP scaffolds and transplanted constructs were prepared as described previously by Xie *et al.* [7]. In brief, 0.5 ml of α -MEM containing 10% FBS was mixed with 0.5 ml of L-PRP or P-PRP to prepare L-PRP or P-PRP scaffolds. For preparation of transplanted constructs, rabbit bone marrow-derived mesenchymal stem cells (rBMSCs) at the third passage were detached, centrifuged and resuspended in α -MEM containing 10% FBS and 1% antibiotics at a concentration of 2.0×10^6 /ml. Then, 0.5 ml of the rBMSCs-containing medium was used to prepare PRP scaffolds as above. The rBMSCs-encapsulated PRP scaffolds were used as transplanted constructs.

Quantification of cytokines released from PRP scaffolds

L-PRP and P-PRP scaffolds were prepared as above, incubated at 37°C in a humidified atmosphere with 5% CO₂ for seven days and spun at 2800 × g for 15 min to collect 0.5 ml of supernatant. The collected supernatant was assayed for concentrations of IL-1 β , TNF- α , PDGF-AB and TGF- β 1 by enzyme-linked immunosorbent assay (ELISA) using commercial kits (Xitang, Shanghai, China) according to manufacturer's instructions.

Animal surgery

Forty-five male mature New Zealand white rabbits (weighing 2.5-3.0 kg) were used to create cartilage defect models as described elsewhere [7]. In brief, after achieving anesthesia with an intravenous injection of ketamine hydrochloride (60 mg/kg) and xylazine (6 mg/kg), a lateral para-patellar skin incision was made. The patella was dislocated medially to expose the knee joint and a full-thickness cylindrical cartilage defect of 5 mm in diameter and 3 mm in depth was created in the patellar groove. The 90 defects in 45 rabbits were divided into 5 groups evenly: they were left unfilled (the control group), or filled with transplanted constructs prepared with autologous L-PRP

(the L-PRP group and L-PRP+CAPE group) or P-PRP (the P-PRP group and P-PRP+CAPE group).

Each rabbit received an intramuscular injection of 8×10^5 IU of penicillin postoperatively. All rabbits were housed in separated cages and had ad libitum access to food and water. Rabbits in the L-PRP+CAPE group and P-PRP+CAPE group received 1 ml of 10 μ mol/kg/day CAPE (caffeic acid phenethyl ester; Sigma-Aldrich, St Louis, MO, USA) intraperitoneally for 7 days to inhibit NF- κ B activation [17], and rabbits in the other groups were injected with 1 ml of saline once a day for 7 days. All rabbits were euthanized with an overdose of anesthesia at 12 weeks postoperatively.

Quantification of IL-1 β and prostaglandin E2 concentrations in the synovial fluid

After rabbits were euthanized, the synovial fluid in the knee joints was collected for measurement of IL-1 β and prostaglandin E2 (PGE2) concentrations by ELISA using commercial kits (Xitang, Shanghai, China) according to manufacturer's instructions.

Macroscopic evaluation

Femoral condyle samples were harvested and fixed with 4% paraformaldehyde for 72 h. Then, macroscopic evaluation of cartilage regeneration in the defects was quantified according to the International Cartilage Regeneration Society (ICRS) macroscopic scores [18].

Micro-computed tomography scanning

Fixed samples were scanned using micro-computed tomography (micro-CT) to evaluate the subchondral bone regeneration in the defects. Micro-CT examination was taken with an 18- μ m resolution using a SkyScan 1076 scanning system (Skyscan, Kontich, Belgium) at 80 kV/300 μ A. NRecon software (Skyscan, Kontich, Belgium) was used to analyze the local bone mineral density (BMD) and bone volume to tissue volume (BV/TV) of the regenerated bone.

Histological evaluation

Samples were decalcified with 10% EDTA for 1 month, dehydrated with graded ethanol solu-

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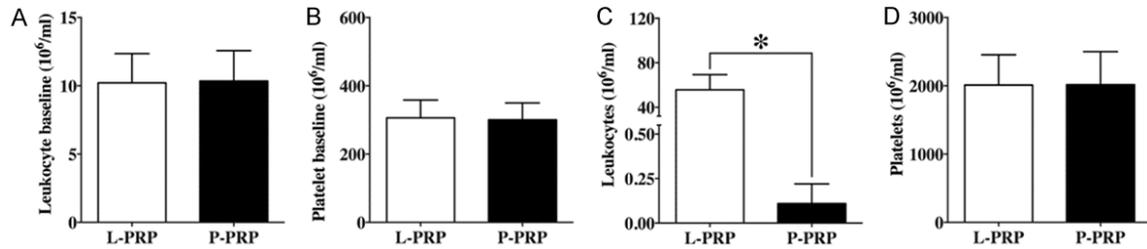


Figure 1. Components of whole blood and platelet-rich plasma formulations. The whole blood samples used for leukocyte- and platelet-rich plasma (L-PRP) and pure platelet-rich plasma (P-PRP) preparation had similar leukocyte concentration (A) and platelet concentration (B), while L-PRP had a significantly higher leukocyte concentration (C) and a similar platelet concentration compared with P-PRP (D). Bars represent the means and standard deviation (n = 10); *P < 0.05.

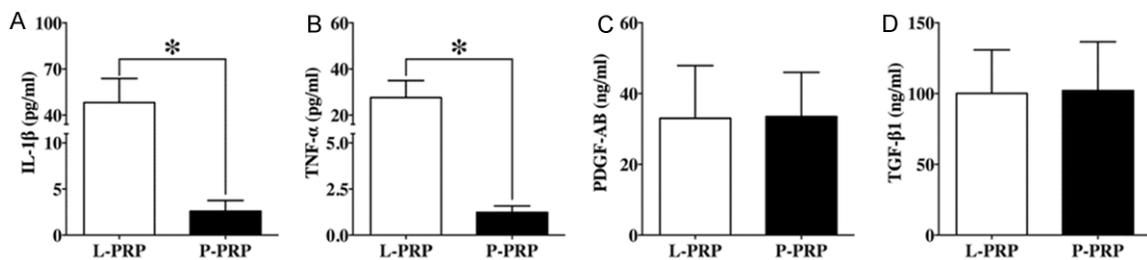


Figure 2. Cytokines released from platelet-rich plasma scaffolds. The supernatant released from leukocyte- and platelet-rich plasma (L-PRP) scaffolds had significantly higher IL-1 β (A) and TNF- α concentrations (B), and similar PDGF-AB (C) and TGF- β 1 concentrations (D) compared with pure platelet-rich plasma (P-PRP) scaffolds. Bars represent the means and standard deviation (n = 10); *P < 0.05.

tions, embedded in paraffin and sectioned at 5 μ m at the central area of the defects. Sections were stained with hematoxylin and eosin (HE) for general histological evaluation or with toluidine blue for evaluation of cartilaginous matrix distribution. Histological evaluation of cartilage regeneration in the defects was quantified according to the ICRS histological scores [19].

Statistical analysis

Data were analyzed using the Statistical Package for Social Sciences version 22.0 (SPSS, Chicago, IL, USA) and presented as means \pm standard deviation. Independent-samples student's t test or one-way analysis of variance and Bonferroni post-hoc test were performed for statistical analysis as appropriate. Pearson correlation analysis was conducted to analyze linear correlations between the cellular components of PRP formulations and cytokines released from PRP scaffolds. A P value less than 0.05 was considered statistically significant.

Results

Cellular components of whole blood, L-PRP and P-PRP

The mean leukocyte and platelet concentrations were $10.22 \pm 2.13 \times 10^6/\text{mL}$ and $306.10 \pm 52.33 \times 10^6/\text{mL}$, respectively, in the whole blood used for L-PRP preparation, and $10.36 \pm 2.22 \times 10^6/\text{mL}$ and $300.50 \pm 49.28 \times 10^6/\text{mL}$, respectively, in the whole blood used for P-PRP preparation. There was no significant difference in the mean leukocyte and platelet concentrations between the whole blood used for L-PRP and P-PRP preparation (**Figure 1A, 1B**). However, the mean leukocyte concentration in L-PRP ($55.68 \pm 13.65 \times 10^6/\text{mL}$) was significantly higher than that in P-PRP ($0.11 \pm 0.11 \times 10^6/\text{mL}$, $P < 0.001$, **Figure 1C**), while the mean platelet concentrations in L-PRP ($2009.80 \pm 443.90 \times 10^6/\text{mL}$) and P-PRP ($2016.30 \pm 483.99 \times 10^6/\text{mL}$) were similar ($P = 0.905$, **Figure 1D**).

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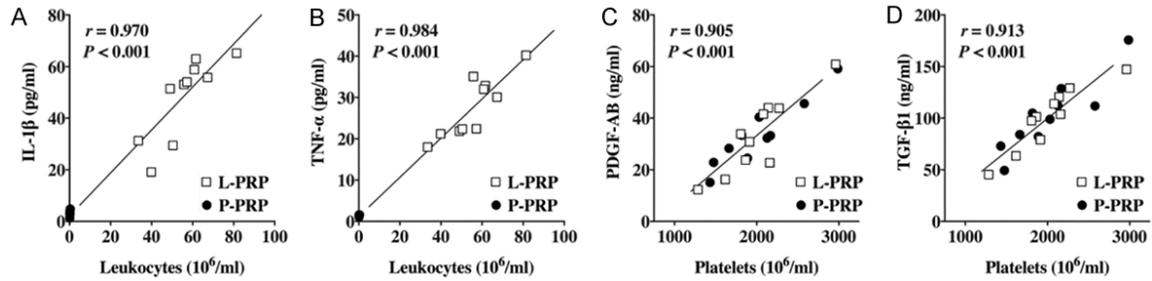


Figure 3. Correlations between cellular components of platelet-rich plasma formulations and cytokines released from platelet-rich plasma scaffolds. There was significantly positive correlations between leukocyte concentration in PRP formulations and IL-1 β (A) and TNF- α released from PRP scaffolds (B), and between platelet concentration in PRP formulations and PDGF-AB (C) and TGF- β 1 released from PRP scaffolds (D). PRP, platelet-rich plasma; L-PRP, leukocyte- and platelet-rich plasma; P-PRP, pure platelet-rich plasma.

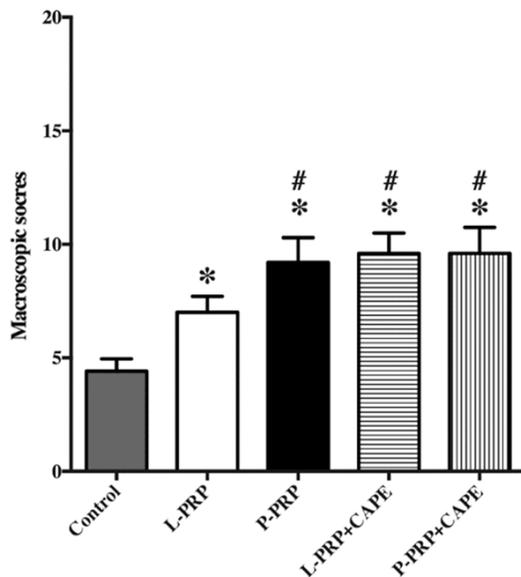


Figure 4. Macroscopic evaluation according to the International Cartilage Regeneration Society macroscopic score. L-PRP, leukocyte- and platelet-rich plasma; P-PRP, pure platelet-rich plasma; CAPE, caffeic acid phenethyl ester. Bars represent the means and standard deviation ($n = 5$); * $P < 0.05$ compared with the control group; # $P < 0.05$ compared with the L-PRP group.

Cytokines released from L-PRP and P-PRP scaffolds

The mean IL-1 β concentration in the supernatant released from L-PRP scaffolds (48.12 ± 15.75 pg/mL) was significantly higher than P-PRP scaffolds (2.60 ± 1.16 pg/mL, $P < 0.001$, **Figure 2A**). Likewise, L-PRP scaffolds also released significantly more TNF- α (27.61 ± 7.37 pg/mL) compared with P-PRP scaffolds (1.23 ± 0.35 pg/mL, $P < 0.001$, **Figure 2B**). But there

was no significant difference between L-PRP and P-PRP scaffolds in PDGF-AB ($P = 0.939$, **Figure 2C**) and TGF- β 1 released ($P = 0.895$, **Figure 2D**).

Correlations between the cellular components of PRP formulations and cytokines released from PRP scaffolds

There was a significantly positive correlation between leukocyte concentration in PRP formulations and IL-1 β ($r = 0.970$, $P < 0.001$, **Figure 3A**), and TNF- α released from PRP scaffolds ($r = 0.984$, $P < 0.001$, **Figure 3B**). Also, the significantly positive correlations were observed between platelet concentration and PDGF-AB ($r = 0.905$, $P < 0.001$, **Figure 3C**), and TGF- β 1 ($r = 0.913$, $P < 0.001$, **Figure 3D**) released.

However, the correlations between leukocyte concentration and PDGF-AB ($r = 0.085$, $P = 0.722$) and TGF- β 1 released ($r = 0.085$, $P = 0.722$), and between platelet concentration and IL-1 β ($r = 0.155$, $P = 0.514$), and TNF- α released ($r = 0.102$, $P = 0.669$) were not significant.

Macroscopic evaluation

Macroscopic evaluation of cartilage regeneration in the defects was quantified according to ICRS macroscopic scores. As shown in **Figure 4**, the mean macroscopic scores of P-PRP group (9.20 ± 1.10), L-PRP+CAPE group (9.60 ± 0.89) and P-PRP+CAPE group (9.60 ± 1.14) were similar ($P > 0.999$), but significantly higher than the L-PRP group (7.00 ± 0.71 ; $P = 0.010$ compared with P-PRP and $P = 0.002$ compared with L-PRP+CAPE and P-PRP+CAPE), which, in

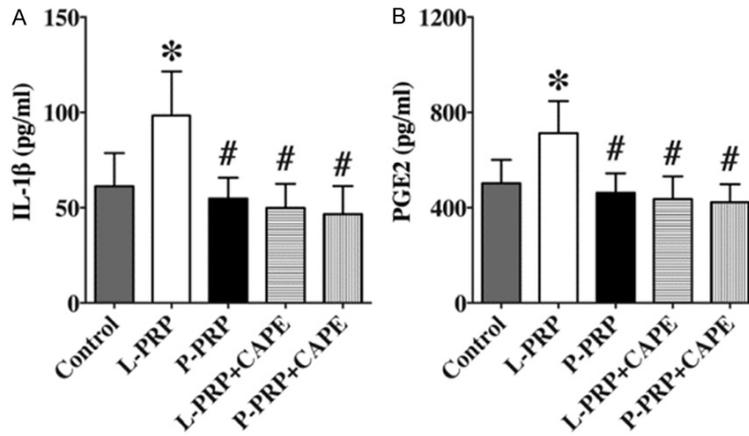


Figure 5. Interleukin 1β (IL-1β) and prostaglandin E2 (PGE2) concentrations in the synovial fluid. IL-1β (A) and PGE2 (B) concentrations in the synovial fluid were quantified by enzyme-linked immunosorbent assay. L-PRP, leukocyte- and platelet-rich plasma; P-PRP, pure platelet-rich plasma; CAPE, caffeic acid phenethyl ester. Bars represent the means and standard deviation (n = 5); *P < 0.05 compared with the control group; #P < 0.05 compared with the L-PRP group.

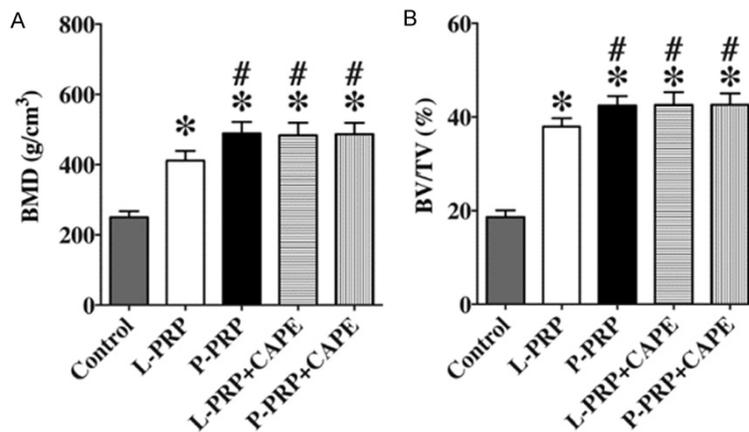


Figure 6. Micro-computed tomography assessment of the mineralized bone formation in the defects. The local bone mineral density (BMD) (A) and bone volume to tissue volume (BV/TV) (B) were used to evaluate the mineralized bone formation in the defects. L-PRP, leukocyte- and platelet-rich plasma; P-PRP, pure platelet-rich plasma; CAPE, caffeic acid phenethyl ester. Bars represent the means and standard deviation (n = 5); *P < 0.05 compared with the control group; #P < 0.05 compared with the L-PRP group.

turn, was significantly higher than the control group (4.40 ± 0.55, P = 0.002).

IL-1β and PGE2 concentrations in the synovial fluid

The synovial fluid collected from rabbits of the L-PRP group had the highest IL-1β concentration compared with the control group (P =

0.018), P-PRP group (P = 0.004), L-PRP+CAPE group (P = 0.001) and P-PRP group (P = 0.001), which had similar IL-1β concentrations compared with each other (P > 0.999, Figure 5A).

A similar trend was observed in the analysis of PGE2 concentrations in the synovial fluid, which demonstrated that the PGE2 concentrations were significantly higher in the synovial fluid collected from the L-PRP group and there was no significant difference in PGE2 concentration between the synovial fluid collected from the control group, P-PRP group, L-PRP+CAPE group and P-PRP+CAPE group (Figure 5B).

Micro-CT assessment

Mineralized bone formation was evaluated using micro-CT with BMD and BV/TV to indicate the subchondral bone regeneration. As shown in Figure 6A, BMD in all PRP groups was significantly higher than the control group (P < 0.001). And BMD in the P-PRP group was significantly higher than the L-PRP group (P = 0.005). Besides that, BMD in the L-PRP+CAPE group was significantly higher than the L-PRP group (P = 0.010), while BMD in the P-PRP group, L-PRP+CAPE group and P-

PRP+CAPE group were similar compared with each other (P > 0.999).

A similar trend was observed in the results of BV/TV analysis, which demonstrated that BV/TV in the P-PRP group, L-PRP+CAPE group and P-PRP+CAPE group were similar (P > 0.999), but significantly higher than the L-PRP group (P = 0.031 compared with P-PRP and P =

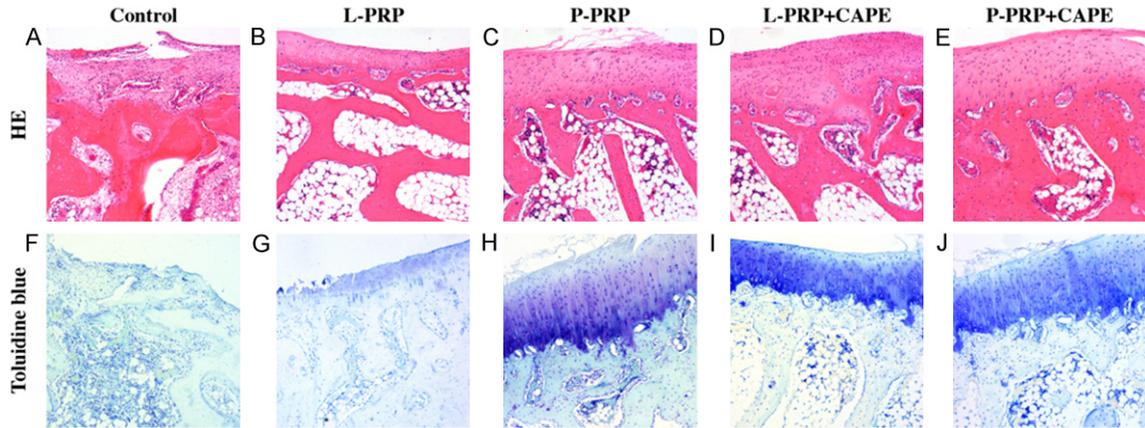


Figure 7. Hematoxylin and eosin staining and toluidine blue staining for histological evaluation. Representative hematoxylin and eosin (HE) stained sections (A-E) and toluidine blue (F-J) stained sections. Defects in the control group were filled with fibrous tissue, while some cartilage-like tissue formation was observed in the defects of the L-PRP group, although the integration was fragile, with splits or cracks. Defects in the P-PRP group, L-PRP+CAPE group and P-PRP+CAPE group were fully filled with cartilage-like tissue and integrated well with the normal cartilage. HE, hematoxylin and eosin staining; L-PRP, leukocyte- and platelet-rich plasma; P-PRP, pure platelet-rich plasma; CAPE, caffeic acid phenethyl ester. Scales represent 200 μ m.

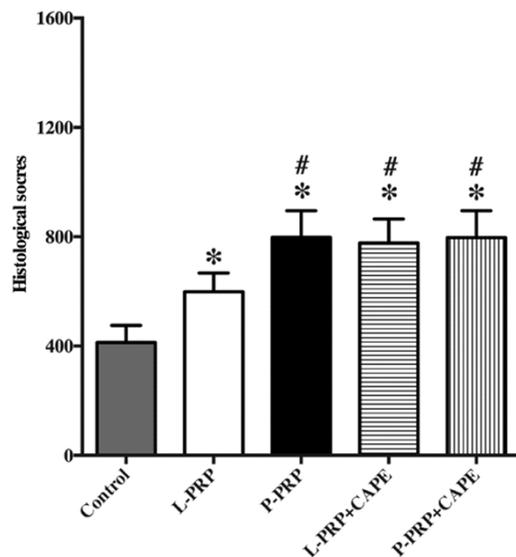


Figure 8. Histological evaluation according to the International Cartilage Regeneration Society histological score. L-PRP, leukocyte- and platelet-rich plasma; P-PRP, pure platelet-rich plasma; CAPE, caffeic acid phenethyl ester. Bars represent the means and standard deviation (n = 5); *P < 0.05 compared with the control group; #P < 0.05 compared with the L-PRP group.

0.023 compared with L-PRP+CAPE and P-PRP+CAPE), which, in turn, was significantly higher than the control group (P < 0.001, **Figure 6B**).

Histological observation

At 12 weeks postoperatively, the defects in the control group were substantially filled with fibrous tissue (**Figure 7A**). Toluidine blue staining also showed no cartilaginous matrix in the defects of the control group (**Figure 7F**). In the defects of the L-PRP group, some cartilage-like tissue formation was observed, but the integration was fragile, with splits or cracks (**Figure 7B, 7G**). In the P-PRP group, L-PRP+CAPE group and P-PRP+CAPE group, the defects were fully filled with cartilage-like tissue and integrated well with the normal cartilage (**Figure 7C-E and 7H-J**).

Histological evaluations of cartilage regeneration in the defects were quantified according to ICRS histological scores. The mean ICRS histological scores were similar in the P-PRP group, L-PRP+CAPE group and P-PRP+CAPE group, lower in the L-PRP group, and the lowest in the control group (**Figure 8**).

Discussion

The rationale for PRP therapy for cartilage defects is largely dependent on the presence of concentrated growth factors in PRP. PDGF-AB and TGF- β 1, which have been detected consistently in concentrated levels in PRP, are the most widely studied growth factors [8, 9]. TGF-

$\beta 1$ has been shown to stimulate chondrogenic cells proliferation and extracellular matrix production, downregulate collagen I expression and recruit chondrogenic progenitor cells [20-22]. PDGF-AB is a chemokine and regulator of cell proliferation and extracellular matrix formation [23]. The *in vivo* studies also demonstrated that injections of these growth factors lead to increased cartilage regeneration [11]. According to the literature, platelets are the major sources of PDGF-AB and TGF- $\beta 1$ in PRP, and therefore PRP formulations that have similar platelet concentrations may release similar amount of PDGF-AB and TGF- $\beta 1$ when they are activated [9, 24]. Hence, concentrations of platelets and growth factors released from the L-PRP and P-PRP used in this study were quantified. Our findings demonstrated that L-PRP and scaffolds used in the current study were similar in growth factors delivery and supported the findings of Sundman and Yin showing the positive correlations between platelet concentration and growth factors delivery. Although the similar growth factors delivery might make the anabolic effects of L-PRP scaffolds and P-PRP scaffolds be similar, the rBMSCs-encapsulated scaffolds prepared with them induced distinct effects on cartilage regeneration, possibly because that these scaffolds differed in IL-1 β and TNF- α delivery.

The use of rBMSCs-encapsulated autologous L-PRP scaffolds has been shown to have positive effects on cartilage regeneration in the cartilage defects in rabbits [7]. However, our results demonstrated that L-PRP concentrated leukocytes and therefore released higher levels of IL-1 β and TNF- α , which might induce harmful effects on cartilage regeneration, compared with P-PRP that had low leukocyte concentration. It has been well established that IL-1 β and TNF- α , which are produced by leukocytes, chondrocytes, osteoblasts and synovial tissues, are associated with the cartilage destruction in the pathophysiology of osteoarthritis [25]. Moreover, antagonists of IL-1 β or TNF- α have been shown effective in countering cartilage destruction [26, 27]. Although growth factors, which are concentrated in PRP, may decrease IL-1 β receptor transcription and binding ability while promoting IL-1 receptor antagonist synthesis [28], an excess of leukocytes, which are the major source of pro-inflammatory cytokines, in L-PRP may overwhelm these abilities [29]. Our results demonstrated that rBMSCs-encapsulated L-PRP and P-PRP scaffolds

induced distinct effects on cartilage regeneration, with P-PRP scaffolds showing greater effects, even though they were similar in growth factors delivery. These findings implied that the concentrated leukocytes in L-PRP might provide increased pro-inflammatory cytokines to induce harmful effects on cartilage regeneration, to counter the beneficial effects of growth factors and result in the inferior effects on cartilage regeneration of L-PRP compared with P-PRP observed here.

The NF- κ B pathway is intimately involved in the disturbed metabolism and enhanced catabolism of cartilage that induced by IL-1 β and TNF- α [30]. Whereas, the effects of PRP formulations on NF- κ B pathway have never been evaluated. Although some authors advocated that growth factors treatment with platelet lysate or PRP clot releasate might inhibit the activation of NF- κ B pathway [31, 32], the absence of viable leukocytes and platelets in these products may result in the distinct biological properties of them compared with PRP formulations used in clinical practice, and therefore the effects of these products on NF- κ B pathway may not reflect that of PRP formulations. Moreover, these findings were based on osteoarthritic chondrocytes under intensely inflammatory conditions. Our results demonstrating the similar concentrations of NF- κ B-dependent inflammatory mediators, IL-1 β and PGE2, in the synovial fluid between the control group, P-PRP group and P-PRP+CAPE group implied that the intensely inflammatory conditions applied in those studies demonstrating the anti-NF- κ B activation potential of PRP formulations were quite different from the local environment of cartilage defects, and P-PRP used in the current study might have the potential of NF- κ B inhibition in the cartilage defects models. However, our results showed that L-PRP treatment induced the increased IL-1 β and PGE2 concentrations in the synovial fluid, which were reversed by inhibition of NF- κ B activation. These findings indicated that the high levels of IL-1 β and TNF- α released from the concentrated leukocytes in L-PRP might induce NF- κ B activation to induce harmful effects on cartilage regeneration.

CAPE, which is the active component of the propolis derived from the hives of honeybees, is a special inhibitor of NF- κ B activation. The use of CAPE has been shown to decrease production of matrix metalloproteinases 9 by

chondrocytes *in vitro* [33] and protect cartilage against experimental induced destruction *in vivo* [34]. But CAPE is not able to enhance cartilage regeneration under physiological conditions. Our results demonstrating the similar therapeutic effects between the P-PRP group and P-PRP+CAPE group also provide substantial proofs for that. However, postoperative delivery of CAPE suppressed the effects on NF- κ B pathway while enhanced the effects on cartilage regeneration of L-PRP scaffolds. These findings implied that the activation of NF- κ B pathway induced by L-PRP might be the mechanisms of the distinct effects of L-PRP and P-PRP on cartilage regeneration. Hence, although L-PRP enhanced cartilage regeneration in the cartilage defects in rabbits compared with the control group, leukocytes in L-PRP might activate NF- κ B pathway via the delivery of IL-1 β and TNF- α to induce the inferior effects of L-PRP compared with P-PRP.

This study investigated the effects of L-PRP and P-PRP on cartilage regeneration and NF- κ B pathway in a rabbit model. Our results implied that leukocytes in L-PRP might activate the NF- κ B pathway via the delivery of IL-1 β and TNF- α to induce harmful effects on cartilage regeneration and result in the inferior effects of L-PRP compared with P-PRP. Hence, P-PRP may be more suitable than L-PRP for the treatment of cartilage defects.

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Disclosure of conflict of interest

None.

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References

[1] Schinhan M, Gruber M, Vavken P, Dorotka R, Samouh L, Chiari C, Gruebl-Barabas R and Nehrer S. Critical-size defect induces unicom-

partmental osteoarthritis in a stable ovine knee. *J Orthop Res* 2012; 30: 214-220.

- [2] Bornes TD, Adesida AB and Jomha NM. Mesenchymal stem cells in the treatment of traumatic articular cartilage defects: a comprehensive review. *Arthritis Res Ther* 2014; 16: 432.
- [3] Solheim E, Hegna J, Oyen J, Harlem T and Strand T. Results at 10 to 14 years after osteochondral autografting (mosaicplasty) in articular cartilage defects in the knee. *Knee* 2013; 20: 287-290.
- [4] Liu J, Nie H, Xu Z, Guo F, Guo S, Yin J, Wang Y and Zhang C. Construction of PRP-containing nanofibrous scaffolds for controlled release and their application to cartilage regeneration. *Journal of Materials Chemistry B* 2015; 3: 581-591.
- [5] Milano G, Sanna Passino E, Deriu L, Careddu G, Manunta L, Manunta A, Saccomanno MF and Fabbriciani C. The effect of platelet rich plasma combined with microfractures on the treatment of chondral defects: an experimental study in a sheep model. *Osteoarthritis Cartilage* 2010; 18: 971-980.
- [6] Altan E, Aydin K, Erkokcak O, Senaran H and Ugras S. The effect of platelet-rich plasma on osteochondral defects treated with mosaicplasty. *Int Orthop* 2014; 38: 1321-1328.
- [7] Xie X, Wang Y, Zhao C, Guo S, Liu S, Jia W, Tuan RS and Zhang C. Comparative evaluation of MSCs from bone marrow and adipose tissue seeded in PRP-derived scaffold for cartilage regeneration. *Biomaterials* 2012; 33: 7008-7018.
- [8] Magalon J, Bausset O, Serratrice N, Giraudo L, Aboudou H, Veran J, Magalon G, Dignat-Georges F and Sabatier F. Characterization and comparison of 5 platelet-rich plasma preparations in a single-donor model. *Arthroscopy* 2014; 30: 629-638.
- [9] Sundman EA, Cole BJ and Fortier LA. Growth factor and catabolic cytokine concentrations are influenced by the cellular composition of platelet-rich plasma. *Am J Sports Med* 2011; 39: 2135-2140.
- [10] Burguera EF, Vela-Anero A, Magalhaes J, Meijide-Failde R and Blanco FJ. Effect of hydrogen sulfide sources on inflammation and catabolic markers on interleukin 1 β -stimulated human articular chondrocytes. *Osteoarthritis Cartilage* 2014; 22: 1026-1035.
- [11] Stewart K, Pabbruwe M, Dickinson S, Sims T, Hollander AP and Chaudhuri JB. The effect of growth factor treatment on meniscal chondrocyte proliferation and differentiation on polyglycolic acid scaffolds. *Tissue Eng* 2007; 13: 271-280.
- [12] Tong W, Geng Y, Huang Y, Shi Y, Xiang S, Zhang N, Qin L, Shi Q, Chen Q, Dai K and Zhang X. *In vivo* identification and induction of articular cartilage stem cells by inhibiting NF- κ B

Leukocyte- and platelet-rich plasma and pure platelet-rich plasma for cartilage

- signaling in osteoarthritis. *Stem Cells* 2015; 33: 3125-3137.
- [13] Henderson B and Pettipher ER. Arthritogenic actions of recombinant IL-1 and tumour necrosis factor alpha in the rabbit: evidence for synergistic interactions between cytokines in vivo. *Clin Exp Immunol* 1989; 75: 306-310.
- [14] Cavallo C, Filardo G, Mariani E, Kon E, Marcacci M, Pereira Ruiz MT, Facchini A and Grigolo B. Comparison of platelet-rich plasma formulations for cartilage healing: an in vitro study. *J Bone Joint Surg Am* 2014; 96: 423-429.
- [15] Rigoglou S and Papavassiliou AG. The NF- κ B signalling pathway in osteoarthritis. *Int J Biochem Cell Biol* 2013; 45: 2580-2584.
- [16] Jia WT, Zhang CQ, Wang JQ, Feng Y and Ai ZS. The prophylactic effects of platelet-leucocyte gel in osteomyelitis: an experimental study in a rabbit model. *J Bone Joint Surg Br* 2010; 92: 304-310.
- [17] Altug ME, Serarslan Y, Bal R, Kontas T, Ekici F, Melek IM, Aslan H and Duman T. Caffeic acid phenethyl ester protects rabbit brains against permanent focal ischemia by antioxidant action: a biochemical and planimetric study. *Brain Res* 2008; 1201: 135-142.
- [18] van den Borne MP, Raijmakers NJ, Vanlauwe J, Victor J, de Jong SN, Bellemans J, Saris DB; International Cartilage Repair Society. International Cartilage Repair Society (ICRS) and Oswestry macroscopic cartilage evaluation scores validated for use in Autologous Chondrocyte Implantation (ACI) and microfracture. *Osteoarthritis Cartilage* 2007; 15: 1397-1402.
- [19] Mainil-Varlet P, Van Damme B, Nesic D, Knutsen G, Kandel R and Roberts S. A new histology scoring system for the assessment of the quality of human cartilage repair: ICRS II. *Am J Sports Med* 2010; 38: 880-890.
- [20] Yaeger PC, Masi TL, de Ortiz JL, Binette F, Tubo R and McPherson JM. Synergistic action of transforming growth factor-beta and insulin-like growth factor-I induces expression of type II collagen and aggrecan genes in adult human articular chondrocytes. *Exp Cell Res* 1997; 237: 318-325.
- [21] Li WJ, Tuli R, Okafor C, Derfoul A, Danielson KG, Hall DJ and Tuan RS. A three-dimensional nanofibrous scaffold for cartilage tissue engineering using human mesenchymal stem cells. *Biomaterials* 2005; 26: 599-609.
- [22] Lee CH, Cook JL, Mendelson A, Moiola EK, Yao H and Mao JJ. Regeneration of the articular surface of the rabbit synovial joint by cell homing: a proof of concept study. *Lancet* 2010; 376: 440-448.
- [23] Heldin CH, Eriksson U and Ostman A. New members of the platelet-derived growth factor family of mitogens. *Arch Biochem Biophys* 2002; 398: 284-290.
- [24] Yin WJ, Xu HT, Sheng JG, An ZQ, Guo SC, Xie XT and Zhang CQ. Advantages of pure platelet-rich plasma compared with leukocyte- and platelet-rich plasma in treating rabbit knee osteoarthritis. *Med Sci Monit* 2016; 22: 1280-1290.
- [25] Kapoor M, Martel-Pelletier J, Lajeunesse D, Pelletier JP and Fahmi H. Role of proinflammatory cytokines in the pathophysiology of osteoarthritis. *Nat Rev Rheumatol* 2011; 7: 33-42.
- [26] Bacconnier L, Jorgensen C and Fabre S. Erosive osteoarthritis of the hand: clinical experience with anakinra. *Ann Rheum Dis* 2009; 68: 1078-1079.
- [27] Grunke M and Schulze-Koops H. Successful treatment of inflammatory knee osteoarthritis with tumour necrosis factor blockade. *Ann Rheum Dis* 2006; 65: 555-556.
- [28] Redini F, Mauviel A, Pronost S, Loyau G and Pujol JP. Transforming growth factor beta exerts opposite effects from interleukin-1 beta on cultured rabbit articular chondrocytes through reduction of interleukin-1 receptor expression. *Arthritis Rheum* 1993; 36: 44-50.
- [29] Pujol JP, Chadjichristos C, Legendre F, Bauge C, Beauchef G, Andriamanalijaona R, Galera P and Boumediene K. Interleukin-1 and transforming growth factor-beta 1 as crucial factors in osteoarthritic cartilage metabolism. *Connect Tissue Res* 2008; 49: 293-297.
- [30] Davidson RK, Jupp O, de Ferrars R, Kay CD, Culley KL, Norton R, Driscoll C, Vincent TL, Donell ST, Bao Y and Clark IM. Sulforaphane represses matrix-degrading proteases and protects cartilage from destruction in vitro and in vivo. *Arthritis Rheum* 2013; 65: 3130-3140.
- [31] Pereira RC, Scaranari M, Benelli R, Strada P, Reis RL, Cancedda R and Gentili C. Dual effect of platelet lysate on human articular cartilage: a maintenance of chondrogenic potential and a transient proinflammatory activity followed by an inflammation resolution. *Tissue Eng Part A* 2013; 19: 1476-1488.
- [32] van Buul GM, Koevoet WL, Kops N, Bos PK, Verhaar JA, Weinans H, Bernsen MR and van Osch GJ. Platelet-rich plasma releasate inhibits inflammatory processes in osteoarthritic chondrocytes. *Am J Sports Med* 2011; 39: 2362-2370.
- [33] Wang Y, Li de L, Zhang XB, Duan YH, Wu ZH, Hao DS, Chen BS and Qiu GX. Increase of TNF α -stimulated osteoarthritic chondrocytes apoptosis and decrease of matrix metalloproteinases 9 by NF- κ B inhibition. *Biomed Environ Sci* 2013; 26: 277-283.
- [34] Elmali N, Ayan I, Turkoz Y, Mizrak B, Germen B and Bora A. Effect of caffeic acid phenethyl ester on cartilage in experimental osteoarthritis. *Rheumatol Int* 2002; 22: 222-226.