Intraarticular administration of platelet-rich plasma with biodegradable gelatin hydrogel microspheres prevents osteoarthritis progression in the rabbit knee

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Abstract

Objective

To investigate the therapeutic potential of administration of gelatin hydrogel microspheres containing platelet-rich plasma (PRP), by examining its effects on progression of osteoarthritis (OA) in a rabbit model.

Methods

PRP and platelet-poor plasma (PPP) were prepared from rabbit blood. Adult rabbit chondrocytes were cultured in the alginate beads with the presence of 3% PRP or 3% PPP. Glycosaminoglycan (GAG) synthesis was quantified using dimethylmethylene blue assay. To confirm the anabolic effect of PRP in vivo*, cartilage matrix gene expression was examined after intraarticular administration of PRP contained in gelatin hydrogel microspheres. The PRP contained in gelatin hydrogel microspheres was administered into the rabbit knee joint twice with an interval of 3 weeks, beginning 4 weeks after anterior cruciate ligament transection (ACLT). Ten weeks after ACLT, gross morphological and histological examinations were performed.*

Results

PRP significantly stimulated chondrocyte GAG synthesis in vitro*. In the knee joint, expression of proteoglycan core protein mRNA in the articular cartilage increased after administration of PRP contained in microspheres. Intraarticular injections of PRP in gelatin hydrogel microspheres significantly suppressed progression of OA in the ACLT rabbit model morphologically and histologically.*

Conclusion

The present findings indicate that sustained release of growth factors contained in PRP has preventive effects against OA progression. These preventive effects appear to be due to stimulation of cartilage matrix metabolism, caused by the growth factors contained in PRP.

Key words

Platelet rich plasma, osteoarthritis, gelatin hydrogel microspheres.

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Introduction

Osteoarthritis (OA) is a progressive disorder based on degeneration of articular cartilage. OA is the most common joint disease, and is one of the main causes to restrict daily activities of people. OA is mainly treated using conservative therapies such as oral administration of non-steroidal anti-inflammatory drugs and intraarticular injection of hyaluronan. Although these therapies are partially effective in controlling the symptoms and relieving pain, they do not suppress progression of cartilage degeneration (1). Recently, so-called disease-modifying OA drugs (DMOADs), such as glucosamine and chondroitin sulfate have attracted attention as a new therapeutic agent of OA. It was shown that an administration of these agents at least partially prevents the joint structure changes and improves symptoms in patients with OA (2, 3). However, the therapeutic effects of these agents are still controversial, and these agents are not satisfactorily effective in preventing the progression of OA. End-stage OA is usually treated surgically, by performing procedures such as total joint replacement. Although clinical results confirm its usefulness, the surgical treatment has problems in terms of invasiveness and high financial burden (4). Thus, there is a great need for an efficient, simple and inexpensive therapeutic modality that prevents cartilage degeneration in the initial stages of OA.

Recent studies have explored regeneration of injured articular cartilage to hyaline cartilage, using various growth factors that accelerate cartilage metabolism or inhibit cartilage degeneration. The results of those studies indicate that basic fibroblast growth factor (bFGF), insulin-like growth factor-1 (IGF-1), transforming growth factor-β1 (TGF-β1) and platelet-derived growth factor (PDGF) are potent mitogens for chondrocytes as well as stimulators of matrix metabolism (5-8). We previously confirmed that controlled release of bFGF into the rabbit knee joint can be achieved by intraarticular injection of gelatin hydrogel microspheres containing bFGF. In addition, we elucidated the inhibitory effects of such injections on OA progression in rabbits that had undergone anterior cruciate ligament transection (ACLT) (9). This method is easy to use and relatively non-invasive, and is thus a promising conservative treatment for OA.

Recently, platelet-rich plasma (PRP) has attracted attention as an autologous source of growth factors. PRP contains a high concentration of platelets in a small volume of plasma, and contains high concentrations of several autologous growth factors, including PDGF, TGF-β1 and IGF-1 (10, 11). PRP has been used clinically in the field of oral surgery and plastic surgery, because it promotes wound healing and tissue regeneration (12-14). A previous study reported that bone regeneration was augmented by administration of gelatin hydrogel microspheres containing PRP, which provided sustained release of growth factors (15). It was also reported that a similar treatment promoted intervertebral disc regeneration (16). PRP has been shown to stimulate proliferation and extracellular matrix metabolism of chondrocytes *in vitro* (17). However, it is unclear what effects intraarticular administration of PRP has on articular cartilage *in vivo* and on models of OA. In the present study, we investigated the therapeutic potential of administration of PRP contained in gelatin hydrogel microspheres, by assessing its effects on progression of OA in a rabbit model.

Materials and methods

Preparation of platelet-rich plasma

Japanese white rabbits ranging in body weight from 1.7 to 2.0 kg were purchased from Oriental BioService (Kyoto, Japan). All animal experiments were conducted in accordance with the guidelines for animal research of Kyoto Prefectural University of Medicine. PRP was prepared using a modified version of a previously reported method (15, 16). Under inhalation anesthesia with 3% isoflurane (Abbott Laboratories, North Chicago, IL), 20 mL of blood was obtained from each rabbit and centrifuged twice. The platelet-poor plasma (PPP) fraction was removed, and 300 μl of PRP was obtained. The platelet count of the whole blood, PPP and PRP was determined (all measurements were performed by FALCO, Japan).

3D culture of chondrocyte in alginate beads and quantification of glycosaminoglycan (GAG)

Chondrocytes were isolated from rabbit cartilage using trypsin and collagenase digestion as described in detail previously (18). The isolated chondrocytes were cultured as monolayers for 2 weeks. Three-dimensional culture of chondrocytes in alginate beads was performed as previously described (19). On the second passage, the cells were embedded in alginate beads. The beads were cultured in DMEM containing 10% FBS, 100 units/mL penicillin, and 100 μg/mL streptomycin (Gibco BRL). PRP and PPP were activated by adding a 10% thrombin (Sigma-Aldrich, St. Louis, MO) solution (1000 U/mL in 100 mM $CaCl_2$). After centrifugation at 1000 × g for 10 min, the supernatant was collected and added to the culture medium (3% v/v). After 3 days or 7 days of culture, the beads were digested, and the amount of GAG in the supernatant was quantified using the dimethylmethylene blue (DMB) assay (20). Chondroitin 6 sulfate (Sigma) was used as a standard. The total amount of GAG was normalized to the total amount of protein.

Preparation of gelatin hydrogel microspheres

The gelatin that was used had an isoelectric point (IEP) of 4.9 (Nitta Gelatin), and was isolated from bovine bone collagen using an alkaline process. Gelatin hydrogel microspheres were obtained from an aqueous gelatin solution dispersed in an oil phase as described (21). The microspheres were dehydrothermally cross-linked at 140°C for 72 h under a vacuum of 0.1 torr (22). The microspheres were then sterilized with ethylene oxide gas. The water content of the microspheres was 95%. The diameter of the microspheres ranged from 32 to 75mm.

Real-time PCR assay for mRNA in knee joint cartilage

The microspheres were impregnated with PRP by adding 100 μl of PRP to 5 mg of microspheres, which were then kept at room temperature for 1 hour. In some rabbits (PRP-M group), gelatin hydrogel microspheres containing PRP, dispersed in 200 μl of PBS, were injected into the left knee. In other rabbits (PRP-only group), 100 μl of PRP in 200 μl of PBS was injected into the left knee. The rabbits were sacrificed 3 and 7 days after the injection (n=2/day), and the cartilage tissue of knee joints was collected. The total RNA was isolated from the cartilage as described (9). First-strand cDNA synthesis was performed for each RNA sample using an ExScript™ RT reagent kit (Takara Bio, Otsu, Japan).

Changes in cartilage matrix gene expression after administration of PRP-M or PRP-only were evaluated by real-time RT-PCR. Quantitative real-time PCR was performed using Biosystem 7300 (Applied Biosystems, Foster City, CA), with monitoring of the increase in reporter fluorescence of the TaqMan probe for the proteoglycan core protein or collagen type II during PCR. The gene-specific primers and TaqMan probes were as follows: proteoglycan core protein forward primer, 5'-CGCCTACCAGGACAAGGT-3'; proteoglycan core protein reverse primer, 5'-GCGCAGGCTCTGGATCTC-3'; proteoglycan core protein probe, 5'-(FAM)TCGCTGCCCAACTAC(TAMRA)-3'; collagen type II forward primer, 5'-CCTGTGCGACGACATAA-TCTGT-3'; collagen type II reverse primer, 5'-GCAGTGGCGAGGTCAGTAG-3'; collagen type II probe, 5'-(FAM) CAGTCCTTGGTGTCTTC (TAMRA)-3' (9). For quantification of changes in gene expression, we used the comparative Ct method to calculate the relative-fold changes normalized against the ribosomal RNA (23). Each value is the mean of 3 samples, and each sample was assayed in duplicate. Gene expression levels were compared with those of untreated control knees.

Treatment and assessment of rabbit OA model

OA was induced in the left knee joints of 33 Japanese white rabbits by performing unilateral transection of the anterior cruciate ligament (ACLT) (24). After ACLT, rabbits were allowed to move freely in the cages. The 33 ACLT rabbits were divided into 4 groups. In the control group (n=8), 300 μL of PBS was injected into the ACLT knee. In the PBS-microspheres group (PBS-M group; n=9), 5 mg of gelatin hydrogel microspheres containing 100 μL of PBS was injected into the ACLT knee. In the PRP-only group (n=8), 100 μL of PRP was injected into the ACLT knee. In the PRP-microspheres group (PRP-M group; n=8), 5 mg of gelatin hydrogel microspheres containing 100 μL of PRP was injected into the ACLT knee. Each injection was performed twice (4 and 7 weeks after ACLT).

Gross morphological changes in the femoral condyles were evaluated 10 weeks after ACLT. The previous study showed that the OA grade of the femoral condyle and opposing tibial plateau were correlated in the ACLT knees (25). Therefore, we evaluated the cartilage lesion of femoral condyles. Findings were classified by 6 grades (grade 1 = intact articular surface; grade 2 = minimal fibrillation; grade 3 = overt fibrillation; grade 4a = erosion of 0-2 mm; grade 4b = erosion of 2-5 mm; and grade 4c = erosion of >5 mm), and the grades were scored from 0 to 5 (24). Three well-trained orthopaedic surgeons evaluated samples independently, and grades were decided by agreement of at least 2 of these surgeons.

For histological examination, the femoral condyles and patella were fixed in 10% formaldehyde for 10 days and decalcified for 7 days with formic acid. After decalcification, the lateral femoral condyles and the synovium surrounding the patella were cut along the sagittal plane, and were then embedded in paraffin. Next, the samples were cut into 3-μm-thick sections, which were stained with hematoxylin and eosin (H&E) and Safranin O. The cartilage was evaluated using Mankin's histological-histochemical grading (26).

Statistical analysis

All results are expressed as the mean±SEM. The differences between the groups in the platelet count and GAG content were determined by one-way ANOVA, and then differences between individual groups were analyzed using Tukey multiple comparison tests. The differences between the groups in

the gross morphological OA score and Mankin's histochemical grading determined by one-way ANOVA, and then differences between individual groups were analyzed using non-parametric Mann-Whitney U test. *P*-values less than 0.05 were considered to indicate statistical significance.

Results

Platelet count of PRP

The number of platelets contained in PPP, whole blood and PRP was $6.0\pm0.5\times10^4/\mu L$, $27.5\pm3.8\times10^4/\mu l$ and $1081.0\pm149.9\times10^4/\mu l$, respectively.

The number of platelets contained in PRP was significantly greater than that of PPP and whole blood ($p<0.01$). The purified PRP contained about 39.4 times the number of platelets contained in whole blood.

Anabolic effect of PRP on cultured chondrocytes in alginate beads

After 3 days of culture, GAG concentrations of the PPP group and PRP group were 116% and 126% greater than that of the control group, respectively. The supernatant of thrombin-activated PPP and PRP significantly increased the GAG concentration, compared to the control group, but there was no significant difference between the PPP and PRP groups. After 7 days of culture, the GAG concentration of the PRP group was 106% greater than the GAG concentration of the control group, but the GAG concentration of the PPP group was the same as that of the control group (Fig. 1). The GAG concentration of the PRP group was significantly higher than those of the control and PPP groups. These results indicate that PRP can significantly increase GAG production by cultured chondrocytes.

In vivo anabolic effects of PRP and gelatin hydrogel microspheres on extracellular matrix (ECM) gene expression

In the cartilage from knee joints treated with PRP-containing microspheres (PRP-M), on days 3 and 7 after administration, the level of proteoglycan core protein mRNA was 2.3- and 1.3-fold greater than that of the control cartilage, respectively. In the cartilage treated with PRP-only, there was no marked change in the level of proteoglycan core protein mRNA, compared to the control cartilage (Fig. 2). These results indicated that administration of PRP-M accelerated the metabolism of articular cartilage, and that this effect continued for 1 week. In contrast, expression of collagen type II mRNA had not markedly changed 7 days after administration of PRP-M, compared to the control cartilage (data not shown).

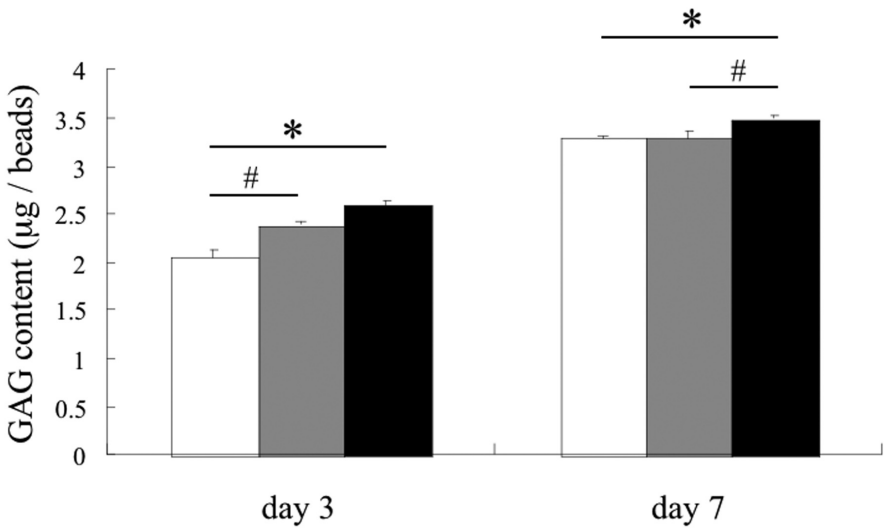

Fig. 1. Amount of glycosaminoglycan (GAG) in alginate beads. The GAG content was quantified using the dimethylmethylene blue (DMB) assay, after 3 and 7 days of incubation with 3% supernatant of thrombin-activated PRP or PPP. Bars represent GAG content (open bars; control group, gray bars; PPP group, black bars; PRP group) (*n=6 in each group*). Values are mean±SEM. * = $p<0.01$; #=$p<0.05$.

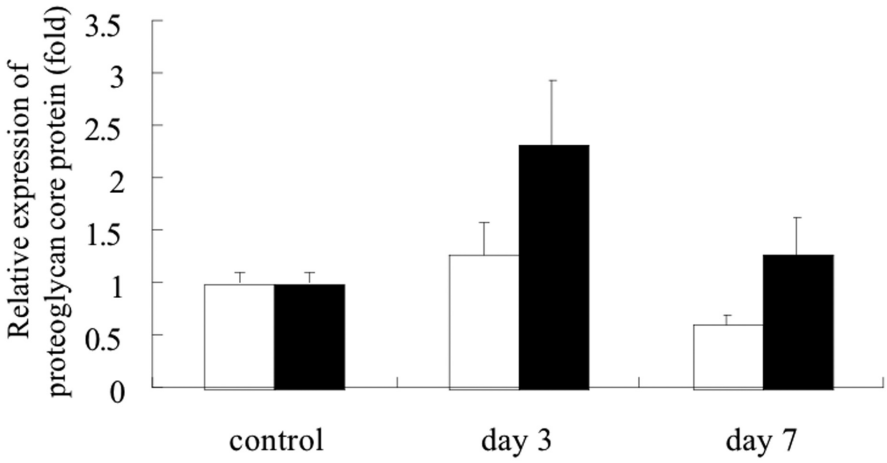

Fig. 2. Time course change of proteoglycan core protein expression after administration of PRP in gelatin hydrogel microspheres *in vivo*. We injected 5 mg of gelatin hydrogel microspheres containing 100 μl of PRP (PRP-M) or 100 μl of PRP (PRP-only) into the left knee joint. At 3 and 7 days after administration, the relative mRNA expression of proteoglycan core protein was examined by real-time PCR. Open bar represents the relative expression of mRNA in the cartilage of the knee joint injected PRP-only. Closed bar represents the relative expression of mRNA in the cartilage of the knee joint injected with PRP-M. Values are mean±SEM.

Gross morphological findings of rabbit OA model

Macroscopically, severe OA (erosion to subchondral bone) was observed in 25% of the control group, 33% of the PBS-M group and 25% of the PRP-only group in lateral femoral condyle. Cartilage erosion also occurred on the tibial plateau of knees in which severe OA were observed on femoral condyles. However, none (0%) of the joints in the PRP-M group showed severe OA. The gross morphological OA score (mea±SEM) was 1.88±0.52

in the control group, 1.77±0.81 in the PBS-M group, 1.50±0.60 in the PRP-only group, and 0.63±0.26 in the PRP-M group. The gross morphological OA score of the PRP-M group was significantly lower than that of the control group ($p<0.05$) (Fig. 3). No marked synovial inflammation or osteophyte formation was observed in the PRP-only or PRP-M group. In some joints with severe OA, synovial thickening was observed in infrapatellar area. However, there were no marked differences between the groups.

Histological examination of rabbit OA model

In some joints of control, PBS-M and PRP-only groups with severe OA, the surface of the articular cartilage was irregular, and the cartilage thickness was decreased in lateral condyles. The laminar structure of articular chondrocytes was destroyed, and clusters of chondrocytes were observed (Fig. 4A, B, C). These findings were observed in all tissues that were macroscopically confirmed to be grade 4. In contrast, in apparently normal cartilage in the PRP-M group, the intensity of Safranin O staining decreased slightly in the surface layer of the articular cartilage, but the surface of the articular cartilage was generally smooth and the laminar structure was maintained (Fig. 4D). None of the joints in the PRP-M group showed severe degeneration of the cartilage. In the PRP-M group, OA was significantly less severe (as indicated by Mankin's histochemical grading) than in the control group ($p<0.05$) (Fig. 4E).

The synovium of joints in the control and PBS-M groups with severe OA exhibited proliferation of synovial cells, infiltration by inflammatory cells, and hypervascularity. Vascular invasion and infiltration by inflammatory cells were also observed in joint synovium of the PRP-only and PRP-M groups, and there were no marked differences between those 2 groups and the control or PBS-M group (data not shown). These findings suggested that inflammation of the synovium was not due to administration of PRP or gelatin hydrogel microspheres, but rather was due to OA.

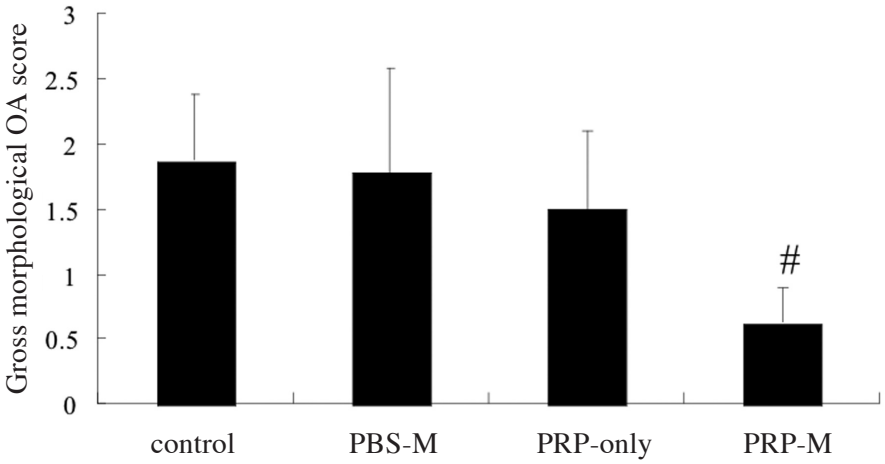

Fig. 3. Gross morphological osteoarthritis (OA) scores (range, 0-5) of rabbit knee joints at 10 weeks after anterior cruciate ligament transaction (ACLT). Values are mean±SEM. Control=phosphate-buffered saline (PBS) injected group (n=8); PBS-M=5 mg of microspheres containing PBS (n=9); PRP-only=100 µl of PRP (n=8); PRP-M=5 mg of microspheres containing 100 µl of PRP (n=8). #=$p<0.05$ versus control group.

Discussion

Previous studies in which growth factors have been administered using various sustained release systems (such as continuous infusion and gene therapy), to assess their effects on repair of localized articular cartilage injury, have generally produced good results (27-29). On the other hand, it is difficult to maintain the effects of growth factors throughout the widespread degenerative cartilage of an OA joint. Maintenance of therapeutic concentrations of growth factors typically requires the administration of large amounts of growth factors and frequent injections. However, administration of such large amounts of growth factors can have unwanted side effects in joints. For example, bFGF plays an important role in the progression of joint inflammation (30). Excessive administration of TGF-β has induced synovial hyperplasia and osteophyte formation (31). Therefore, it is important to precisely regulate the concentration of growth factors in joints. Consequently, there has been intensive research aimed at developing effective drug delivery systems. Some biomaterials have been used as the sustained release systems for therapeutic agents. We have previously reported on our research into gelatin hydrogel microspheres (32). Gelatin hydrogel microspheres can be impregnated with growth factors, which are then continuously released as the gelatin degrades *in vivo*. This system does not require complicated techniques, and can be administered via intraarticular injection.

In the present study, we used PRP as a safe and effective source of growth factors. A great advantage of PRP is that it is made from autologous blood, and thus does not induce an immune reaction (33). In addition, previous studies indicate that the biological effect of multiple growth factors on tissue regeneration and repair is greater than that of a single growth factor (15). In this study, PRP was prepared according to the method which is described by Nagae *et al*. The PRP contained 35.0 times as many platelets as whole blood, and that produced 23.0 times as much TGF-β1 and 70 times as much PDGF-BB as whole blood (16). It has been reported that TGF-β plays a significant role in promotion of chondrocyte anabolism (7). Moreover, PDGF stimulates chondrocyte proliferation and upregulates proteoglycan synthesis (8). Importantly, intraarticular injection of PDGF-BB did not induce inflammation or fibrosis (34). Studies indicate that platelets present in PRP secrete several growth factors as a result of α granule degradation, and exhibit various biological activities (35). Thus, specific substances with certain properties were needed to activate the growth

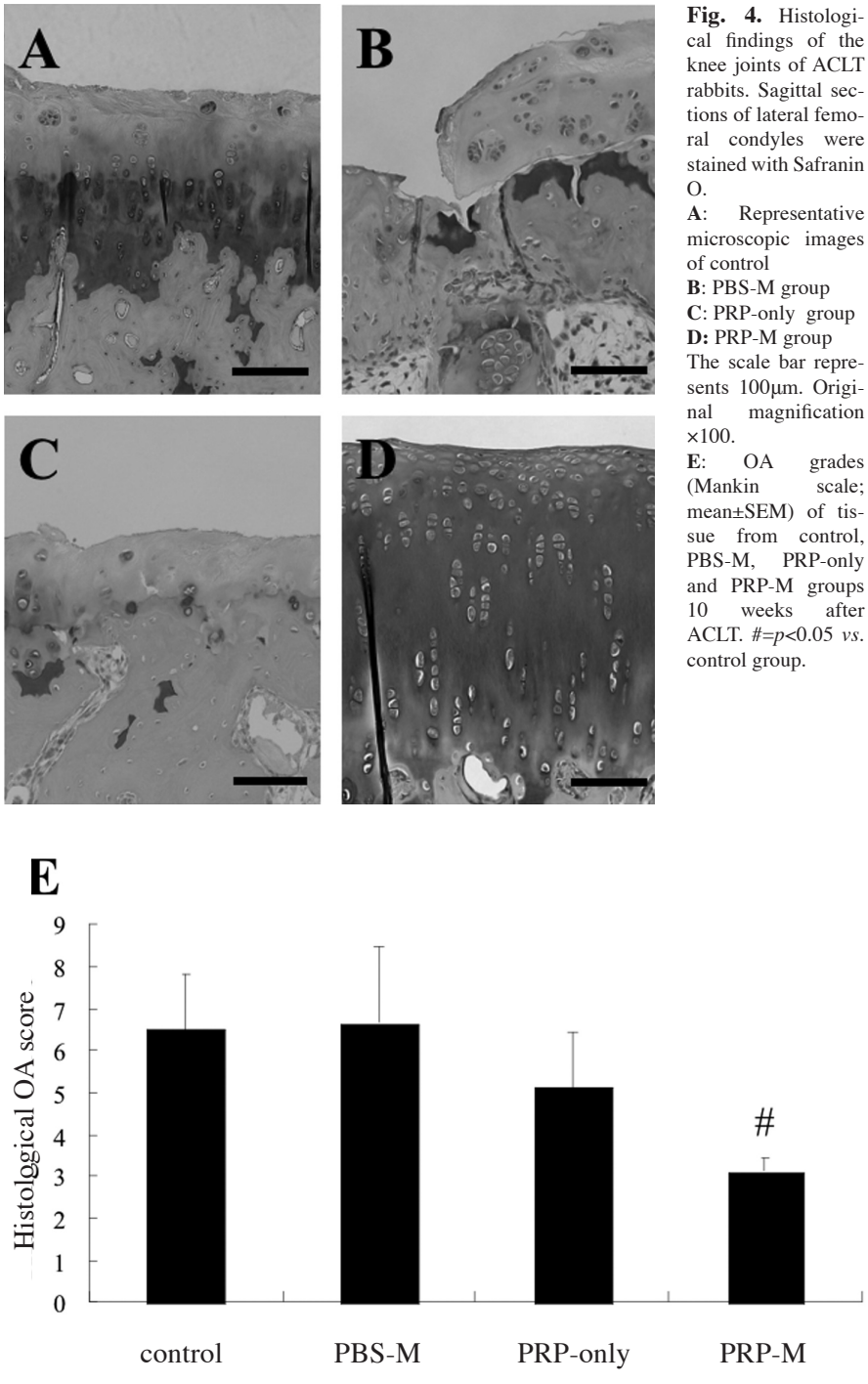

Fig. 4. Histological findings of the knee joints of ACLT rabbits. Sagittal sections of lateral femoral condyles were stained with Safranin O.
A: Representative microscopic images of control
B: PBS-M group
C: PRP-only group
D: PRP-M group
The scale bar represents 100μm. Original magnification ×100.
E: OA grades (Mankin scale; mean±SEM) of tissue from control, PBS-M, PRP-only and PRP-M groups 10 weeks after ACLT. *#=p*<0.05 *vs.* control group.

factors contained in PRP. Hokugo *et al*. reported that gelatin hydrogel was capable of activating platelet growth factors such as TGF-β1 and PDGF (15). Given these findings, we concluded that PRP and dehydrothermally cross-linked gelatin hydrogel microspheres are harmless and effective materials.

It is well known that joint bleeding or hemarthrosis leads to severe cartilage damage. Rosendaal *et al*. reported that monocytes or macrophages within the mononuclear cell population, together with red blood cells present in the blood, can cause irreversible inhibition of matrix synthesis (36, 37). However, the PRP used in the present study contained only a high concentration of platelets, and no other blood components (16). Thus, there is no reason to expect PRP to have any adverse effects on cartilage. In the present study, PRP increased GAG synthesis of chondrocytes cultured in alginate beads. The present results suggest that this stimulative effect was due to the synergistic effects of growth factors in PRP. In the knee joint, expression of proteoglycan core protein mRNA in the articular cartilage increased after administration of microspheres containing PRP. In addition, only administration of microspheres containing PRP had a preventive effect against OA. These findings suggest that the preventive effect of PRP-microspheres against OA progression is the result of stimulation of cartilage matrix metabolism, which is induced by sustained release of growth factors contained in PRP. On the other hand, when PRP was administered independently, growth factors contained in PRP were thought to be degraded immediately and did not have stimulative effect on cartilage. As a result, PRP without microspheres did not have preventive effect against OA. The present stimulative effect on matrix metabolism and preventive effect against OA of microspheres containing PRP were slightly less pronounced than those previously reported for sustained release of bFGF (9). However, the safety of using PRP and gelatin hydrogel makes it particularly easy to apply to clinical settings.

In present study, intraarticular injection of PRP with gelatin hydrogel had inhibitory effects on OA progression in rabbit model. However, we did not administer this substance to the joints affected severe OA. Therefore, the regenerative effects of this treatment against advanced OA are unclear, and require further study. Furthermore, the possibility of adverse effects of this treatment, such as osteophyte formation or synovial inflammation, must be thoroughly explored. However, the present results suggest that intraarticular injection of PRP in gelatin hydrogel could be a simple and safe conservative treatment for OA.

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