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연골재생을 위한 혈소판 풍부 혈장의 보관조건, 조성 및 적용

The storage condition, composition and application of platelet-rich plasma for cartilage regeneration

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서울대학교 대학원

의학과 정형외과학 전공

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The storage condition, composition and application of platelet-rich plasma for cartilage regeneration

by

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A thesis submitted to the Department of Medicine in partial fulfillment of the requirements for the Degree of Doctor of Philosophy in Medicine (Orthopedic Surgery)
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Abstract

The storage condition, composition and application of platelet-rich plasma for cartilage regeneration

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Purpose: The purpose of this study was to investigate the practical use of platelet-rich plasma (PRP) for cartilage regeneration. For this purpose, the author evaluated 1) the concentrations of growth factors in PRP, depending on the storage conditions; 2) the effects of leukocyte concentrations in PRP on the proliferation and chondrogenesis of synovial membrane-derived mesenchymal stem cells (SDSCs) and chondrocytes; and 3) *in vivo* effectiveness of PRP with a hyaluronic acid (HA) hydrogel for cartilage regeneration.

Materials and Methods: To evaluate growth factor concentration in PRP based on storage conditions, PRP samples were stored at 24℃ (room temperature group), 4℃ (refrigerator group), and −70℃ (deep-freezer group). In each temperature, four aliquots were prepared based on the time of analysis (immediately, 1, 3, 7 days after preparation). After storage, concentrations of platelet-derived growth factor-AA (PDGF-AA), transforming growth factor-β (TGF-β), vascular endothelial growth factor (VEGF), insulin-like growth factor-1 (IGF-1), and fibroblast growth factor-basic (FGF-B) were assessed with/without activation using Quantikine colorimetric sandwich immunoassay kits. PRP was activated with 10% Triton-X

for PDGF-AA, VEGF, FGF-B, IGF-1 measurement and sonication for TGF-β1 measurement.

To evaluate the effect of leukocyte concentrations in PRP on the proliferation and chondrogenesis of SDSCs and chondrocytes, we prepared two PRP formulations: (1) leukocyte-poor PRP (P-PRP) and (2) leukocyte-rich PRP (L-PRP). SDSCs and chondrocytes were obtained by enzymatic digestion of synovial tissues and the cartilage from a knee joint undergoing total knee arthroplasty. The primary cells were expanded in Dulbecco's modified Eagle's medium supplemented with fetal bovine serum (FBS), L-PRP, or P-PRP. Cell proliferation was measured using the MTT assay. SDSCs and chondrocytes were cultured with chondrogenic medium (CM) only, CM with L-PRP, or CM with P-PRP using a high-density pellet culture system for 3 weeks. The expression of chondrogenesis-related genes (type II collagen, type X collagen, aggrecan, and SOX-9) were analyzed by RT-qPCR. Pellets were stained with safranin-O for proteoglycan detection. The expression of type II collagen was detected by immunohistochemical staining.

To evaluate the effect of PRP in combination with the HA hydrogel on *in vivo* cartilage regeneration, eighteen rabbit osteochondral defect models (round shape defect in the femoral trochlear groove with 4 mm in radius and 3 mm in depth) were made and divided into 3 groups: control group, in which the defect was left untreated; HA group, in which the defect was filled with the HA hydrogel; and HA-PRP group, in which the defect was filled with HA hydrogel and PRP. After 12 weeks, tissue specimens were assessed by macroscopic examination, histological evaluation, and by measuring the glycosaminoglycan (GAG) content.

Results: Regarding growth factor concentration in PRP based on storage conditions, PDGF-AA concentration was highest on day 7 in the room temperature group without activation. With activation, the concentration of PDGF-AA was

constant over the observation period at all temperatures. Without activation, the TGF-β1 concentration remained negligible over the observation period at all temperatures. However, with activation, TGF-β1 concentration was highest on day 7 at all temperatures. Over the observation period, VEGF and IGF-1 concentration were constant with and without activation at all temperatures. Without activation, FGF-B concentration was highest on day 7 in the deep-freezer group. With activation, FGF-B concentration decreased after day 1 in the room temperature group.

Regarding the effect of the leukocyte concentration in PRP on the proliferation and chondrogenesis of SDSCs and chondrocytes, L-PRP showed a stronger proliferative effect on both types of cells than P-PRP and FBS. Meanwhile, RT-qPCR revealed higher cartilage-related gene expression in SDSCs and chondrocytes in the P-PRP group compared with that in the L-PRP and CM groups. However, SDSCs and chondrocytes in both PRP groups showed weaker safranin-O staining than those in the CM group. In immunohistochemical analysis, positive staining of SDSCs were not observed in either of the PRP groups and staining of chondrocytes in both PRP groups were weaker than those in the CM group.

Regarding the effect of PRP combined with the HA hydrogel on *in vivo* cartilage regeneration, the macroscopic ICRS scores were not different among the three groups. The HA and HA-PRP groups showed significantly higher microscopic O'Driscoll scores and a significantly higher GAG content than the control group, although the values were not different between the HA and HA-PRP groups.

Conclusion: The growth factor concentrations significantly differed in PRP, depending on the storage temperature, duration of storage, and activation. Regardless of the leukocyte concentration, PRP showed a negative effect on chondrogenesis of SDSCs and chondrocytes. In addition, a combined use of the

HA hydrogel and PRP did not show better chondrogenic effects *in vivo* compared with those of the HA hydrogel alone. Considering the results of our study, a further study is necessary to clarify the ideal composition and application of PRP for cartilage regeneration.

Keywords: platelet rich plasma, storage, leukocyte, synovial stem cell, chondrogenesis, hyaluronic acid

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Introduction

Platelet-rich plasma (PRP) is a derivative of whole blood obtained by the process of centrifugation. It contains a high concentration of platelets that release several growth factors, such as platelet-derived growth factor (PDGF), transforming growth factor-beta (TGF-β), vascular endothelial growth factor (VEGF), insulin-like growth factor-1 (IGF-1), and fibroblast growth factor-basic (FGF-B) contained in the constituent alpha (α) granules upon activation (1-3). Recent studies have shown that these growth factors participate in tissue regeneration by fostering cellular growth, proliferation, and differentiation (3-5), leading to an increase in the popularity of PRP and its widespread application in orthopedic field.

However, the effectiveness of PRP in cartilage regeneration remains controversial due to inconsistent clinical outcomes. There are several reasons for this inconsistency, among which differences in the PRP storage, composition, and application are one of the most important aspects (6-9). Indeed, the method for effective use of PRP for cartilage regeneration has not been well established. Therefore, we investigated three important aspects for practical use of PRP, namely, its storage, composition, and application.

In the first part of this study, we evaluated the effect of PRP storage conditions on growth factor concentration. While PRP can be used immediately after its preparation, in practice, it may be stored for several reasons. One such reason is for modifying growth factor release kinetics. PRP contains several growth factors, each of which plays a unique role by acting on specific tissue components to promote tissue regeneration (10-13). Depending on the purpose of the PRP usage, some of the growth factors in PRP may be beneficial or harmful after application. Additionally, timing and duration of action of growth factors are important considerations for optimal effect.

Several studies (14-17) have reported that differences in storage conditions of time and temperature of PRP can alter growth factor release. Therefore, by controlling storage conditions of time and temperature, it may be possible to maximize or minimize the amount of specific growth factors in PRP, depending on the intended clinical use. Further, storage of PRP may be needed for the purpose of repeated injections at specific time intervals. Several studies (18, 19) have shown that repeated PRP injections are associated with better clinical outcomes than a single injection. Therefore, the effect of storage conditions, including time and temperature, on PRP needs to be elucidated.

Moreover, it is unclear whether activation of PRP, following a period of storage, prior to its use is beneficial. Activation of platelets releases growth factors in the α-granules and causes gelation of PRP, facilitating its application. Hence, some physicians prefer to activate PRP before its use. However, others prefer it without activation as *ex vivo* activation is associated with concerns of early washout of the released growth factors, whereas PRP activation by the native collagen of connective tissue *in vivo* results in slow release of growth factors (20-24). Thus, to clarify the effect of PRP, it is important to evaluate growth factor concentration with and without activation under different storage conditions.

While earlier studies (14-16, 25-27) have examined growth factor concentration, data on the effect of various storage conditions on the concentration of various growth factors in PRP are limited. In addition, none of these studies compared the effect of storage and activation on growth factor concentrations in PRP.

Therefore, the purpose of the first topic was to evaluate growth factor concentration in PRP based on storage conditions, with or without activation.

In the second part of this study, we evaluated the effect of leukocyte

concentrations in PRP on the proliferation and chondrogenesis of synovial membrane-derived mesenchymal stem cells (SDSCs) and chondrocytes. There are various methods of PRP preparation; however, research on the characteristics of PRP obtained by different methods is insufficient (26-28). In particular, differences in leukocyte concentrations in PRP may affect PRP properties (29-33), leading to different cartilage regeneration results. However, there is a paucity of the literature data regarding this aspect.

Furthermore, there is a growing interest in the use of mesenchymal stem cells (MSCs) for the treatment of articular cartilage damage. As multipotent cells, MSCs have the abilities to self-renew and differentiate into articular cartilage (34, 35). MSCs can be obtained from several tissues, such as the bone marrow, synovial membrane, and adipose tissue. Among them, SDSCs have higher proliferation and chondrogenic differentiation potential compared to MSCs derived from other tissues (36, 37). Moreover, because the synovial membrane is located near the articular cartilage of the knee, SDSCs can be easily obtained through a minimal incision, without donor site morbidity.

However, MSCs that are directly obtained from tissues are too small to be directly applied for cartilage repair. Therefore, before their clinical use, expansion of MSCs is necessary through *in vitro* culture. There are some reports (38-41) indicating that PRP enhances the proliferation and chondrogenesis of MSCs. However, most studies used stem cells obtained from the bone marrow or adipose tissue. Meanwhile, only one study (42) investigated the effect of PRP on SDSCs; however, a platelet concentrate was used in this study. To the best of our knowledge, there has been no study that evaluated the effect of the leukocyte concentration in PRP on the proliferation and chondrogenesis of SDSCs and chondrocytes. Therefore, the purpose of the second topic was to investigate the effect of leukocyte concentrations in PRP

on the proliferation and chondrogenesis of SDSCs and chondrocytes.

In the last part of this study, we evaluated the effect of PRP, in combination with a hyaluronic acid (HA) hydrogel, on *in vivo* cartilage regeneration. The articular surface is composed of the hyaline cartilage, which is unable to heal spontaneously, owing to its avascular nature and the inability to support chondrocyte migration to an injured site (43, 44). Various surgical procedures have been used for cartilage repair, including microfracture (45), osteochondral autograft or allograft transplants (OATs) (46), and autologous chondrocyte implantation (ACI) (47). The microfracture method is used for relatively small cartilage lesions (< 2 cm^2), whereas OATs or ACI is used for larger cartilage lesions (> 2 cm^2). However, the microfracture technique has the disadvantage of regenerating a fibrous cartilage rather than the hyaline cartilage. Furthermore, OATs and ACI have the disadvantages of causing donor site morbidity and requiring two invasive surgeries, respectively (48, 49).

Recent studies (50-53) have shown superior results of scaffold-assisted cartilage regeneration techniques. Scaffolds may provide a suitable environment for bone marrow stem cell (BMSC) infiltration, proliferation, and differentiation, thus leading to better cartilage regeneration (54, 55). Among various currently used scaffolds, HA-based hydrogels are especially promising for cartilage regeneration because HA is a major natural macromolecular component of the extracellular matrix (ECM) of the articular cartilage and has excellent biocompatibility and mechanical properties (56-58). The benefits of scaffold application for cartilage regeneration may be further enhanced by the use of PRP, owing to the presence of various growth factors, which can stimulate the proliferation and chondrogenesis of BMSCs and chondrocytes (10-13). Therefore, it is important to ensure that adding PRP

would lead to superior cartilage healing compared with that supported by a hydrogel alone. Hence, the purpose of the last topic was to evaluate the effect of PRP, in combination with an HA hydrogel, on *in vivo* cartilage regeneration.

Chapter I.

Effect of storage conditions and activation on growth factor concentration in platelet-rich plasma

I-1. Materials and Methods

Preparation of PRP

From donated human fresh blood from six donors (Asian males, 34.2 ± 5.3; range: 30–43 years), 320 ml of peripheral venous blood was collected and mixed with the anticoagulant 10% acid-citrate dextrose A (ACD-A). To prepare PRP, 25ml of this blood was transferred to the PRP separation kit (Prosys PRP Kit, Prodizen, Korea) and centrifuged at 2,800 rpm for 3 min. The upper layer of plasma was separated and subjected to a second round of centrifugation at 3,300 rpm for 3 min following which, the lower 2ml of plasma was collected as PRP.

Storage and Activation of PRP

The PRP samples from each donor were stored at three different storage temperatures: 24℃ (room temperature group), 4℃ (refrigerator group), and −70℃ (deep-freezer group). From the PRP stored at each temperature, four aliquots were prepared based on the time of analysis. While one aliquot was analyzed immediately after preparation, the remaining three were stored for 1, 3, and 7 days. After the specified storage time, the aliquots in the deep-freezer were thawed to room temperature prior to analysis. After storage, PRP activation was done with 10% Triton-X followed by incubation for 1 h at 37℃ for PDGF-AA, VEGF, FGF-B, and IGF-1 measurement and sonication (Digital Sonifier 450; Branson, Danbury, CT) for TGF-β1 measurement. Sonication was performed with a total of 10 repetitions of the sonicating-cooling cycle (1 min for 1 cycle) for 10 min.

Evaluation of Platelet Count and Growth Factor Concentrations in PRP

The platelet, white blood cell (WBC), and red blood cell (RBC) counts of PRP were performed before the analysis of serial platelet count and growth factor concentration, using a cell count machine (ADVIA 2120i; Siemens, Erlangen, Germany). After storage, the samples were assayed in duplicate, and the platelet count and concentrations of growth factors in each aliquot were assessed. The platelet count of each aliquot was determined using a cell counter machine. Concentrations of growth factors PDGF-AA, TGF-β1, VEGF, IGF-1, and FGF-B in each aliquot were measured with and without activation, using commercially available Quantikine colorimetric sandwich immunoassay kits (R&D Systems, Minneapolis, MN).

Statistical Analysis

Data are presented as mean and standard deviation (SD). The Friedman test was used to determine whether the platelet count and growth factor concentration changed significantly during the time of storage in each group. The concentrations of growth factors at the end of each specified storage time were analyzed using the Kruskal–Wallis test (nonparametric one-way analysis of variance). Bonferroni post hoc analysis was used to compare the values of samples at each of the storage temperatures. $p < 0.05$ was considered significant.

I-2. Results

Cellular Concentration of PRP

The mean platelet, WBC, and RBC counts of PRP were 1,588 ± 176.9 × 10^3/μl, 13.5±3.3 × 10^3/μl, 1.5±1.0 × 10^6/μl, respectively.

Platelet Count

In the deep-freezer group, the platelet count was significantly decreased on day 1 from 1,588 ± 176.9 × 10^3/μl to 1,325 ± 278.2 × 10^3/μl ($p < 0.05$), but remained constant thereafter until day 7. The platelet counts in the room temperature and refrigerator groups stayed constant (Figure 1).

Growth Factor Concentrations Under Various Storage Conditions and Activation

PDGF-AA

Without activation, the release of PDGF-AA during storage was constant over the observation period in the refrigerator and deep-freezer groups. However, in the room temperature group, the highest PDGF-AA concentration was observed on day 7 ($p < 0.05$), which was significantly greater than that in the refrigerator

($p < 0.05$) and deep-freezer ($p < 0.05$) groups. With activation, the release of PDGF-AA was constant over the observation period at all three temperatures (Figure 2).

TGF-β1

Without activation, the release of TGF-β1 during storage were negligible over the observation period at all three temperatures. With activation, the concentration of TGF-β1 increased at each time point and was highest on day 7 at all three temperatures ($p < 0.05$ for all three temperatures) (Figure 3).

VEGF and IGF-1

Without activation, the release patterns of VEGF (Figure 4) and IGF-1 (Figure 5) during storage were similar to the levels remaining constant over the

observation period at all three temperatures. With activation, the release of VEGF and IGF-1 were also constant over the observation period at all three temperatures.

FGF-B

Without activation, the release of FGF-B during storage significantly increased in the deep-freezer group, with levels reached highest concentration on day 7 ($p < 0.05$), whereas FGF-B levels were negligible in the room temperature and refrigerator groups. Therefore, FGF-B concentration in the deep-freezer was significantly higher on days 1, 3, and 7 than that in the room temperature ($p < 0.05$ on days 1, 3, and 7) and refrigerator ($p < 0.05$ on days 1, 3, and 7) groups. With activation, the release of FGF-B remained constant over the observation period in both the refrigerator and deep-freezer groups, while significantly decreasing after day 1 in the room temperature group ($p < 0.05$), such that on days 3 and 7, FGF-B concentration was significantly lower in the room temperature group than that in the refrigerator ($p < 0.05$ on days 3 and 7) and deep-freezer groups ($p < 0.05$ on days 3 and 7) (Figure 6).

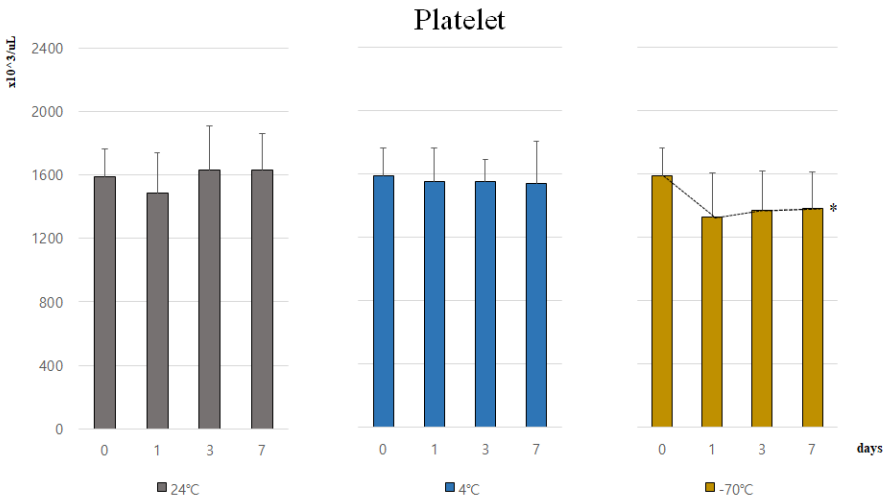

Figure 1. Changes in platelet count at different storage temperatures. The bars show the mean platelet count at each time point of the specified temperature (n = 6), vertical lines represent standard errors of the means, (*) represents statistically significant time-dependent release pattern in each group.

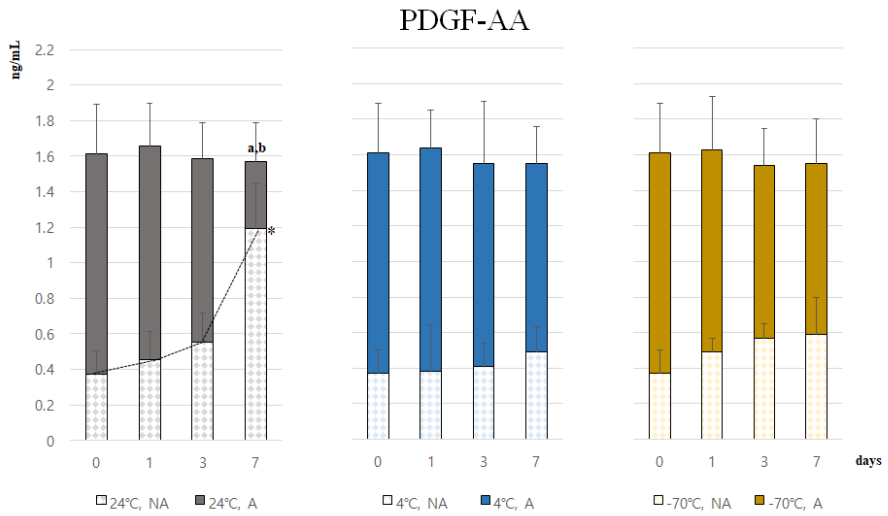

Figure 2. Growth factor release profile of platelet-derived growth factor (PDGF)-AA at different storage temperatures without (dotted bars) and with activation (solid bars). The bars show the mean values of growth factors at each time point of the specified temperature group (n = 6): dotted bars show PDGF-AA concentration without activation and solid bars show total amount of PDGF-AA release with activation. Vertical lines represent standard errors of the means, (*) represents statistically significant time-dependent changes in each group, (a) represents $p < 0.05$ compared with the refrigerator group, (b) represents $p < 0.05$ compared with the deep-freezer group at the same time points. NA; Not activated, A; Activated.

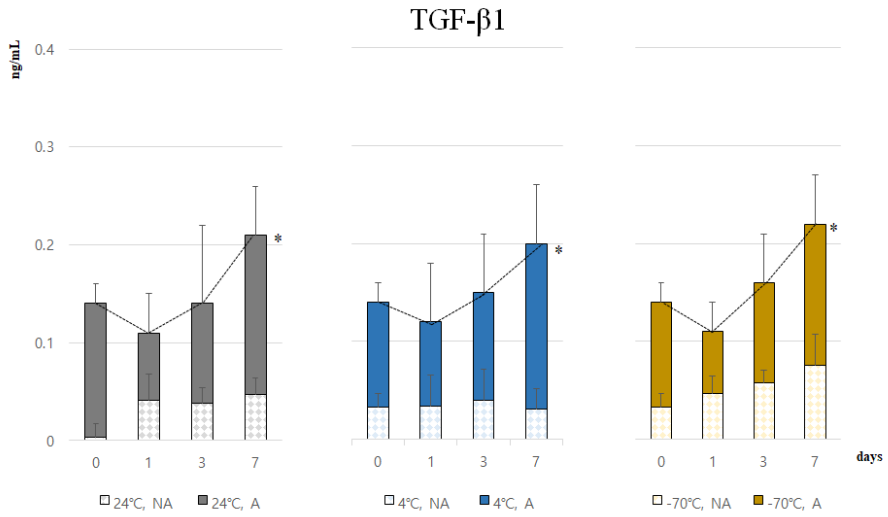

Figure 3. Growth factor concentration of transforming growth factor (TGF)-β1 at different storage temperatures without (dotted bars) and with activation (solid bars). The bars show the mean values of growth factors at each time point of the specified temperature group (n = 6): dotted bars show TGF-β1 concentration before activation and solid bars show total amount of TGF-β1 release with activation. Vertical lines represent standard errors of the means, (*) represents statistically significant time-dependent changes in each group. NA; Not activated, A; Activated.

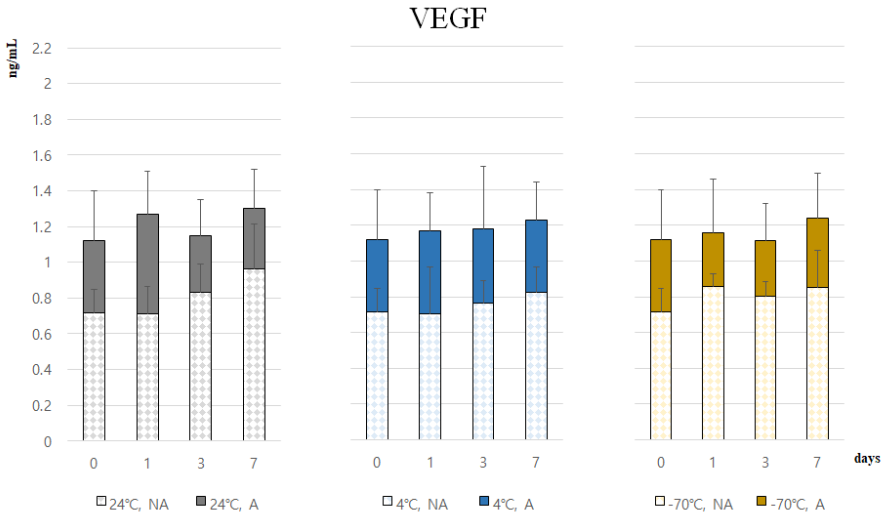

Figure 4. Growth factor concentration of vascular endothelial growth factor (VEGF) at different storage temperatures without (dotted bars) and with activation (solid bars). The bars show the mean values of growth factors at each time point of the specified temperature group (n=6): dotted bars show VEGF concentration without activation and solid bars show total amount of VEGF release with activation. Vertical lines represent standard errors of the means. NA; Not activated, A; Activated.

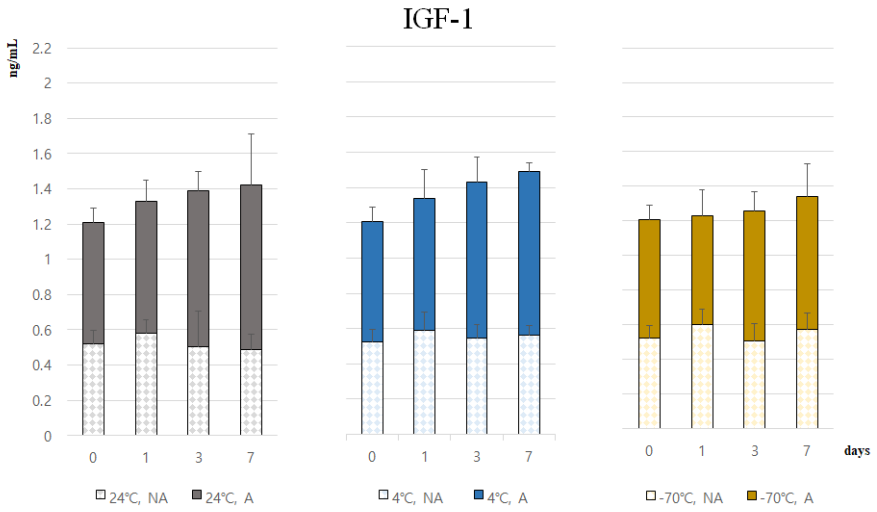

Figure 5. Growth factor concentration of insulin-like growth factor-1 (IGF-1) at different storage temperatures without (dotted bars) and with activation (solid bars). The bars show the mean values of growth factors at each time point of the specified temperature group (n=6): dotted bars show IGF-1 concentration without activation and solid bars show total amount of IGF-1 release with activation. Vertical lines represent standard errors of the means. NA; Not activated, A; Activated.

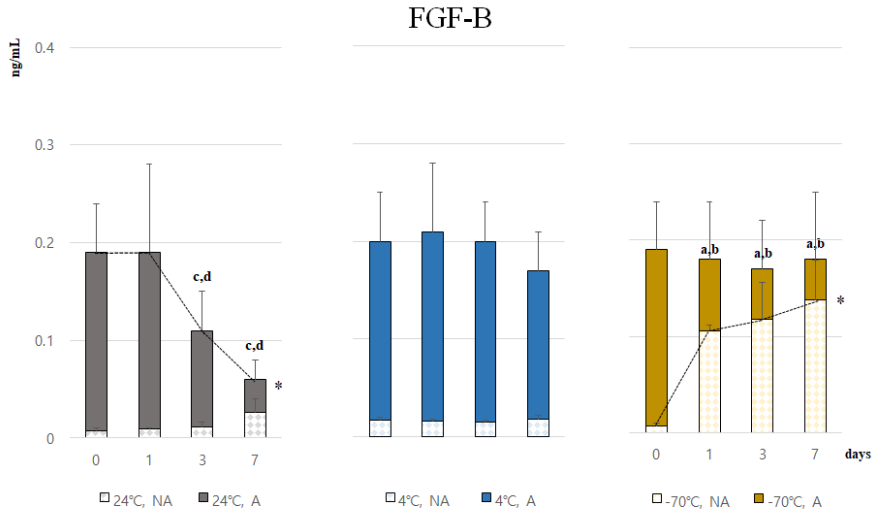

Figure 6. Growth factor concentration of fibroblast growth factor-basic (FGF-B) at different storage temperatures without (dotted bars) and with activation (solid bars). The bars show the mean values of growth factors at each time point of the specified temperature group (n = 6): dotted bars show FGF-B concentration without activation and solid bars show total amount of FGF-B release with activation. Vertical lines represent standard errors of the means, (*) represents statistically significant time-dependent changes in each group. (a) represents $p < 0.05$ compared with the room temperature group and (b) represents $p < 0.05$ compared with the refrigerator group at the same time points. (c) represents $p < 0.05$ compared with the refrigerator group and (d) represents $p < 0.05$ compared with the deep-freezer group at the same time points. NA; Not activated, A; Activated.

I-3. Discussion

This study found that growth factor concentration in PRP differed significantly based on storage temperature, duration of storage, and method of *ex vivo* activation used in this study. Without activation, while the concentration of TGF-β1 was negligible, that of VEGF and IGF-1 remained constant over the seven-day observation period. Also, PDGF-AA in the room temperature group and FGF-B in the deep-freezer group reached their highest concentrations on day 7. With activation, most growth factors showed a constant release pattern over 7 days, with TGF-β1 reaching its highest concentration on day 7. An exception to this was FGF-B, which showed poor release pattern in the room temperature group. Therefore, depending on the intended clinical use, it is necessary to consider the role of appropriate storage conditions and activation to maximize the effect of PRP.

Care should be taken when interpreting the growth factor concentration of PRP stored in the deep-freezer. Unlike PRP stored at room temperature or in the refrigerator, thawing is necessary to analyze growth factors in PRP stored in the deep-freezer. Although this process does not involve a repeated freeze/thaw cycle, it can still result in unintentional PRP activation. In our study, platelet count and FGF-B concentration were greatly influenced by the thawing process. Platelet count decreased significantly on day 1 in PRP stored in the deep-freezer, possibly owing to cell lysis during thawing. Moreover, before iatrogenic activation, the concentration of FGF-B significantly increased on day 1 in PRP stored in the deep-freezer, which may be attributed to the activation effect of thawing. With regard to the concentrations of other growth factors, there was no significant increase between day 0 and day 1 before iatrogenic activation, suggesting that the activation effect of thawing was nonsignificant as compared to that seen in FGF-B.

Among the various growth factors contained in the α-granules, PDGF-AA, TGF-β1, VEGF, IGF-1, and FGF-B play key roles in tissue regeneration (59, 60). Given their important roles in tissue regeneration, we selected the aforementioned growth factors to be evaluated in our study. The growth factor concentration was assessed at four time points: 0, 1, 3, and 7 days after storage. Considering that the life span of platelets is 8 to 9 days, we assumed that the day 1 assay would detect early depletion of growth factors, day 3 assay would detect intermediate depletion, and day 7 assay would confirm sustained release of growth factors.

While PRP can be used immediately after its preparation, in practice, it may be needed to be stored until the time of its use. It is known that platelets remain active for 5 to 7 days at room temperature or up to 10 days at temperatures of 0°C to 6°C (61, 62). However, the number of studies dealing with growth factor release kinetics in PRP stored under different conditions is limited. Bausset et al. (63) reported that the concentrations of PDGF-AB and VEGF were well-sustained at room temperature up to 6 hours. Similarly, Wilson et al. (17) reported that TGF-β1 could be used without loss of activity up to 4 hours at room temperature. There are studies that observed changes in growth factor concentration over a few days. Moore et al. (15) measured the levels of PDGF-BB over 8 days and showed that PRP intended for tissue regeneration could be stored at room temperature for at least 5 days, without loss of efficacy. Similarly, Wen et al. (16) reported that the concentrations of VEGF, epidermal growth factor (EGF), FGF-B, IGF-1, and PDGF-AA were not only sustained but also showed an increase after 7 days at storage temperature of 22°C. However, Hosnuter et al. (14) found that the levels of EGF, VEGF, PDGF, IGF-1, and TGF-β1 were significantly decreased after 7 days in PRP stored at -20°C. In our study, with activation, the concentration

of all growth factors (except for FGF-B) were similar at each time point tested during 7 days of storage, regardless of storage temperature, with TGF-β1 reaching its highest concentration on day 7.

Activation of PRP causes degranulation of α-granules and triggers the clotting system through cleavage of fibrinogen resulting in gelation of PRP, which facilitates its application (21). For this purpose, many physicians prefer to activate PRP using various activation methods, such as use of calcium chloride, collagen, thrombin, calcium chloride/thrombin mixture, or freeze/thaw cycle (25, 27). However, these methods are associated with concerns of early washout of the released growth factors, prompting other physicians to use non-activated PRP, thereby relying on native collagen at the desired site to promote activation, resulting in slow growth factor release (20, 22-24). To clarify the effect of activation on growth factor release, we evaluated the growth factor concentration with and without activation in the present study. In accordance with the findings of Oh et al. (26), our study showed that some degree of growth factor existed before the intentional exogenous activation of PRP. This is possibly owing to pre-existed growth factor in normal plasma, inevitable activation during centrifugation in the preparation phase, or due to platelet aging and consequent loss of membrane integrity during the storage phase.

There are numerous PRP activation methods, each with a varying effect on the timing and amount of growth factors released. When calcium chloride or collagen is used for activation, a low level of endogenous thrombin is formed, enabling sustained growth factor release for a long period of time (25, 64). However, when thrombin is used as an activator, it rapidly aggregates platelets and results in immediate release of growth factors that lasts only for a brief period (65, 66). While it is known that the total amount of growth

factors released is affected by the activation method used, there is no consensus as to which method results in the release of the highest amount of specific growth factors. Kim et al. (67) compared four activation methods and reported that calcium chloride alone was the best activator for FGF-B, Triton-X and calcium chloride/thrombin mixture for PDGF, and thrombin/calcium chloride/collagen mixture for VEGF release. Regarding TGF-β1, there are several reports that the amount of TGF-β1 released by sonication is higher than that with other activation methods (68, 69). Liao et al. (68) reported in their systematic review that the concentration of TGF-β1 was 10 to 120 ng/mL when activation was done using thrombin or thrombin/calcium chloride mixture, while the levels were nearly three times as much (300 ng/mL) with the use of sonication. In this study, we aimed to measure the highest concentration of the growth factor present in PRP following activation, at each of the time points of interest. To this end, we performed a pilot study to determine the method of activation associated with the greatest amount of growth factors released. In this pilot study, three aliquots of 300 μL of human PRP from three donors were activated using calcium chloride/thrombin mixture (14 mg/mL of calcium chloride with 135 unit/mL of thrombin powder), 10% Triton-X, and sonication, respectively. The release of PDGF-AA and TGF-β1 was measured and we found that activation using Triton-X yielded the highest concentration of PDGF-AA, while sonication was associated with highest levels of TGF-β1. Therefore, we used these activation methods in the present study. However, the reason for the difference in best activator for specific growth factor is not well known. Considering that all growth factors are contained in the same α-granules in the platelet, it is certainly difficult to explain. However, the mechanism of growth factor release from α-granules is not a simple degranulation process. Before platelet

activation, growth factors in α-granules remain in an inactive form. However, after activation, α-granules fuse with the platelet membrane, and the inactive growth factors are converted to their bioactive forms via addition of histones and carbohydrate side chain and are finally released from the platelet (70, 71). We assume that the method of activation differently influenced the activation process of each growth factor and led to the difference in the total amount of growth factors measured. Nonetheless, further studies are warranted to investigate the exact mechanism for the observed difference in the best activator for specific growth factor.

This study has several limitations, which must be considered while interpreting the findings. First, the activation methods used in this study (determined from the pilot study) were chosen based on their ability to release the highest amount of PDGF-AA and TGF- β1 at the initial time point. Therefore, the activation protocol used here may not be the best protocol for later time points after storage and for other growth factors (except PDGF-AA and TGF- β1). Secondly, this study was conducted *in vitro*. However, growth factor concentrations are subjected to influences from various cellular interactions *in vivo*. Therefore, a further well-designed *in vivo* study is needed to verify the findings of our study. Third, properties of PRP may be influenced by inter-individual variability. The donors in our study were young male individuals from the Asian population, and growth factor release patterns might be different for older individuals, female individuals, and individuals of other ethnicities. Finally, the PRP preparation method used in this study yielded PRP containing some amount of white blood cells, which could have influenced growth factor concentration.

Chapter II.

The effect of leukocyte concentration in platelet-rich plasma on the proliferation and chondrogenesis of synovium-derived mesenchymal stem cell and chondrocytes

II-1. Materials and Methods

Preparation of P-PRP, L-PRP

Using fresh blood from six Asian male donors (35.1 ± 4.7 years; range: 32–43 years), we prepared two PRP formulations: (1) PRP with a very few leukocytes (Leukocyte poor PRP, P-PRP) and (2) PRP with high concentrations of leukocytes (Leukocyte rich PRP, L-PRP). To prepare these PRP formulations, 25 mL of whole blood was transferred to a Prosys PRP separation kit (Prodizen) and centrifuged at 2,800 rpm for 3 min. The upper layer of the plasma was separated with a buffy coat (L-PRP) or without a buffy coat (P-PRP) and subjected to a second round of centrifugation at 3,300 rpm for 3 min. Finally, the lower 2 mL of the plasma was collected as PRP. Platelet and WBC counts were estimated in both PRP formulations using a cell counting machine (ADVIA 2120i). To activate the plasma, we added a thrombin powder (135 U/mL) and calcium chloride (14 mg/mL).

Harvest of Synovial and Cartilage Tissues

Synovial tissues were harvested from the suprapatellar pouch, and cartilage tissues were harvested from the lateral femoral and tibial condyles of six female patients undergoing total knee arthroplasty. The average age of the patients was 72.2 years (range: 67–75 years). All patients had severe medial compartmental osteoarthritis (OA), and therefore, the cartilages were relatively intact in the lateral femoral and tibial condyles. The exclusion criteria were inflammatory arthritis such as rheumatoid arthritis, prior infective arthritis, and a major knee trauma.

Isolation and In Vitro Expansion of SDSCs and Chondrocytes

After removal of adipose and periosteal tissues, synovial tissues were finely minced in phosphate-buffered saline and then digested with 0.02% collagenase (Sigma–Aldrich, St. Louis, MO, USA) overnight at 37℃. The cartilage was digested with 0.2% pronase (Sigma–Aldrich) for 1 h and with 0.2% collagenase (Sigma–Aldrich) overnight at 37℃. The resultant cells were filtered through a 70-μm sieve to remove undigested tissue and then centrifuged at 1,500 rpm for 5 min. Afterward, the cells were cultured in low-glucose Dulbecco's modified Eagle's medium (LG-DMEM; Gibco, Paisley, UK) containing 10% fetal bovine serum (FBS) and a 1% antibiotic–antimycotic solution (100 U/mL penicillin, 100 mg/mL streptomycin, and 0.25 mg/mL amphotericin B; Gibco) in an incubator at 37℃ with 5% CO_2. The culture medium was changed every 3 days. When the passage 0 (P0) cell population reached 80–90% confluence (after 10–14 days), the cells were separated using trypsin–EDTA (0.25% trypsin, 0.53 mM EDTA; Life Technologies, Rockville, MD, USA) and plated at a density of 2×10^5 cells/150-mL dish (P1). After repeated passage, as described above, cells (P2) were used for pellet culture.

SDSC and Chondrocyte Proliferation Assay

Primary (P0) SDSCs and chondrocytes were seeded onto 96-well plates (Nunc, Roskilde, Denmark) at a density of 5×10^3 per well. Cells were grown in LG-DMEM supplemented with 10% FBS, P-PRP, or L-PRP for 48 h at 37°C with 5% CO_2. The PRP concentrations were selected based on our previous study (42). For proliferation analysis, cell viability was measured after 48 h using the MTT assay. Briefly, 20 mL of the CellTiter 96® AQ_{ueous} One Solution reagent (Promega, Fitchburg, WI, USA), containing the MTS tetrazolium

compound and an electron coupling reagent (phenazine ethosulfate), was added to each well containing 100 μL of LG-DMEM and incubated for 2 h. Absorbance was measured at 490 nm using a microplate reader (VersaMax; Molecular Devices, Sunnyvale, CA, USA), with the SoftMax® ver. 5.2 program (Molecular Devices). The assay was repeated three times.

In Vitro Chondrogenesis Using Pellet Culture

Cell suspensions (P2) containing 5×10^5 chondrocytes or SDSCs were centrifuged at 1,500 rpm for 5 min to obtain cell pellets. The cell pellets were cultured in chondrogenic medium (CM) or CM supplemented with 10% L-PRP or P-PRP. The name and the composition of the groups are shown in Table 1. The chemically defined CM was composed of LG-DMEM containing 0.1 mmol/L ascorbic acid 2-phosphate, 100 nmol dexamethasone, 40 μg/mL proline, 100 U/mL penicillin, 100 μg/mL streptomycin, ITS Premix (BD Biosciences, Bedford, MA, USA), and 10 ng/mL TGF-β1. The cell pellets were cultured at 37°C with 5% CO_2, and the medium was changed every other day. The cell pellets were harvested for evaluation of chondrogenesis after 21 days.

Reverse Transcription–Quantitative Polymerase Chain Reaction (RT-qPCR)

After 21 days in culture, total RNA was extracted from cell pellets using the TRIzol reagent (Invitrogen, Carlsbad, CA, USA), and complementary DNA was synthesized using the RevertAid™ first-strand cDNA synthesis kit (Thermo Scientific, Waltham, MA, USA). Expression of chondrogenic marker genes, including those encoding aggrecan, Sry-type high-mobility-group box transcription factor-9 (Sox-9), type II collagen, and type X collagen, was assessed using RT-qPCR. The Primer Express software version 1.5

(Applied Biosystems, Abingdon, UK) was used for RT-qPCR analysis, and glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) was used as a housekeeping gene for normalization of the results. Gene expression levels were analyzed using an ABI Prism 7000 sequence detection system with a relative standard curve method.

Histology and Immunohistochemistry

For histological evaluation of glycosaminoglycan (GAG) synthesis, cell pellets from each group were stained with safranin-O/fast green on day 21, as described in our previous study (72). The expression of type II collagen was analyzed by immunohistochemical staining on day 21, as described in our previous study (72). Positive results were defined as a dense brown color surrounded by a blue color.

Statistical Analysis

The Kruskal–Wallis test, followed by multiple significance analyses, involving the Mann–Whitney test, was used to compare the scores and gene expression levels among the groups. A value of $p < 0.05$ was considered significant.

Table 1. Name and medium composition of the groups

Group	Composition
CM group	Chondrogenic medium only
P-PRP group	Chondrogenic medium + 10% P-PRP
L-PRP group	Chondrogenic medium + 10% L-PRP

CM, chondrogenic medium; P-PRP; Leukocyte poor PRP; L-PRP, Leukocyte rich PRP

II-2. Results

Cellular Concentration of L-PRP, P-PRP

The mean platelet counts were not different between L-PRP and P-PRP (1,621 ± 152.7 × 10^3/μL vs. 1,425 ± 134.6 × 10^3/μL, respectively). However, the WBC counts were significantly higher in L-PRP than in P-PRP (10.5 ± 2.6 × 10^3/μL vs. 0.1 ± 0.2 × 10^3/μL, respectively; $p < 0.05$).

Proliferation of SDSCs and Chondrocytes in Different Media

Proliferation of SDSCs and chondrocytes was significantly higher in the L-PRP group than in the FBS and P-PRP groups ($p < 0.05$ for all comparison). However, it was not different between the FBS and P-PRP groups (Figure 7).

Gene Expression of Chondrogenic Markers

The gene expression of chondrogenic markers were measured by RT-qPCR on day 21. In SDSCs, the gene expression levels of aggrecan, type II collagen, and type X collagen were significantly higher in the P-PRP group than in the CM and L-PRP groups ($p < 0.05$ for all comparison). The gene expression levels of Sox-9 were significantly higher in the CM and P-PRP groups than in the L-PRP group ($p < 0.05$ for all comparison) but were not different between the CM and P-PRP groups (Figure 8). In chondrocytes, the gene expression levels of aggrecan, type II collagen, and Sox-9 were significantly higher in the P-PRP group than in the CM and L-PRP groups ($p < 0.05$ for all comparison). The gene expression levels of type X collagen were significantly higher in the P-PRP and L-PRP groups than in the CM group ($p < 0.05$ for all comparison) but were not different between the P-PRP and L-PRP groups (Figure 9).

Histological Analysis

The presence of proteoglycans was assessed using safranin-O/fast green staining on day 21. In SDSCs and chondrocytes, the densest positive staining was observed in the CM group. Although better staining was observed in the P-PRP group than in the L-PRP group, both groups showed weaker staining than did the CM group for both SDSCs and chondrocytes (Figure 10).

Immunohistochemical Analysis

Immunohistochemistry was used to detect type II collagen on day 21. In SDSCs, the densest positive staining was observed in the CM group, while no positive staining was observed in either P-PRP or L-PRP group. In chondrocytes, although better staining was observed in the P-PRP group than in the L-PRP group, it was weaker than that in the CM group (Figure 11).

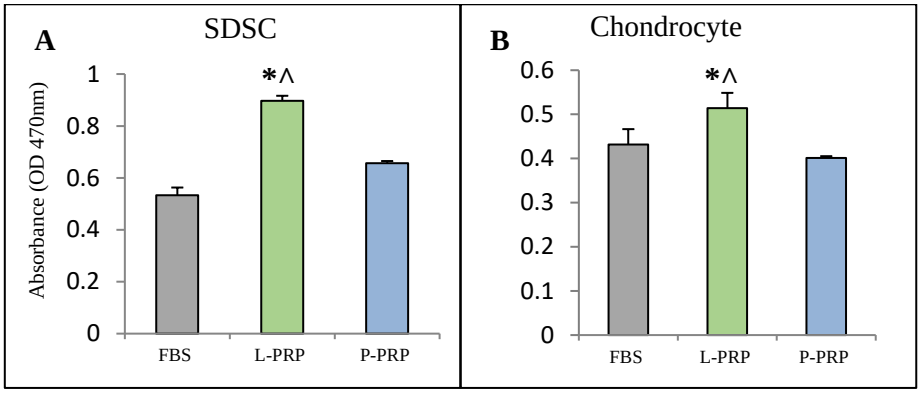

Figure 7. Proliferation of SDSCs (A) and chondrocytes (B) in different medium after 48 hours. SDSCs and chondrocytes proliferation were significantly higher in L-PRP group compared to P-PRP and FBS groups. Results are presented as mean ± SD (n = 6). * and ^ indicate significant difference compared to FBS group and P-PRP group, respectively ($p < 0.05$).

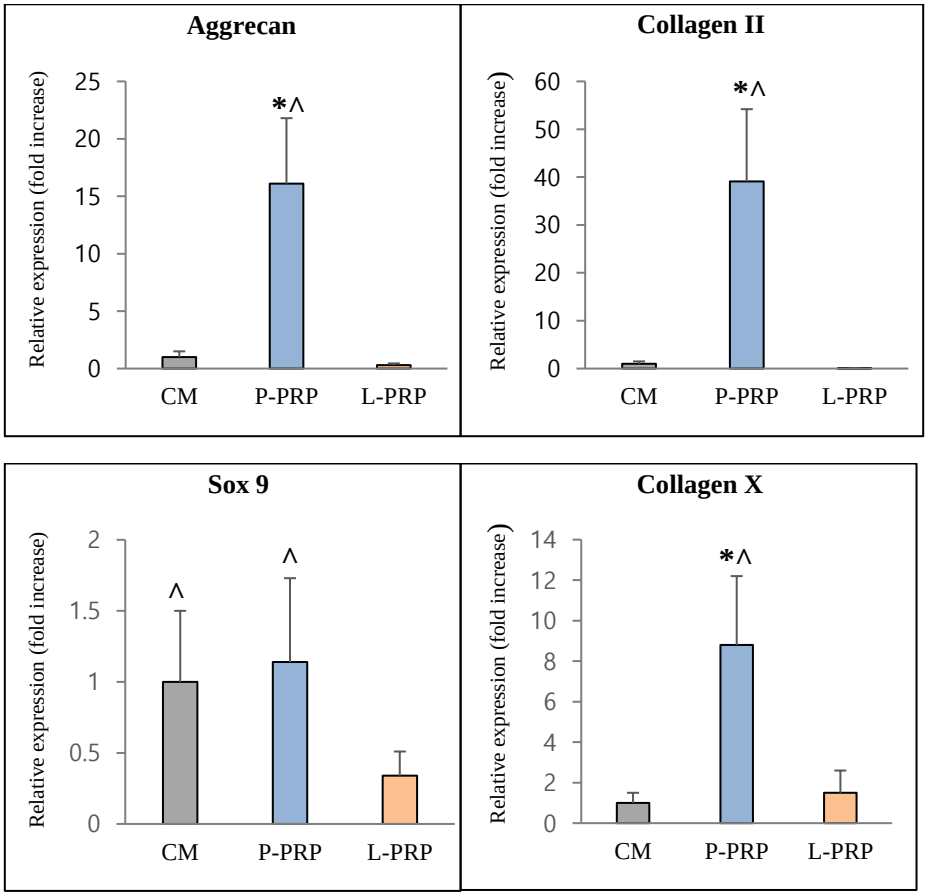

Figure 8. RT-PCR analysis for chondrogenic marker gene expression in SDSCs after 21 days of culture period. Results are presented as mean ± SD (n = 6). * and ^ indicate significant difference compared to CM group and L-PRP group, respectively ($p < 0.05$). CM: chondrogenic medium; P-PRP: Leukocyte poor PRP; L-PRP: Leukocyte rich PRP

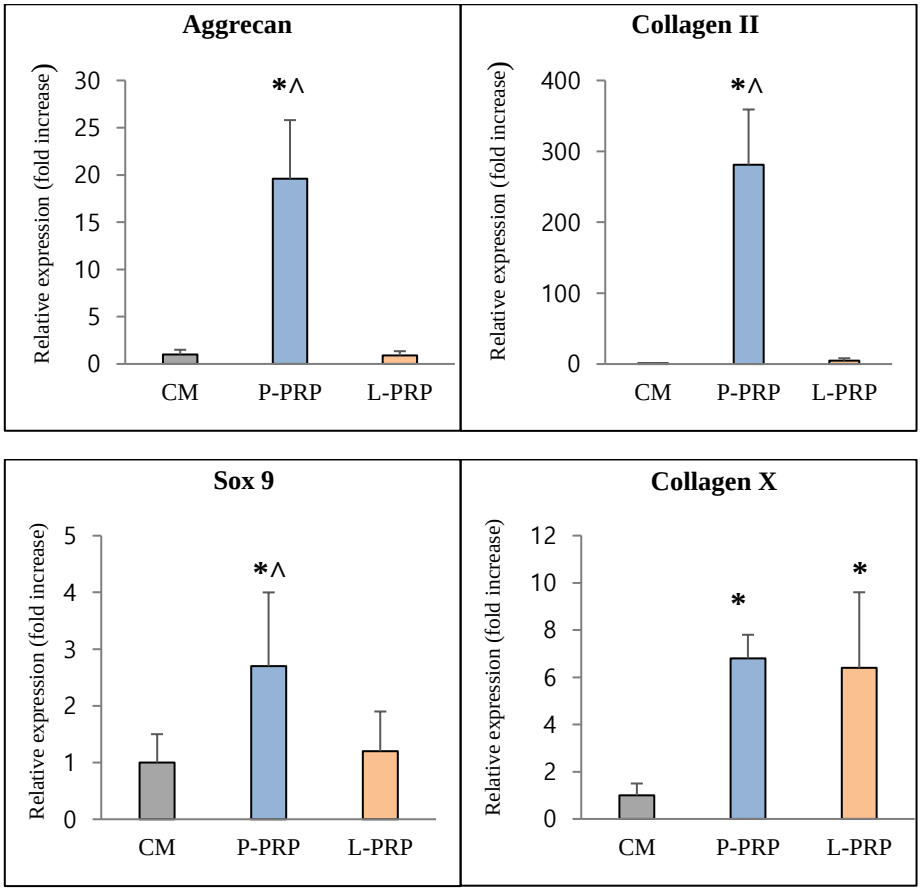

Figure 9. RT-PCR analysis for chondrogenic marker gene expression in chondrocytes after 21 days of culture period. Results are presented as mean ± SD (n = 6). * and ^ indicate significant difference compared to CM group and L-PRP group, respectively ($p < 0.05$). CM: chondrogenic medium; P-PRP: Leukocyte poor PRP; L-PRP: Leukocyte rich PRP

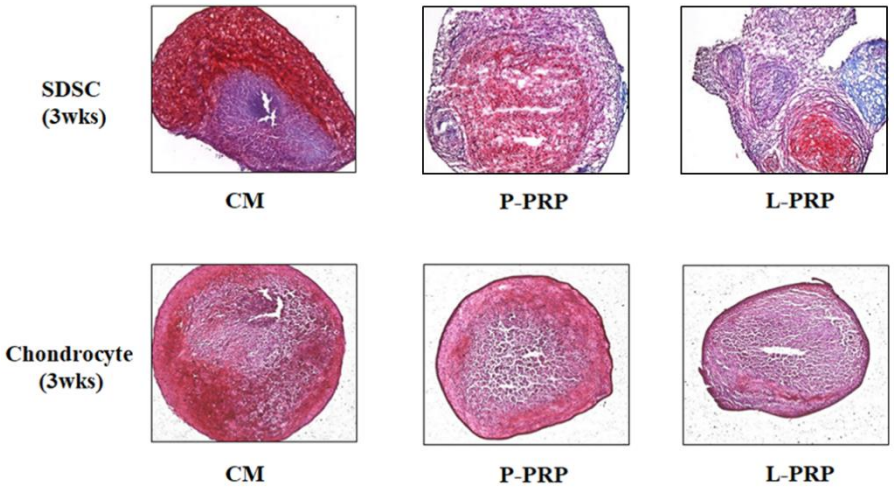

Figure 10. Histological evaluation of proteoglycan in the pellets with Safranin O-fast green staining. Pellets formed by SDSCs and chondrocytes were differentiated with various mediums and evaluated at day 21. Magnification, x100. CM: chondrogenic medium; P-PRP: Leukocyte poor PRP; L-PRP: Leukocyte rich PRP

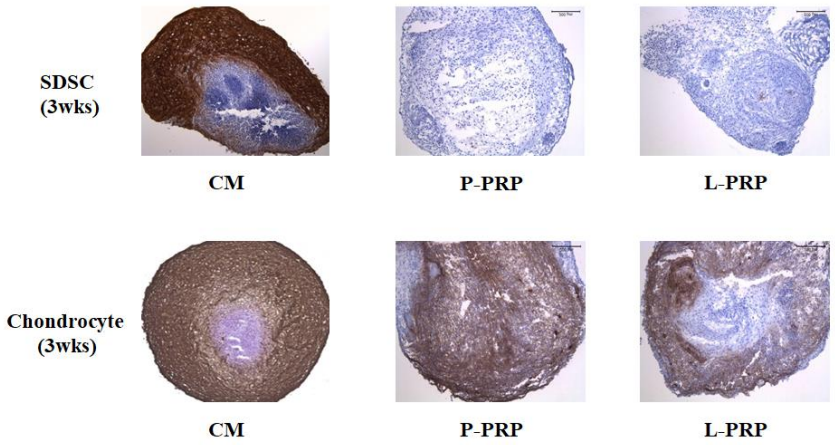

Figure 11. Immunohistochemistry evaluation of type II collagen in the pellets. Pellets formed by SDSCs and chondrocytes were differentiated with various mediums and evaluated at day 21. Magnification, x100. CM: chondrogenic medium; P-PRP: Leukocyte poor PRP; L-PRP: Leukocyte rich PRP

II-3. Discussion

The most important findings in this study were that (1) L-PRP showed better proliferative effects on SDSCs and chondrocytes than P-PRP and (2) both L-PRP and P-PRP showed negative effects on chondrogenesis of SDSCs and chondrocytes.

Recently, DeLong et al. (73) have proposed the PAW (Platelets, Activation, and White cells) classification system based on three components: (1) the absolute number of platelets; (2) the method of activation; and (3) the leukocyte concentration in PRP. Among the three components, the most important distinguishing characteristic of PRP is the leukocyte concentration. L-PRP has several advantages and disadvantages compared with P-PRP. Regarding advantages, leukocytes contain cytokines involved in an immune response to infection (33). Also, leukocytes can increase the total amount of growth factor released in the PRP (29). Regarding disadvantages, leukocytes can increase the levels of proinflammatory cytokines such as IL-1 and TNF-α, which may adversely affect cartilage regeneration (31, 32). Moreover, leukocytes also increase the levels of matrix metalloproteinases (MMPs), which accelerate the degradation of ECM (30).

Several studies have reported the effects of PRP on cartilage regeneration (39, 40, 42, 74-78); however, most of these studies have not clearly indicated the leukocyte concentration in PRP. Only two studies directly compared L-PRP and P-PRP in terms of their effects on cartilage regeneration. Cavello et al. (38) compared the effects of L-PRP and P-PRP on human chondrocytes *in vitro* and showed that both formulations stimulated chondrocyte proliferation. However, regarding chondrogenesis, P-PRP stimulated type II collagen and aggrecan gene expression, whereas L-PRP induced higher expression of the HA synthase-2 gene than P-PRP did. Therefore, the authors concluded that

optimization of the leukocyte concentration might result in a more suitable PRP for the treatment of cartilage lesions. In addition, Xu et al. (41) performed an *in vitro* and *in vivo* study using rabbits and have reported that better proliferation and chondrogenic differentiation of rabbit BMSCs, as well as better cartilage regeneration in a rabbit osteochondral defect model, were observed in the presence of P-PRP than with L-PRP; therefore, the authors concluded that P-PRP might be more suitable for cartilage regeneration. However, no study compared P-PRP and L-PRP in terms of their effects on cartilage regeneration using human SDSCs.

In our study, L-PRP showed better proliferative effects on SDSCs and chondrocytes than P-PRP did. However, although RT-qPCR revealed higher gene expression of chondrogenic markers in SDSCs and chondrocytes cultured with P-PRP than in those cultured with L-PRP, histological and immunohistochemical findings suggested that both types of PRP had negative effects on chondrogenesis of SDSCs and chondrocytes. Our findings are certainly difficult to explain, but opposite effects of PRP on the proliferation and chondrogenesis were also observed in other studies (74-76). Drengk et al. (74) reported that PRP showed a proliferative effect on sheep BMSCs, while downregulated type II collagen gene expression. The explanation was that the proliferation and differentiation could not occur at the same time, and therefore, increased proliferation led to a decrease in the differentiation potential of BMSCs. Additionally, Kaps et al. (76) reported that PRP stimulated chondrocyte proliferation but failed to stimulate chondrocytes to redifferentiate in three-dimensional cultures. Therefore, the authors concluded that PRP might be suitable for chondrocyte expansion but not for the maturation of a tissue-engineered cartilage. Similar findings were reported in a study by Gaissmaier et al. (75), who found that PRP enhanced the

chondrocyte proliferation in monolayer cultures but induced dedifferentiation of chondrocytes toward a fibroblast-like phenotype.

Regarding better proliferative effects of L-PRP, several reports (29, 38) showed that leukocytes in PRP could further increase the levels of anabolic growth factors such as TGF-β, PDGF-AB, and VEGF. We assume that the abundance of growth factors in L-PRP may result in better proliferative effects on SDSCs and chondrocytes compared with those of P-PRP. Regarding the negative effects of PRP on chondrogenesis, regardless of the presence of leukocytes, several studies (42, 77, 78) showed similar results. Liou et al. (77) investigated the effect of PRP on chondrogenic differentiation of human MSCs derived from the infrapatellar fat pad and bone marrow and found that PRP did not improve chondrogenesis of MSCs of different origins. Moreover, chondrogenesis was inhibited with increasing PRP concentrations and the duration of exposure; therefore, the researchers concluded that PRP was unlikely to enhance the MSC-mediated hyaline cartilage regeneration. Furthermore, Lee et al. (42) showed that PRP had negative effects on chondrogenic differentiation of SDSCs and PRP alone did not induce SDSC differentiation. In addition, O'Donnell et al. (78) showed that the PRP obtained from older patients with knee OA downregulated the type II collagen and Sox-9 gene expression while upregulating that of TNF-α and MMP-9 in chondrocytes. Therefore, the authors concluded that PRP might suppress the synthesis of the chondrocyte matrix and promote inflammation.

However, several studies (39, 40) have reported opposite results. Mishra et al. (40) showed that PRP induced 10-fold increases in the Sox-9 and aggrecan gene expression in MSCs (origin unreported) compared with that in the control and concluded that PRP enhanced chondrogenic differentiation of MSCs. Additionally, Jeyakumar et al. (39) reported that PRP increased type 2

collagen gene expression in chondrocytes and concluded that PRP enhanced redifferentiation of chondrocytes. However, these studies are difficult to compare with our study, owing to different experimental conditions, especially properties of PRP. As described by Russell et al. (79), the time and speed of centrifugation, method of PRP activation, time interval between PRP preparation and application, and method of application all play an important role in the efficacy of PRP. Indeed, Kreuz et al. (28) showed that the use of PRP samples prepared using different methods resulted in different proliferation abilities and chondrogenic differentiation potential of BMSCs. Therefore, the availability of standardized methods of PRP preparation and application is crucial to minimize variability between studies and determine the actual effect of PRP on chondrogenesis.

This study has several limitations. First, we only evaluated the effects of L-PRP and P-PRP on SDSCs, while those on MSCs from the bone marrow or adipose tissue may still be different. Second, there were inconsistencies; chondrogenic marker genes were markedly upregulated, whereas, type II collagen and proteoglycan were not synthesized well in the presence of P-PRP. As protein-coding genes are eventually translated into proteins, it is usually accepted that there is a positive correlation between gene and protein expression levels. There are three possible explanations for the poor correlation between gene and protein expression. First of all, there are many complex and diverse posttranscriptional mechanisms involved in mRNA translation into a protein, which are not yet fully understood (80). Furthermore, protein expression levels at the time of measurement can be influenced by the protein turnover rate, which can be greatly affected by cell culture conditions (81). Lastly, there is the possibility of measurement errors in detection of both gene and protein expression (82). However, considering

that cell culture conditions and method of measurement did not vary much among the three groups, we assume that our result is mainly the outcome of potential negative effects of some growth factors in PRP on protein synthesis during posttranscriptional mechanism. Third, we obtained chondrocytes and SDSCs from older female patients, who had advanced knee OA. Considering that the proliferation and chondrogenic potential of chondrocytes and SDSCs are largely affected by the age of the donor and the grade of knee OA (83), older OA patients undergoing total knee arthroplasty may not be an ideal source of these cells for experiments. However, we used this alternative source because obtaining sufficient volumes of the cartilage and synovium from a young healthy donor may have raised ethical issues. Fourth, the evaluation of chondrogenesis using pellet culture was assessed at only one time point, namely 3 weeks after culture. However, several studies (84-86) have shown that although the expression of early stage chondrogenic marker genes and corresponding protein (Sox-9, aggrecan, type II collagen) synthesis did not appear distinctly at 1 week, these genes and proteins were increased at 2 weeks, and were considerably higher at the end of 3 weeks of differentiation. Moreover, Gong et al (84) showed that between 2 to 3 weeks of differentiation, late chondrogenic marker gene such as type X collagen, which causes cartilage hypertrophy and calcification, increased significantly. Therefore, we believe that measuring at 3 weeks after culture best reflects the chondrogenesis of the pellet.

Chapter III.

The effect of platelet-rich plasma with hyaluronic acid hydrogel on *in vivo* chondrogenesis in a rabbit osteochondral defect model

III-1. Materials and Methods

Preparation of PRP

Nine milliliters of whole blood was obtained under anesthesia from New Zealand white rabbits, and the blood was anticoagulated with 1 mL of a 3.8% sodium citrate solution. The tube was centrifuged at 2,000 rpm for 5 min, and the upper layer of plasma was separated and subjected to a second round of centrifugation at 3,000 rpm for 10 min, followed by the collection of the lower 1 mL of plasma as PRP.

Surgical Procedure

The knee joints were exposed through a medial parapatellar incision, and the patella was everted laterally to expose the trochlear groove of the rabbits. A full-thickness cartilage defect of 4 mm in diameter and 3 mm in depth was made in the trochlear groove using a hand drill. Before transplantation, the joints were thoroughly irrigated with a sterile saline solution. Eighteen male New Zealand white rabbits were divided into three groups (n = 6 each) as follows: control group, in which the defect was left untreated; HA group, in which the defect was filled with an HA hydrogel (200 μL/defect); and HA-PRP group, in which the defect was filled with HA hydrogel and 10% PRP (200 μL/defect). To make HA hydrogel, HA solution (10 mg/mL, LG CI) was mixed with fibrinogen (120 mg/mL, Greenplast kit; Green Cross) and then thrombin (10 mg/mL) containing 40 mM $CaCl_2$ to form a gel. After the soft tissue and skin were closed, the rabbits were housed in separate cages and allowed to move freely without joint immobilization after surgery.

Macroscopic Examination

The rabbits were sacrificed at postoperative 12 week by intravenous overdose of a ketamine solution (15 mg/kg). The distal femurs were dissected, and the macroscopic appearance of the defects was evaluated using the International Cartilage Repair Society (ICRS) Visual Assessment Scale for cartilage repair (Table 2).

Histological Examination

After macroscopic examination, the distal femurs were fixed in 10% buffered formalin for 5 days, then decalcified using the Decal Rapid decalcifying agent (National Diagnostics, Atlanta, GA, USA) for 2 days, dehydrated with a graded series of ethanol solutions, embedded in paraffin, and cut into 5-mm-thick sagittal sections. The sections were stained with hematoxylin and eosin (H&E) for morphological evaluation and with safranin-O/fast green for proteoglycan content evaluation. The sections were evaluated by three trained observers to determine O'Driscoll histological cartilage repair scores (Table 3).

Determination of the GAG Content

Total glycosaminoglycan (GAG) contents were assessed at 12 weeks after surgery using the dimethylmethylene blue (DMB) assay. Samples were digested with a papain buffer at 65°C for 18 h. After centrifugation at 6,000 rpm for 5 min, 50μL aliquots were placed on 96-well plates with 250μL of the DMB solution. Absorbance of these extracts was measured at 530 and 590 nm using a plate reader, and the values were standardized by the absorbance of the chondroitin-6-sulfate standard. The total GAG per gram of wet weight of the cartilage was calculated for each sample.

Statistical Analysis

The Kruskal–Wallis test, followed by multiple significance analyses involving the Mann–Whitney test, was used to compare scores among the groups. A value of $p < 0.05$ was considered significant.

Table 2. ICRS macroscopic evaluation of cartilage repair.

Categories	**Score**
Degree of defect repair	
In level with surrounding cartilage	4
75% repair of defect depth	3
50% repair of defect depth	2
25% repair of defect depth	1
0% repair of defect depth	0
Integration with border zone	
Complete integration with surrounding cartilage	4
Demarcating border <1 mm	3
3/4 of graft integrated, 1/4 with a notable border >1 mm width	2
1/2 of graft integrated with surrounding cartilage. 1/2 with a notable border >1 mm	1
From no contact to 1/4 of graft integrated with surrounding cartilage	0
Macroscopic appearance	
Intact smooth surface	4
Fibrillated surface	3
Small, scattered fissures or cracks	2
Several, small or few but large fissures	1
Total degeneration of grafted area	0
Overall repair assessment	
Grade I: normal	12
Grade II: nearly normal	11-8
Grade III: abnormal	7-4
Grade IV: severely abnormal	3-1

Table 3. O'Driscoll histological scores

Characteristics	**Score**
1. Cell morphology	
Hyaline like articular cartilage	4
Partial differentiated hyaline cartilage	2
Fibrous tissue	0
2. Integrity of surface	
Surface smooth and intact	3
Surface horizontal fibrillation	2
Surface shows fissures to 25–100% of the depth of the cartilage	1
Serious deep interruption of the surface and many deep fibrillations	0
3. Thickness	
100% of normal host cartilage	2
50–100% of normal cartilage	1
0–50%	0
4. Surface of area filled with cells	
100–75%	3
75–50%	2
50–25%	1
25–0%	0

5. Chondrocyte clustering	
None at all	2
<25% of the cells	1
25–100% of the cells	0
6. Degenerative changes	
Normal cell quantity, no clustering, normal staining with proteoglycan specific stain	3
Normal cell quantity, some cluster formation, moderate staining	2
Clearly less cells, poor staining	1
View cells, no or very little staining	0
7. Restoration of the subchondral bone	
Normal and straight	4
Slight contour changes	2
Larger interruptions in subchondral bone	1
Defect	0
8. Integration	
Both sides of repair tissue integrated with host cartilage	2
One side integrated	1
No integration	0
Total maximal score	23

III-2. Results

Results of Macroscopic Examination

At 12 weeks after operation, a partially healed cartilage was observed within the defect, but the margin was clearly distinguished from the surrounding intact cartilage in all three groups (Figure 12). There were no differences in the macroscopic ICRS scores among the three groups (Figure 13).

Results of Histological Examination

At 12 weeks after operation, the HA-PRP group showed better histological features with both H&E and safranin-O/fast green staining than did the control and HA groups (Figure 14). However, the HA and HA-PRP groups showed significantly higher O'Driscoll scores than did the control group ($p < 0.05$ for all comparison), whereas the scores were not different between the HA and HA-PRP groups (Figure 15).

GAG Content

The GAG content was significantly higher in the HA and HA-PRP groups than in the control group ($p < 0.05$ for all comparison) but was not different between the HA and HA-PRP groups (Figure 16).

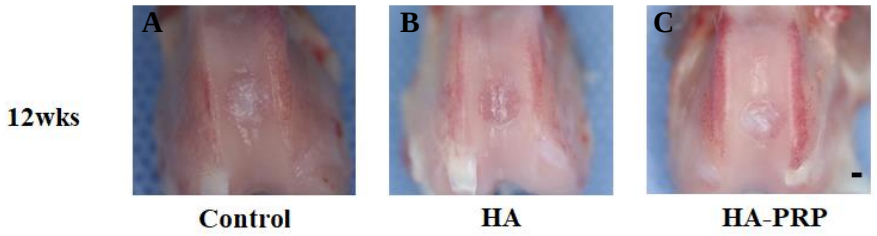

Figure 12. Macroscopic appearance of defects in trochlear groove (4 mm in diameter) at 12 weeks after surgery in control group (A), HA group (B), HA-PRP group (C) (scale bar, .7 mm).

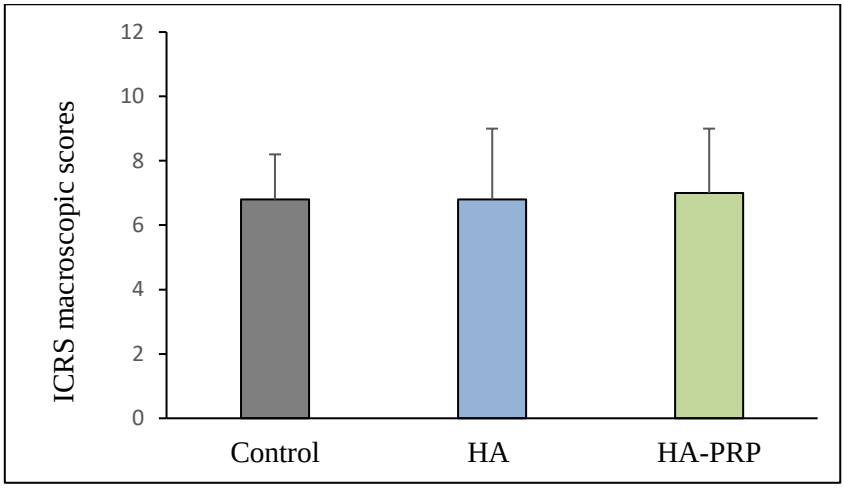

Figure 13. ICRS macroscopic scores for control group, HA group, HA-PRP group at postoperative 12 weeks. There was no difference among three groups.

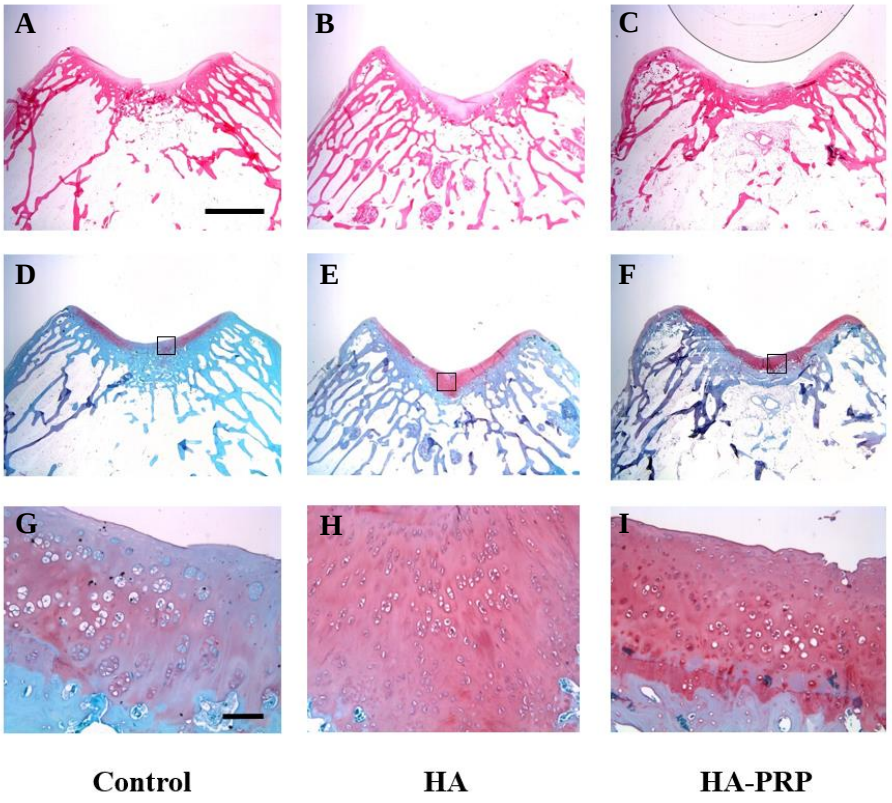

Figure 14. Histologic appearance of defects in trochlear groove (4 mm in diameter) with H&E staining (A-C) and Safranin-O staining (D-I) at 12 weeks after surgery in control group (A, D, G), HA group (B, E, H), HA-PRP group (C, F, I). G, H and I are magnified views of the boxes in D, E, and F. The scale bar equals 2 mm for A-F; 200 μm for G-I.

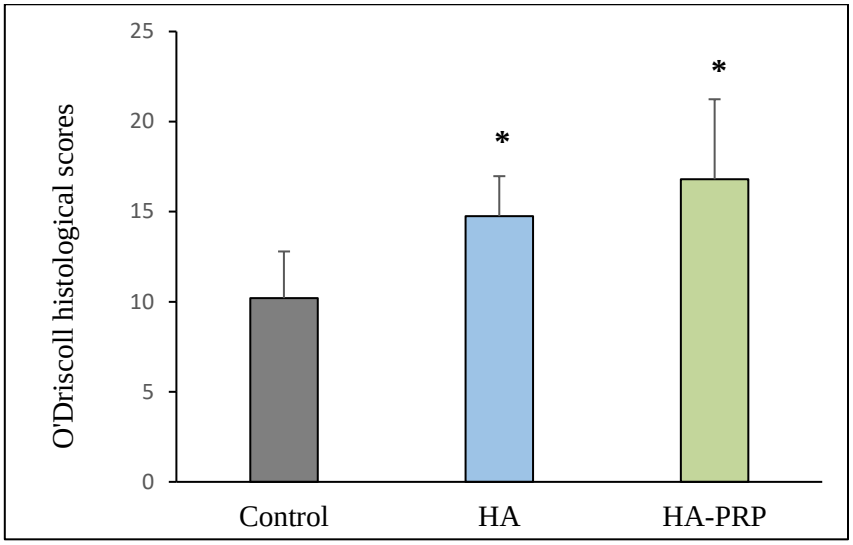

Figure 15. O'Driscoll histologic scores for control group, HA group, HA-PRP group at postoperative 12 weeks. HA and HA-PRP group showed significantly higher scores compared with that in the control group, but it was not different between HA and HA-PRP group. Results are presented as mean ± SD (n = 6). * indicate significant difference compared to control group ($p < 0.05$).

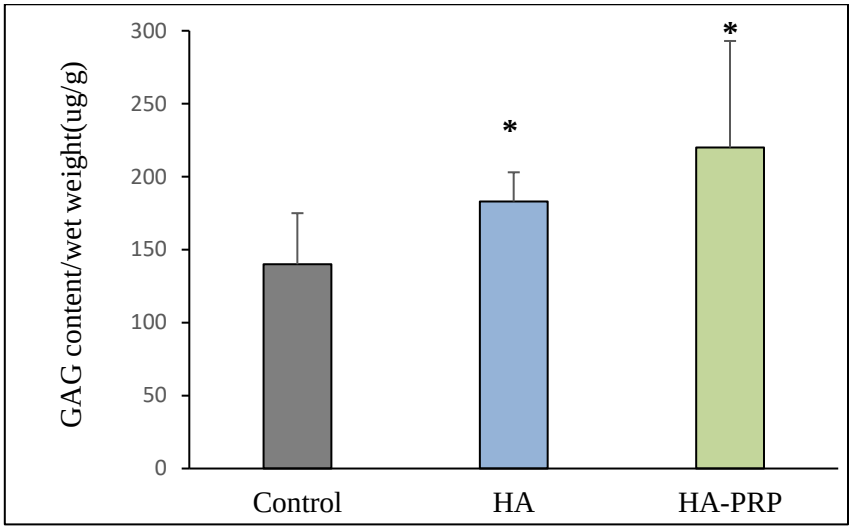

Figure 16. GAG content for control group, HA group, HA-PRP group at postoperative 12 weeks. The GAG content was significantly higher in the HA and HA-PRP group compared to that in the control group, but it was not different between the HA and HA-PRP group. Results are presented as mean ± SD (n = 6). * indicate significant difference compared to control group ($p < 0.05$).

III-3. Discussion

The most important findings in this study were that (1) the macroscopic ICRS scores were not different among the three groups and (2) the HA and HA-PRP groups showed significantly higher histological scores and a significantly higher GAG content than the control group, although the values were not different between the HA and HA-PRP groups. Therefore, we concluded that the addition of PRP did not provide an additional effect on cartilage regeneration *in vivo* compared with that of the scaffold alone.

The most critical limitation of articular cartilage regeneration techniques has been an inability to restore the normal biochemical and mechanical function of the native hyaline cartilage, which is essential (43, 44). The bone marrow stimulation technique involves the recruitment of endogenous MSCs from the bone marrow to a cartilage lesion, and cartilage regeneration is possible because mesenchymal progenitor cells have high proliferation potential and a high ECM synthesis capacity (87, 88). However, the mesenchymal progenitor cells recruited via the bone marrow stimulation technique are too small, and there is a possibility of cell leakage because the endogenous fibrin clot formed by this technique cannot effectively hold these cells. To overcome the shortcomings of the bone marrow stimulation technique, various scaffolds have been developed, which provide a suitable environment for MSC expansion and chondrogenic differentiation (54, 55). Several studies have shown more favorable outcomes when the bone marrow stimulation technique was used in combination with scaffolds rather than alone (50, 51, 53).

PRP contains various growth factors, including PDGF, TGF-β1, VEFG, IGF, and FGF, which can promote cartilage formation. PDGF is a strong mitogen, and it helps chondrocytes proliferate and induce ECM synthesis (12). Furthermore, TGF-β1 plays a role in chondrogenic differentiation of MSCs

and in the regulation of activity of catabolic growth factors such as IL-1β and MMP-9 (11). VEGF is the most potent proangiogenic growth factor, whereas IGF-1 is one of the main anabolic growth factors in the cartilage, which inhibits IL-1β-induced inflammation (13). FGF stimulates the proliferation and chondrogenic differentiation of MSCs (10). Therefore, theoretically, using PRP-augmented scaffolds in combination with the bone marrow stimulation technique seems advantageous and may provide an additional benefit by accelerating cartilage regeneration.

However, in our study, there was no difference in histological scores and the GAG content between the HA and HA-PRP groups, indicating that there was no additional effect of PRP on cartilage regeneration. There are several possible reasons, one of which is the type of the scaffold. In this study, an HA-based hydrogel was selected as a scaffold because HA is a major component of the articular cartilage, and several studies have shown that HA can provide a suitable microenvironment for chondrogenesis (56, 58). However, there are various types of scaffolds, and conflicting results have been reported depending on which scaffold was used in combination with PRP for cartilage regeneration (89-93). Kon et al. (91) found that PRP combined with type I collagen scaffolds decreased the cartilage regeneration capability in a sheep osteochondral defect model. However, Sun et al. (93) showed that the addition of PRP to a polyglycolic acid scaffold additionally stimulated cartilage regeneration in a rabbit osteochondral defect model. Moreover, not only the type of the scaffold but also the presence of chondrocytes or stem cells in the scaffold is an important factor. Sermer et al. (94) conducted a systematic review and found that the use of PRP with cell-induced scaffolds generally showed superior cartilage healing over that of PRP with cell-free scaffolds. In cell-induced scaffolds, cells directly

differentiate to chondrocytes or produce ECM, without the need for native MSCs to migrate into the scaffold, whereas in the case of cell-free scaffolds, cartilage regeneration exclusively relies on migrated MSCs. The other factors contributing to the conflicting results are characteristics of the PRP used. The quality and quantity of growth factors in PRP depend on the method of PRP preparation, storage conditions, and method of activation (26, 27, 66, 79). Considering that cartilage regeneration is a complex process, which involves various molecular pathways, even small differences in growth factors in PRP can result in differences in cartilage regeneration.

This study has several limitations. First, *in vivo* chondrogenesis was assessed at only one time point, 12 weeks after operation, which we considered to be a sufficient time for chondrogenesis to proceed and be evaluated. However, in a rabbit osteochondral defect model, Shapiro et al. (95) showed that degeneration of repaired tissue could occur after postoperative 12 weeks. Therefore, a longer evaluation period may be needed to verify long-term effects on chondrogenesis. Second, we did not evaluate the effect of PRP alone on *in vivo* chondrogenesis, as this study mainly aimed to evaluate an additional effect of PRP in combination with an HA hydrogel. Moreover, when PRP is used alone, the growth factors, which are only released in an early period, may then be washed away by bodily fluids under *in vivo* conditions (23, 24).

Conclusion

Growth factor concentrations in PRP significantly differed depending on the storage temperature, duration of storage, and activation. Regardless of the leukocyte concentration, PRP showed a negative effect on chondrogenesis of SDSCs and chondrocytes. In addition, combined use of an HA hydrogel and PRP did not show additional chondrogenic effects *in vivo* compared with those of the HA hydrogel alone. Considering the results of our study, a further study is necessary to clarify the ideal composition and application of PRP for cartilage regeneration.

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국문 초록

목 적: 본 연구에서는 혈소판 풍부 혈장을 이용한 연골재생에 대해 알아보고자 한다. 이를 위해 먼저 보관조건에 따른 혈소판 풍부 혈장 내 성장인자 농도를 조사하고, 혈소판 풍부 혈장 내 백혈구 농도 차이가 인체활막세포와 연골세포에 미치는 영향을 알아본 후, 마지막으로 혈소판 풍부 혈장과 지지체의 복합체 형성이 연골재생에 미치는 영향을 알아보았다.

대상 및 방법: 보관조건에 따른 혈소판 풍부 혈장 내 성장인자 농도를 조사하기 위해, 혈소판 풍부 혈장을 24도, 4도, −70도에서 7일 동안 보관한 후 활성화 전후로 PDGF-AA, TGF-β, VEGF, IGF-1, FGF-B 농도를 ELISA를 이용하여 측정하였다. 또한 혈소판 풍부 혈장 내 백혈구 농도 차이가 인체활막세포와 연골세포에 미치는 영향을 알아보기 위해, 백혈구 풍부 (Leukocyte−rich)와 순수 (Leukocyte−poor) 혈소판 풍부 혈장을 제조하고 이들을 각각 인체활막세포, 연골세포와 같이 3주간 pellet 배양하였다. 이후 세포 증식을 MTT assay로 평가하고, 연골형성능은 RT−PCR로 연골형성에 관련된 유전자 (Type II collagen, Type X collagen, SOX−9, aggrecan) 발현을 측정한 후 조직학/면역학적 염색을 시행하였다. 마지막으로 혈소판 풍부 혈장과 지지체의 복합체 형성이 연골재생에 미치는 영향을 조사하기 위해, 토끼 대퇴 활차에 골연골 결손을 만들고 대조군, 지지체군, 복합체군 (혈소판 풍부 혈장+지지체)으로 나눈 후 12주에 ICRS macroscopic score와 Modified O' Driscoll score를 얻고 DMB assay를 통해 GAG 합성을 평가하였다.

결 과: 보관조건에 따른 혈소판 풍부 혈장 내 성장인자 농도의 경우, 활성화 전 PDGF-AA 농도는 24도에서 7일째 가장 높았고, TGF-β는 모든 온도에서 7일 동안 잘 검출되지 않았으며, VEGF와 IGF-1 농도는 모든 온도에서 7일 동안 일정하게 유지되고, FGF-B 농도는 −4도에서 7일째 가장 높았다. 활성화 후 PDGF-AA, VEGF, IGF-1 농도가 모든 온도에서 7일 동안 일정하게 유지된 것에 반해, TGF-β 농도는 모든 온도에서 7일에 가장 높았고, FGF-B 농도는 24도에서 1일 후 급격히 감소하는 양상을 보였다. 혈소판 풍부 혈장 내 백혈구 농도 차이가 인체활막세포와 연골세포에 미치는 영향의 경우, 백혈구 풍부 혈소판 풍부 혈장에서 인체활막세포와 연골세포 모두 보다 큰 증식능력을 보였으나, RT−PCR의 경우 두 세포 모두 백혈구 순수 혈소판 풍부 혈장에서 보다 많은 유전자 발현이 관찰되었다. 하지만 인체활막세포와 연골세포의 조직학/면역학적 염색상, 백혈구 농도에 관계없이 두 혈소판 풍부 혈장 모두에서 연골 분화가 억제되는 양상이 관찰되었다. 혈소판 풍부 혈장과 지지체의 복합체 형성이 연골재생에 미치는 영향의 경우, 세군 모두에서 ICRS macroscopic score에 차이가 없었다. Modified O’ Driscoll score와 GAG 농도의 경우, 지지체군과 복합체군 모두 대조군보다 높았으나, 두 군 간에는 차이가 관찰되지 않았다.

결 론: 혈소판 풍부 혈장에서 방출되는 성장인자들의 농도는 보관온도, 보관기간, 활성화 여부에 따라 큰 차이가 있었다. 또한 혈소판 풍부 혈장 내 백혈구 농도에 관계없이 혈소판 풍부 혈장은 인체활막세포와 연골세포의 연골형성에 부정적인 영향을 보였으며, 혈소판 풍부 혈장과 지지체의 복합체 사용은 지지체 단독 사용에 비해 향상된 연골형성으로 이어지지

못했다. 이번 연구결과들을 기반으로 연골재생을 위한 최적의 혈소판 풍부 혈장 사용에 대한 추가연구가 필요 할 것으로 생각된다.

색인 단어: 혈소판 풍부 혈장, 보관, 백혈구, 활막줄기세포, 연골세포, 지지체

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