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Comparison of the Cellular Composition and Cytokine-Release Kinetics According to Different Preparations of Platelet-rich Plasmas (PRPs)

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Comparison of the Cellular Composition and Cytokine-Release Kinetics According to Different Preparations of Platelet-rich Plasmas (PRPs)

By Roh, Young Hak

A thesis submitted to the Department of Orthopedics in partial fulfillment of the requirements for the Degree of Doctor of Philosophy in Medical Science at Seoul National University College of Medicine

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ABSTRACT

Comparison of the Cellular Composition and Cytokine-Release Kinetics According to Different Preparations of Platelet-rich Plasmas (PRPs)

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Introduction: Variations in formulations used to prepare or activate platelet-rich plasmas (PRPs) result in differences in the cellular composition and biological activity of the platelets. This study evaluates the cellular composition and the cytokine release kinetics of PRP according to differences in the preparation and activation protocols.

Methods: Blood samples were obtained from 14 healthy subjects, and five preparation procedures were performed for each donor including two methods that used manual procedures [single-spin (SS) at 900 g for 5 min; double-spin (DS) at 900 g for 5 min and then 1500 g for 15 min] and three methods with commercial kits (Arthrex ACP, Biomet GPS, and Prodizen Prosys). Each PRP preparation was divided into four aliquots

and was incubated for 1 h, 24 h, 72 h, and 7 days. The cytokine release kinetics were evaluated by assessing the platelet-derived growth factor (PDGF), transforming growth factor (TGF), vascular endothelial growth factor (VEGF), fibroblast growth factor (FGF), interleukin-1 (IL-1), and matrix metalloproteinase-9 (MMP-9) concentrations with commercially available bead-based sandwich immunoassay kits. The SS and DS PRP preparations were tested for platelet activation with three different conditions: no activation, activation with calcium only, or calcium with a low dose of thrombin.

Results: The DS PRP had a higher concentration of platelets (x 4) and leukocytes (x 9) than the SS PRP. Both PRP preparations exhibited an increase in PDGF, TGF, VEGF, and FGF release when compared to whole-blood samples, and the IL-1 and MMP-9 concentrations of the SS or DS PRP preparations were lower than those of whole-blood samples during the 7 days of the experiment. The cytokine release kinetics varied for the different cytokines, and the FGF and the TGF release occurred quickly, with maximum concentrations detected within 1 h, and then decreased over time. The PDGF

and VEGF release was constant and was sustained over 7 days. The PDGF and VEGF concentrations were higher in the DS PRP than in the SS PRP, whereas the TGF and FGF concentrations were higher in the SS PRP than in the DS PRP.

With respect to the commercial PRP kits, Arthrex ACP had cytokine release kinetics similar to those of SS PRP, and Prodizen Prosys had kinetics similar to those of DS PRP. Among the three commercial kits that were used, Biomet GPS had the highest platelet and leukocyte concentrations and the highest VEGF and MMP-9 concentrations but the lowest TGF concentration. Arthrex ACP had the highest FGF concentration but the lowest PDGF concentration.

Ca-only activation had a significant effect on the DS PRP preparations (where the VEGF, FGF, and IL-1 concentrations were sustained) while Ca/thrombin activation had significant effects on both SS and DS PRPs (where the PDGF and VEGF concentrations were sustained and the TGF and FGF concentrations were not sustained). The IL-1 content showed a significant increase with Ca only or Ca/thrombin activation while Ca only or Ca/thrombin activation did not increase the MMP-9 concentration.

Conclusions: The PRP preparations showed different qualities in terms of their cellular composition and their cytokine release kinetics, depending on the preparation and activation protocols that were used. The DS method generally led to a higher concentration of PLT relative to the SS method, and it was considered to be preferable for an efficient local cellular response of the PRP. However, the cytokine content was not necessarily proportional to the cellular composition of the PRPs since greater cytokine content could be different between the SS or the DS method, depending on the type of cytokine evaluated. In terms of the PRP activation, a low dose of thrombin/calcium activation increased the overall cytokine release of the PRP preparations over 7 days relative to that with a calcium-only supplement or non-activation.

Keywords: platelet rich plasma, preparation, activation, cytokine, cell composition, kinetics

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INTRODUCTION

Platelet-rich plasma (PRP) treatments promote tissue healing and regeneration and have gained increasing popularity in various areas of orthopedic surgery (Charousset et al., 2014, Hsu et al., 2013, Mishra et al., 2014, Jo et al., 2012). The basis for this treatment has historically centered on maximizing the growth factor (GF) found in platelet a-granules - such as the platelet-derived growth factor (PDGF), the transforming growth factor-B (TGF-B), and the vascular endothelial growth factor (VEGF). The goal is to regulate wound-healing processes and to promote an anabolic environment at the injury site (Werner and Grose, 2003). However, in vitro and in vivo studies using PRP preparations have produced results that are difficult to interpret in terms of the dose-dependent relationship between the load of the platelets/GFs delivered to the injury site and actual healing (Vo et al., 2012) because sample variability poses some methodological challenges to investigators.

PRP preparations have been prepared or activated from various formulations and have resulted in differences in the cellular

composition and biological activity of the platelets, which are sensitive to any type of process–related stress (Cavallo et al., 2014, Mazzucco et al., 2009, Mazzocca et al., 2012). According to previous studies, PRP can be classified according to three components (DeLong et al., 2012): (1) the absolute number of platelets, (2) the manner in which platelet activation occurs, and (3) the presence or absence of white cells. However, the cell count cannot comprehensively predict the GF content for some individual preparation, and variability in the isolation methods, the centrifuge speed/time, and the activation level of the PRP preparation can produce plasma preparations with differences in the quantity or quality of cytokine.

It is crucial to understand the GF release kinetics of PRP in order to determine the practical methods of the PRP application, such as the dose and the number of PRP injections. Most GFs that are contained within platelets have a short life span, and several clinical studies have suggested that a repeated injection of PRP improves clinical outcomes (Charousset et al., 2014). Although the platelet concentration or the cellular composition of PRP is widely used to assess PRP preparations (Cavallo et al., 2014, Braun et al., 2014), a description of the biological

characteristics of PRP may also be necessary to interpret study outcomes according to the preparation or activation method.

This study presents an in vitro analysis of the release of platelet-derived factors from PRP according to different preparation and activation protocols. Several studies have demonstrated differences in the platelet and in cytokine concentrations in PRP systems (Magalon et al., 2014, Mazzocca et al., 2012, Sundman et al., 2011), but these have only focused on comparing the platelet or cytokine concentration after an uncertain incubation period. The present study hypothesizes that PRP preparations are different in terms of their cellular composition and quality of bioactive molecules as a result of the differences in their preparation and activation protocols. In general, single-spin (SS) methods result in a lower concentrations of platelets and white blood cells, and double-spin (DS) methods result in a higher concentrations of platelets and white blood cells, so this study (1) determines the cytokine release kinetics of PRP between SS and DS methods, (2) evaluates the effects of Ca-only or Ca/thrombin activation on cytokine release kinetics in SS and DS PRP preparations, and

(3) compares the results against those of obtained with commercially available PRP kits.

MATERIALS AND METHODS

1. Subjects

Blood samples were obtained from 14 healthy subjects (7 females, 7 males; mean age ± standard deviation of 34.6 ± 6.6 years; range from 26 to 50 years). The Institutional Review Board approved this study, and all patients signed an informed consent form. The inclusion criteria included healthy subjects between the ages of 18 and 65 years without any known blood dyscrasia, and the exclusion criteria included medical history of any blood-derived illness or medication known to affect platelet or bone marrow function for a minimum of two weeks prior to testing.

2. Platelet-Rich Plasma Preparation

Each of the preparation procedures was performed for each subject to minimize the effect of inter-donor variability. Approximately 290 mL of peripheral venous blood was drawn from each subject and was collected into acid-citrate dextrose A (ACD-A) anticoagulant with a ratio of 1 ml ACD-A to 9 mL of whole blood. ACD-A binds with calcium and prevents blood

clotting with no known interference to platelet function. Then, the blood was divided and transferred to different protocol systems (Table 1).

3. Single-spin or double-spin PRP preparations

The optimal separating centrifugation condition (first spin) has been suggested to be of 900 g for 5 min with a recovery ratio of 92.0 + 3.1%, and the optimal condensation centrifugation condition (second spin) is of 1500 g for 15 min with a recovery ratio of 84.3 + 10.0% (Jo et al., 2013). In this study, the same protocols were used for the SS and the DS PRP preparations. For the SS PRP preparation, 30 ml of whole blood was centrifuged at 900 g for 5 min (Fig. 1A). Then the top plasma layer was separated, and the lower 3 ml of volume of the plasma were used as the SS PRP preparation. For the DS PRP preparation, the top plasma layer after the single spin was separated and was centrifuged a second time under 1500 g for 15 min, and the superficial plasma layer was removed, and finally, the lower 3 ml of volume of the plasma were used as the DS PRP preparation (Fig. 1B).

4. SS/DS PRP activation

Three different conditions were tested for exogenous activation: no activation, calcium gluconate only, and calcium and thrombin (Dede R Thrombin reagent, Siemens, Germany). The concentration of the GFs released from PRP varies over time and is influenced by the amount of calcium and thrombin (Lacoste et al., 2003, Roussy et al., 2007). Activation with a high dose of thrombin has been reported to induce an immediate release of GFs from platelets (Foster et al., 2009), whereas that with a low dose of calcium and thrombin reduces the immediate GF release (Lacoste et al., 2003). Calcium gluconate (2.084g/20ml) was added to PRP at a ratio of 1:10, and thrombin was added at 50 IU per 1 ml volume of PRP, which is less than that reported in a previous study (Berghoff et al., 2006, Han et al., 2009), to prevent any early depletion of GFs and to induce a sustained cytokine release over 7 days.

5. Comparison with three commercial kits

Three commercially available kits were used according to the manufacturer's instructions: Arthrex ACP (SS, plasma technique), Prodizen Prosys (DS, plasma technique), and

Biomet GPS III (SS, buffy coat method). None of these three commercial kits were exogenously activated by Ca or thrombin. The Arthrex ACP Double Syringe (Arthrex, Naples, Florida) used the SS method with a relatively lower concentration of platelets and white blood cells. Here, 15 mL of blood were filled into a double syringe to produce 3 mL of PRP. The syringes were centrifuged at 1500 rpm (1012.5 g) for five min to separate erythrocytes from the remaining plasma components. The top portion of the plasma was drawn up using an inner syringe without disrupting the erythrocyte layer (Fig. 1C). The GPS III Platelet Concentrate System (Biomet, Warsaw, Indiana) used the SS buffy coat extraction method, and 54 mL of whole blood were required to produce approximately 6 mL of PRP. The tubes were centrifuged for 15 min at 3200 rpm (2011.42 g) according to the manufacturer's protocols. After the specific construction of these tubes, it was possible to draw a portion of the PRP into a 6 mL syringe according to the manufacturer's instructions (Fig. 1D). The Prosys kit (Prodizen, Seoul, Korea) used DS methods with relatively higher concentrations of platelets and white blood cells. Here, 30 mL of citrated blood were filled in a tube and were centrifuged for 3 min at 3000

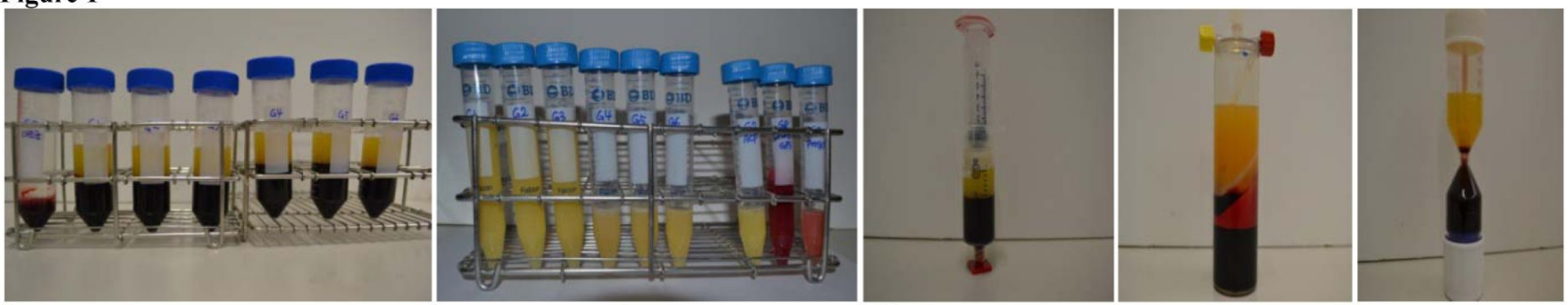
rpm (1660g) in the case of the male samples and 2800 rpm (1446g) in the case of the female samples. The upper plasma and the buffy coat layer after the first spin were separated and were centrifuged a second time under 3000 rpm (2008g) for 3 min, and the lower 3 mL of the plasma were used as the PRP (Fig. 1E)

Table 1. Five preparation protocols: two manual methods and three commercial kits

Preparations	Centrifugations		Isolation	Final vol./ WB vol.
	#1	#2		
Control				3 mL
SS	900g - 5min		Plasma layer	3 mL / 30 mL
DS	900g - 5min	1500g – 15min	Plasma layer	3 mL / 30 mL
Arthrex ACP	1012.5g-5min		Plasma layer	3 mL / 15 mL
Biomet GPSIII	2011g - 15min		Buffy coat layer	6 mL / 54 mL
Prodizen Prosys	1660g – 3min 1446g – 3min	2008g - 3min	Plasma layer	3 mL / 30 mL

SS = single spine method; DS = double spin method; WB = whole blood.

Figure 1



A. B. C. D. E.

Blood samples from 14 healthy subjects [7 females and 7 males with a mean age of 34.6 years (range, 26 – 50)] were obtained, and five preparation procedures were performed in each donor: single-spin (SS, 900 g / 5 min, Fig. 1A) and double-spin (DS, 900 g / 5 min and 1500 g/ 15 min, Fig 1B) manual methods and three commercial kits [Arthrex ACP (Fig. 1C), Biomet GPS (Fig. 1D), and ProdizenProsys (Fig. 1E)].

6. Evaluation of the cellular composition and kinetics of cytokine release

The platelet, WBC, and RBC counts were performed before cytokine quantification by using a cell count machine (XE-2100, Sysmex, Ill., USA). Most hematology analyzers are designed to operate within ranges found in whole blood, and PRP may exceed the upper limit of the linear range of platelets that can be counted. Therefore, PRP was suspended with normal saline at a ratio of 1:5, and the cellular content of PRP was analyzed.

Each PRP preparation or whole-blood (control) sample was divided into four aliquots and was incubated for 1 h, 24 h, 72 h and 7 days at 37 ℃ in 5% CO2. The samples were snap-frozen in individual aliquots after a given incubation period and were stored at -80 ℃ for a cytokine assay. Then these samples were assayed in duplicate, and PDGF-BB, TGF-b1, VEGF, bFGF, IL-1b, and MMP-9 concentrations were evaluated using commercially available bead-based sandwich immunoassay kits (Bio-Rad Laboratories, Hercules, CA, USA).

7. Statistical analysis

The measured data are presented as the arithmetic mean and the standard deviation (SD). The concentrations of the cytokines in each of the incubation periods were analyzed using a Kruskall-Wallis test (nonparametric ANOVA), a Bonferroni post-hoc test was conducted to compare multiple values from each of the preparation conditions, and linear correlations between the cell count and cytokine content were analyzed via Pearson correlations. A p-value of .05 was considered significant.

RESULTS

Cellular concentrations and compositions

The mean platelet concentration of the control samples was of 147 ± 59 x10^3/uL. The platelet concentration of the SS PRP at 311 ± 72 x 10^3/uL is significantly higher than that of control ($p < 0.01$), and that of the DS PRP is even higher at 1145 ± 244 x10^3/uL ($p < 0.01$)(Fig 2A). The WBC concentration of the DS PRP was of about 10 times higher than that of the SS PRP ($p < 0.01$), but both concentrations were lower than that of the control samples (Fig. 2B).

The commercial preparations exhibited wide variations in their ability to concentrate platelets and leukocytes. Of the three commercial kits, Biomet GPS had the highest platelet and WBC concentrations at 1076 ± 333 x10^3/uL and 32 ± 15 x10^3/uL, respectively, and Prodizen (DS method) had higher concentrations of the platelets and leukocytes than Arthrex ACP (SS method; $p < 0.01$ and < 0.01, respectively) (Fig. 2A, 2B). The whole-blood mean RBC concentration was significantly higher than that of any PRP system ($p < 0.01$ for all systems), and among all PRP systems, Biomet produced a PRP preparation with the highest mean RBC concentration in

comparison to Prodizen Prosys ($p = 0.03$) and Arthrex ACP ($p < 0.01$) (Fig. 2C).

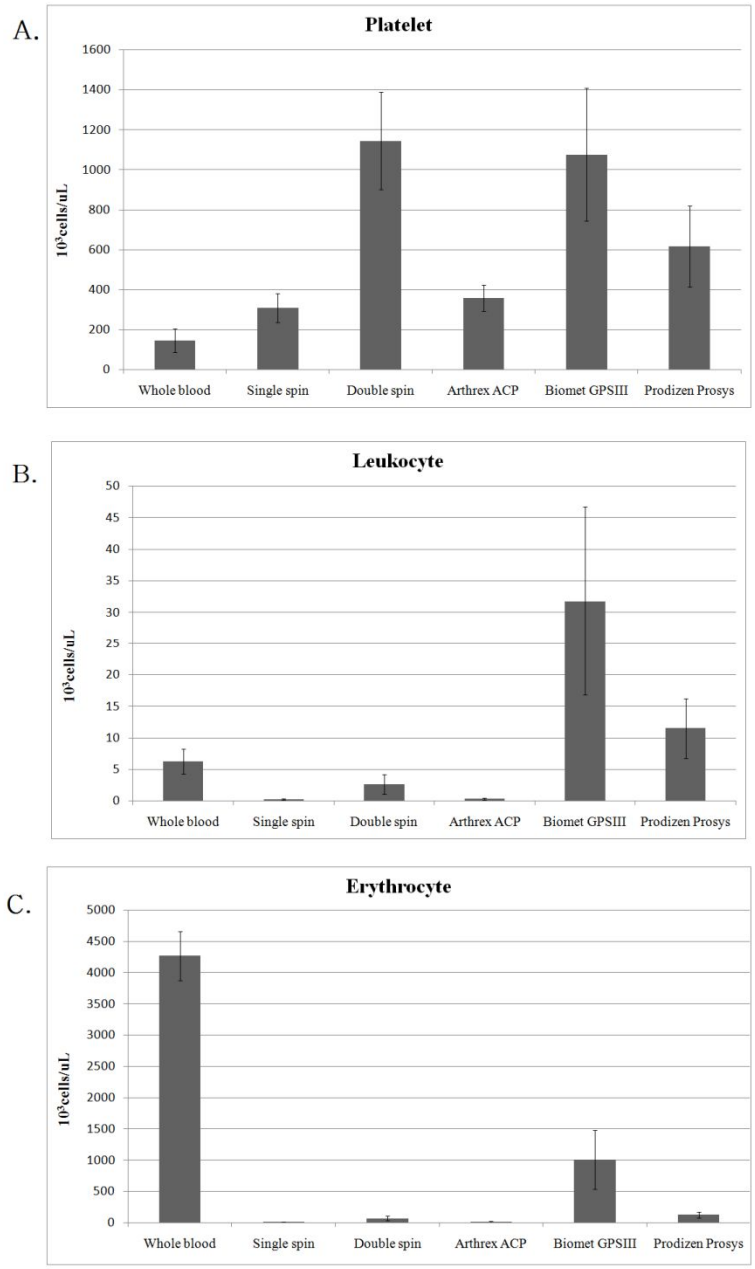


Figure 2. The platelet concentration of the SS PRP at 311 ± 72 x 10^3/uL is significantly higher than that of control ($p < .01$), and that of

the DS PRP is even higher at 1145 ± 244 x10^3/uL ($p < .01$) (Fig. 2A) The WBC concentration of the DS PRP was of about 10 times higher than that of the SS PRP ($p < 0.01$), but both concentrations were lower than that of the control samples. Biomet GPS had the highest platelet and WBC concentrations at 1076 ± 333 x103/uL and 32 ± 15 x103/uL, respectively, and Prodizen had higher concentrations of the platelets and leukocytes than Arthrex ACP (Fig. 2A, 2B). The whole-blood mean RBC concentration was significantly higher than that of any PRP system ($p < 0.01$ for all systems, Fig. 2C).

Time-sequential cytokine release

PDGF-BB

The PDGF release was constant and was sustained over 7 days. The PDGF concentrations of PRP were correlated with platelet concentrations ($r = 0.71$, $p = 0.02$), and the PDGF concentrations of the DS PRP were 2 to 3 times higher than those of the SS PRP over a 7-day period ($p < 0.01$ for all concentrations, Fig 3a). Ca-only activation did not significantly increase the PDGF concentration for both the SS and the DS PRP preparations (Fig 3b), whereas Ca/thrombin activation induced an immediate and sustainable increase in the PDGF concentration for both SS and DS PRPs over the 7-day period

(p < 0.01 for all measured times, Fig 3c). For the three commercial PRP kits, the PDGF concentrations of Prodizen exceeded those of Arthrex at 1 h, 24 h, 72 h and 7 days (p = 0.04, 0.01, 0.04, and 0.02, respectively), and those of Biomet exceeded those of Arthrex at 1 h, 24 h and 72 h (p = 0.04, <0.01, 0.07, and 0.26, respectively). However, no differences in the PDGF concentration were observed between Biomet and Prodizen over a 7-day period (Fig 3d).

TGF-b1

The TGF release was rapidly induced, and a maximum concentration was detected within 1 h with no significant differences in the TGF concentration between the SS and DS PRP preparations over the 7-day period (Fig 4a). The TGF-b1 concentration was not correlated with the platelet concentration of the PRP. Ca-only activation did not increase the TGF concentration of the SS or the DS PRP (Fig 4b). Ca/thrombin activation immediately but not-sustainably increased the TGF concentration of SS PRP at 1 h and 24 h (p <0.01 and 0.02, respectively) and that of DS PRP at 1 h and 24 hour (p < 0.01 and 0.02, respectively, Fig 4c). With respect to the three

commercial PRP kits, the TGF concentration of Arthrex exceeded that of Biomet at all measured times ($p = <0.01$, <0.01, 0.02, and <0.01, respectively), and there was no significant difference between Arthrex ACP and Prodizen Prosys (Fig 4d).

VEGF

The VEGF concentration of the PRP was correlated with the platelet concentration ($r = 0.71$, $p = 0.02$) and was constant and sustained over 7 days. The VEGF concentration of the DS PRP exceeded that of SS PRP at 72 h and after 7 days ($p = 0.06$ and < 0.01, respectively, Fig 5a). The Ca activation immediately and sustainably increased the VEGF concentration of the DS PRP at all measured times ($p < 0.01$ for all measured times, Fig 5b). The Ca/thrombin activation sustainably increased the VEGF concentration of DS PRP ($p <0.01$, <0.01, <0.01, 0.04 at 1 h, 24 h, 72 h, and 7 days, respectively) but increased that of SS PRP only at 1 h and 24 h ($p < 0.01$ and 0.02, respectively, Fig 5c). For the three commercial kits, the VEGF concentration of Biomet exceeded that of Arthrex at 24 h, 72 h, and 7 days ($p = 0.04$, < 0.01 and < 0.01, respectively)

and that of Prodizen at 24 h and 72 h ($p = 0.04$ and 0.04, respectively). On the other hand, there were no significant differences in the VEGF concentration between Arthrex and Prodizen at all of the measured times (Fig 5d).

FGF

The FGF release was rapidly induced and a maximum concentration was detected within 1 h, but the FGF concentration was not correlated with the platelet concentration. The FGF concentration of the SS PRP exceeded that of DS PRP at 24 h, 72 h, and 7 days ($p < 0.01$, $< .01$, and 0.04, respectively, Fig 6a). The Ca-only activation increased the FGF concentration of DS PRP at 24 h, 72 h and 7 days ($p < 0.01$, < 0.01, and 0.03, respectively), but reduced that of the SS PRP at all measured times ($p < 0.01$ for all measured times, Fig 6b). The Ca/thrombin activation immediately but not sustainably increased the FGF concentration of SS PRP ($p < 0.01$ at 1 h) and that of DS PRP ($p = 0.01$ and < 0.01, at 1 and 24 hours, respectively, Fig 6c). For the three commercial kits, the FGF concentration of Arthrex exceeded that of Biomet and Prodizen for all measured times ($p < 0.01$ for all measured times, Fig 6d).

IL-1

IL-1 release was constant and was sustained over 7 days. There were no significant differences in the IL-1 concentration between the SS and DS PRP preparations (Fig 7a). The IL-1 concentration was not correlated with the WBC or the PLT concentration, and the Ca activation led to a 10 to 20 times increase in IL-1 concentration for DS PRP at 24 h, 72 h, and 7 days ($p = 0.02$, < 0.01, and < 0.01, respectively, Fig 7b). The Ca/thrombin activation led to a 2 to 10 times increase in this concentration for the SS PRP over 7 days ($p < .01$ for all measured times, Fig 7c). Regarding the three commercial kits, the IL-1 concentration for Prodizen exceeded that of Arthrex and Biomet at 3 and 7 days ($p < 0.01$ and 0.01, respectively), but these were lower than the mean level of the control samples (Fig 7d).

MMP-9

The MMP-9 concentration was lower than 50 pg/mL over 7 days for both the SS and DS PRP preparations, and these values were lower than that of control (Fig 8a). The MMP-9

concentration was strongly correlated with the WBC concentration ($r = 0.938$, $p < 0.01$). There were no significant differences in the MMP-9 concentration between the SS and the DS PRP preparations. Ca-only or Ca/thrombin activation did not increase the MMP-9 concentration for both the SS and the DS PRP preparations (Fig 8b and 8c). For the three commercial kits, the MMP-9 concentration for Biomet exceeded that of Arthrex for all measured times ($p < 0.01$ for all measured time) and that of Prodizen at 1 h, 24 h, and 7 days ($p < 0.01$, 0.04, and < 0.01, respectively, Fig 8d).

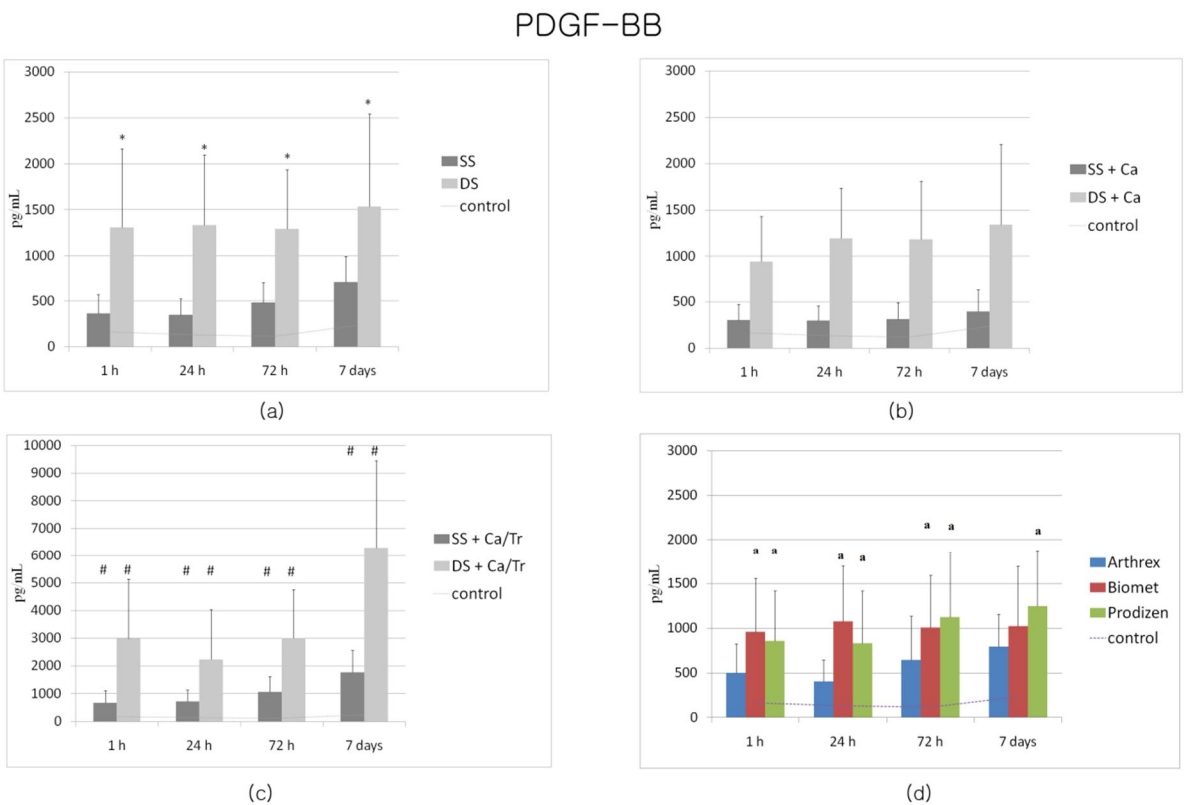


Figure 3. (a) The PDGF release was sustained over 7 days. (b) Ca-only activation did not significantly increase the

PDGF concentration for both the SS and the DS PRP preparations, (c) whereas Ca/thrombin activation induced an immediate and sustainable increase in the PDGF concentration for both SS and DS PRPs over the 7-day period. (d) The PDGF concentrations of Prodizen exceeded those of Arthrex at 1 h, 24 h, 72 h and 7 days and those of Biomet exceeded those of Arthrex at 1 h, 24 h and 72 h. (* represents $p < 0.05$ compared the SS group, # represents $p < 0.05$ compared to the non-activation group, a represents $p < 0.05$ compared to the Arthrex group.)

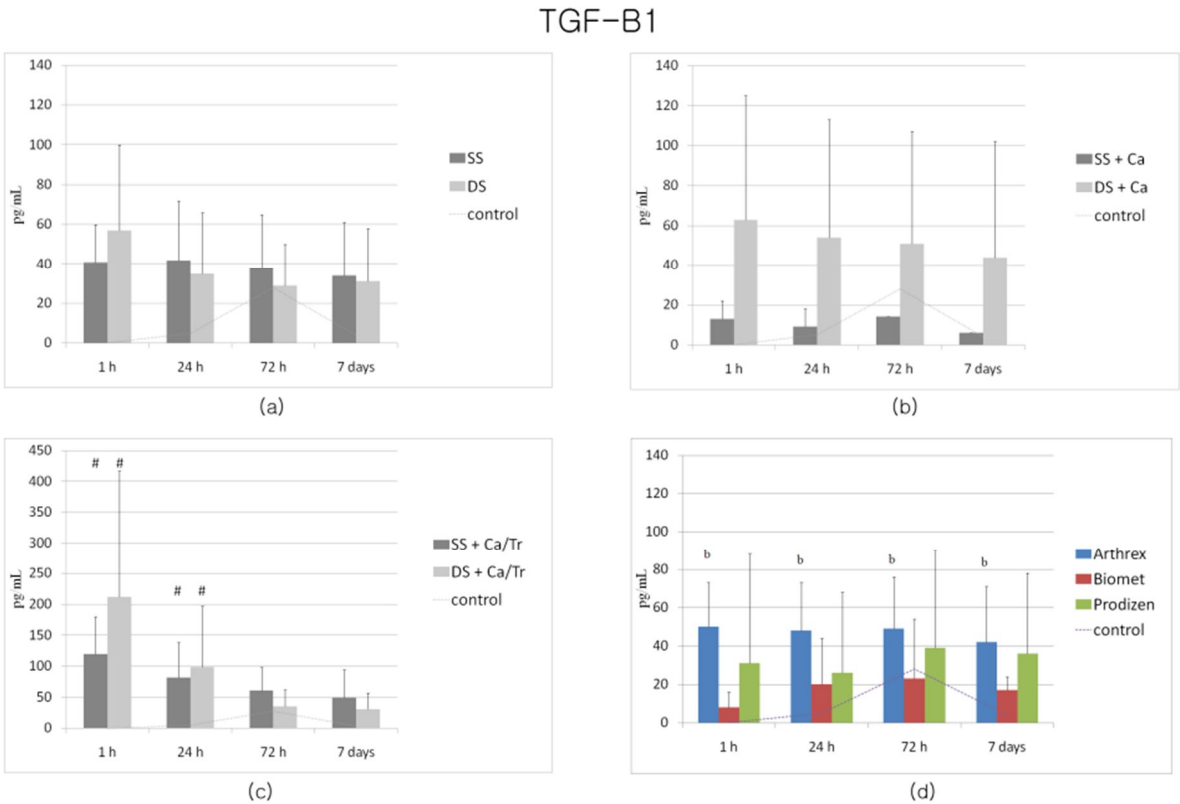


Figure 4. (a) The TGF release was rapidly induced, and a maximum concentration was detected within 1 h. (b) Ca-

only activation did not increase the TGF concentration of the SS or the DS PRP, and (c) Ca/thrombin activation immediately but not-sustainably increased the TGF concentration of SS PRP at 1 h and 24 h and that of DS PRP at 1 h and 24 hour. (d) The TGF concentration of Arthrex exceeded that of Biomet at all measured times (* represents $p < 0.05$ compared the SS group, # represents $p < 0.05$ compared to the non-activation group, b represents $p < 0.05$ compared to the Biomet group.)

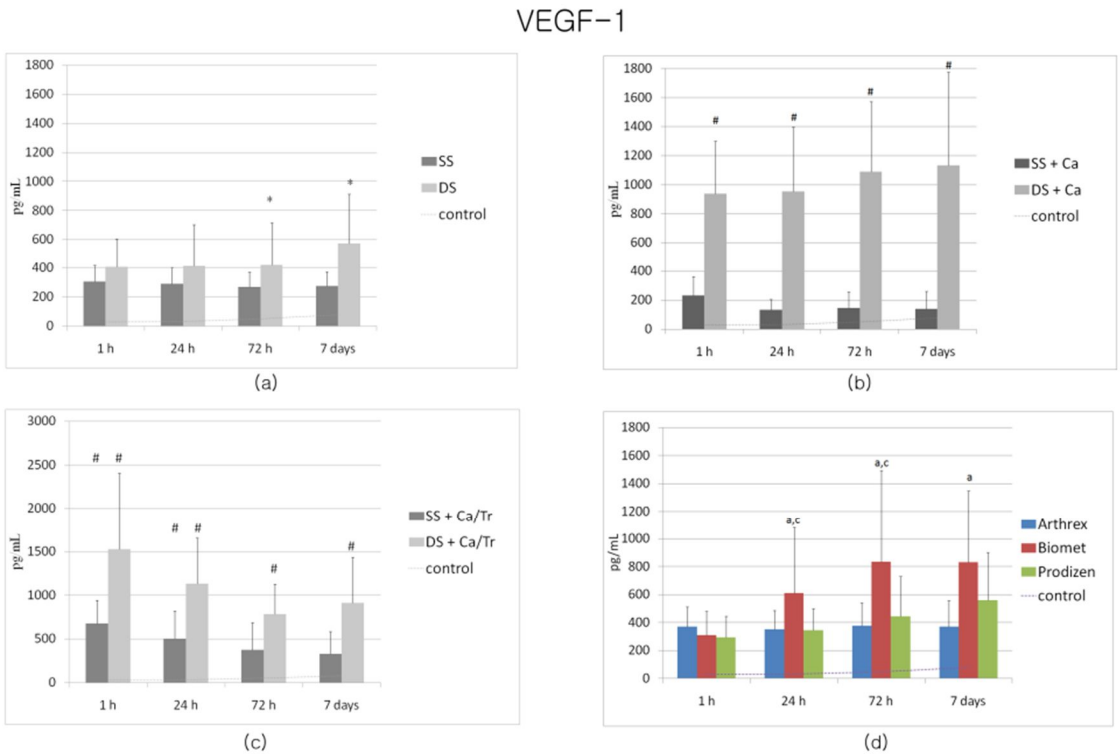


Figure 5. (a) The VEGF release was constant and sustained over 7 days. (b) The Ca activation immediately and

sustainably increased the VEGF concentration of the DS PRP at all measured times.(c) The Ca/thrombin activation sustainably increased the VEGF concentration of DS PRP, but increased that of SS PRP only at 1 h and 24 h. (d) The VEGF concentration of Biomet exceed that of Arthrex at 24 h, 72 h, and 7days and that of Prodizen at 24 h and 72 h. (* represents $p < 0.05$ compared the SS group, # represents $p < 0.05$ compared to the non-activation group, a represents $p < 0.05$ compared to the Arthrex group, c represents $p < 0.05$ compared to the Prodizen group.)

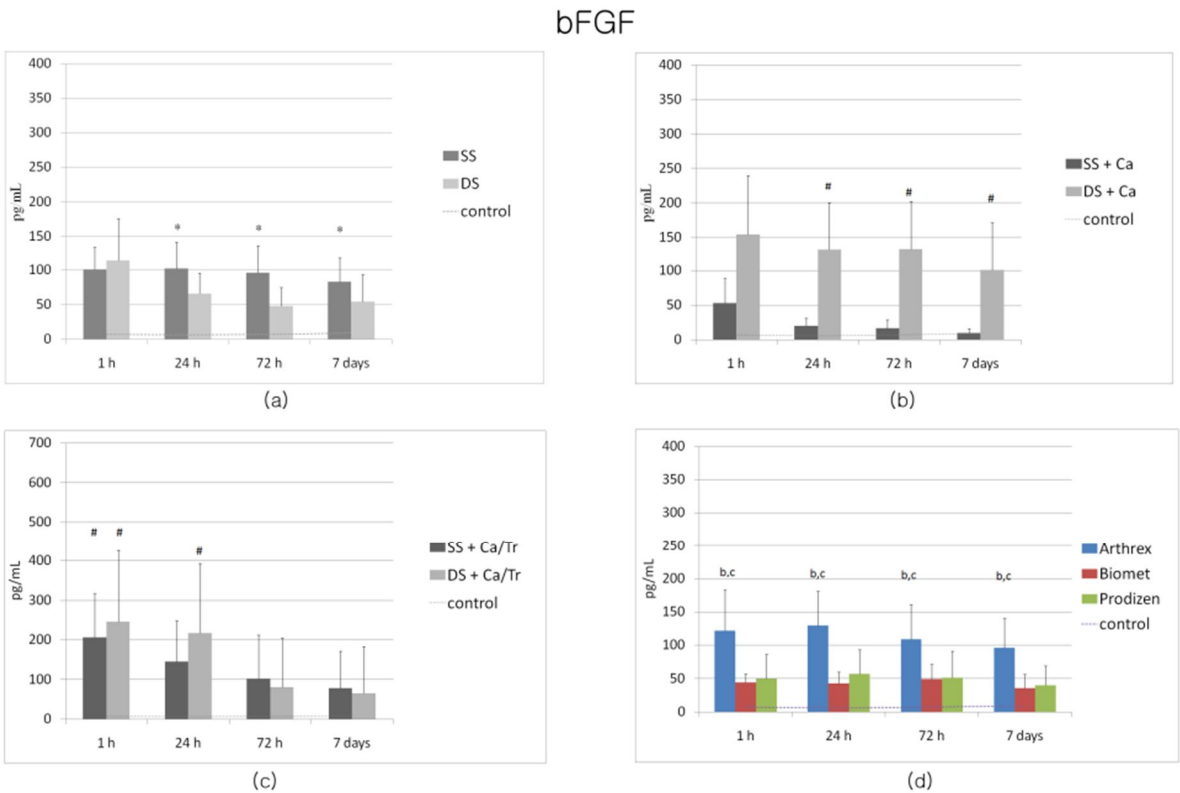


Figure 6. (a) The FGF release was rapidly induced and a maximum concentration was detected within 1 h. (b) The

Ca-only activation increased the FGF concentration of DS PRP at 24 h, 72 h and 7 days. (c) The Ca/thrombin activation immediately but not sustainably increased the FGF concentration of SS PRP and that of DS PRP. (d) The FGF concentration of Arthrex exceeded that of Biomet and Prodizen at all measured time. (* represents $p < 0.05$ compared the SS group, # represents $p < 0.05$ compared to the non-activation group, b represents $p < 0.05$ compared to the Biomet group, c represents $p < 0.05$ compared to the Prodizen group.)

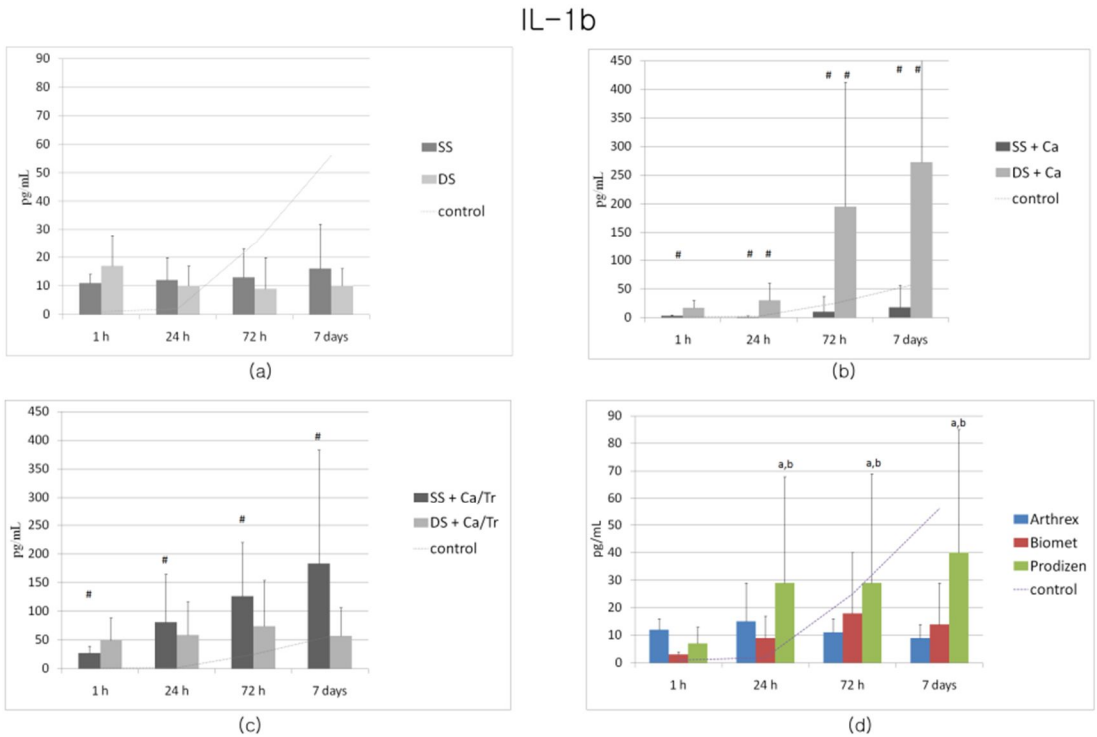


Figure 7. (a) There were no significant differences in the IL-1 concentration between the SS and DS PRP

preparations. (b) The Ca activation led to 10 to 20 times increase in IL−1 concentration for the DS PRP at 24 h, 72 h, and 7 days. (c) The Ca/thrombin activation led to 2 to 10 times increase in this concentration for the SS PRP over 7 days. (d) The IL−1 concentration for Prodizen exceeded that of Arthrex and Biomet at 3 and 7 days. . (* represents $p < 0.05$ compared the SS group, # represents $p < 0.05$ compared to the non−activation group, a represents $p < 0.05$ compared to the Arthrex group, b represents $p < 0.05$ compared to the Biomet group.)

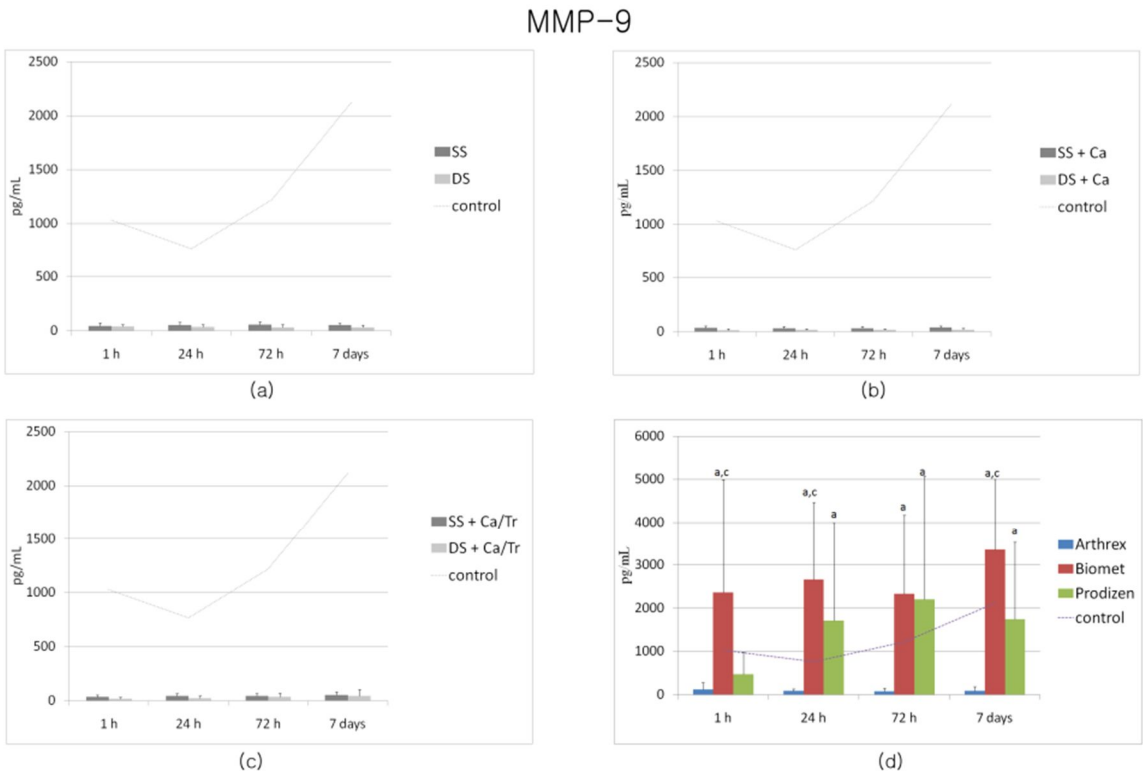


Figure 8. (a) The MMP-9 concentration was lower than 50pg/mL over 7 days for both the SS and DS PRP

preparations. There were no significant differences in the MMP-9 concentration between the SS and the DS PRP preparations. (b, c) Ca-only or Ca/thrombin activation did not increase the MMP-9 concentration for both the SS and the DS PRP preparations. (d) The MMP-9 concentration for Biomet exceeded that of Arthrex at all measured times and that of Prodizen at 1 h, 24 h, and 7 days. (* represents $p < 0.05$ compared the SS group, # represents $p < 0.05$ compared to the non-activation group, a represents $p < 0.05$ compared to the Arthrex group, c represents $p < 0.05$ compared to the Prodizen group.)

DISCUSSION

Despite the increase in the use of the PRP for local tissue healing and regeneration in human and animal studies, little is currently known about the bio-molecular characteristics of the PRP in terms of the cytokine content. It is important to determine the method of local application as well as to evaluate the effectiveness of the procedure, and to this end, this study evaluated the different qualities of bioactive molecules in PRP according to different preparation and activation protocols. The cytokine content is a prerequisite for PRP effectiveness, and it differed between the SS and the DS methods while neither was superior in terms of GF content. The effect of the activation was shown to be dependent on the preparation method as well as on the type of cytokine, and accordingly, proper PRP components with certain platelet concentrations and activation methods should be selected by considering their bio-molecular characteristics and patient indications.

The optimal platelet concentration of PRP for tissue healing and regeneration is believed to be 3 to 5 times higher than that of whole blood, and 1000 x 10^3/ uL is widely considered to be an effective platelet concentration to induce an efficient local

cellular response (Marx, 2004, Everts et al., 2006, Mazzocca et al., 2012). On the other hand, a platelet concentration of six times higher than that of whole blood has been reported to have an inhibitory effect on healing (El-Sharkawy et al., 2007). Conversely, the effect of highly concentrated WBCs in PRP preparation has been widely debated (Dohan Ehrenfest et al., 2009). Leukocytes in PRP may play a valuable antimicrobial role (Moojen et al., 2008) but may impede tissue healing by producing biologically active catabolic cytokines (Braun et al., 2014). The effect of the leukocyte may depend on the concentration of the leukocytes or the biological state of the injured tissue, indicating a need for further research about the role that leukocytes of PRPs play in different patient indications. In this study, the DS method generally produced higher concentrations of platelets and leukocytes, and similar outcomes were observed when using commercial kits. More specifically, Prodizen Prosys produced higher concentrations of platelets and leukocytes than Arthrex ACP. It is noteworthy that Biomet GPS showed the highest concentrations of platelet and leukocytes, which may be due to direct PRP extraction from the buffy coat layer after a single round of centrifugation.

This suggests that a direct buffy coat isolation method requires close attention in terms of its high leukocyte content.

The PDGF, TFG-B, VEGF, and FGF concentrations in PRP are known to play a crucial role in cell proliferation, chemotaxis, cell differentiation, and angiogenesis (Sanchez-Gonzalez et al., 2012). PDGF is a powerful mitogen for fibroblasts and smooth muscle cells, and it is involved in all three phases of wound healing, including angiogenesis, the formation of fibrous tissue, and re-epithelialization (Hosgood, 1993). In this study, PDGF-BB, which is a prevalent circulating isoform, was evaluated whereas normal unstimulated cells of the osteoblast lineage primarily synthesize PDGF-AA. TGF β stimulates the proliferation of undifferentiated mesenchymal stem cells and the chemotaxis of endothelial cells and angiogenesis (Civinini et al., 2013), and it exists as three known subtypes in humans (TGFβ1, TGFβ2, and TGFβ3). VEGF was originally referred to as the vascular permeability factor, and it increases angiogenesis and vascular permeability (Hoeben et al., 2004). In this study, VEGF-1, which is the most important member of the VEGF family that stimulates endothelial cell mitogenesis and cell migration, was evaluated. FGF plays a key role in the

proliferation and differentiation of a wide variety of cells and tissues (Cao et al., 2003), and bFGF mediates the formation of new blood vessels during the wound healing of normal tissue and tumor development.

IL-1b and MMP-9 are catabolic cytokines that are known to play roles in inflammation or matrix degradation (Sundman et al., 2011). Interleukin-1b is a primary cytokine during inflammation and matrix degradation, and it is a common target to reduce inflammation by manipulating IL-1ra (Thampatty et al., 2007). Injured human rotator cuffs have been shown to result in an increase in the concentration of IL-1b in the tendon, along with other proinflammatory cytokines (Voloshin et al., 2005). MMP-9 is known to degrade collagen and other extracellular matrix molecules and has been implicated as a predictor of poor healing (Chakraborti et al., 2003). Elevated MMP concentrations, which are found in acute injured and denegerative tissue and postoperative tissue, have been widely observed in tens to hundreds of nanograms per milliliter. Several studies have shown these concentrations to be a component of non-healing or poorly healing wounds (Voloshin et al., 2005, Liu et al., 2009).

The results of this study indicate that the relationship between cytokine concentrations and cellular compositions was mainly dependent on the type of cytokine. The DS method generally resulted in higher concentrations of platelets and leukocytes and was supposed to produce more GFs than the SS method. However, the resulting GF or cytokine concentrations were not necessarily proportional to the cellular count. The PDGF and VEGF concentrations for PRP were correlated with the platelet count, but the TGF and FGF concentrations were higher in the SS method than in the DS method, despite the lower platelet count. In addition, the three commercial PRP kits provided similar results with higher TGF and FGF concentrations in Arthrex ACP than in Prodizen Prosys. Some studies have reported a correlation between the platelet concentration and the GF concentration in PRP (Sundman et al., 2011, Castillo et al., 2011) while others have not (Mazzucco et al., 2009, Weibrich et al., 2002). In addition, Martineau et al. (Lacoste et al., 2003) found no correlations between the concentrations of various GFs within the same donor with the highest bFGF concentration and the lowest VEGF concentration. Several factors, including inter-donor content variability of cytokine or

GF-absorbing proteins, manipulation-induced platelet stress, the variable susceptibility of platelets, and micro-aggregation in PRP may contribute to differences in the correlations.

The DS method showed higher concentrations of IL-1 and MMP-9 than the SS method, but these concentrations were similar or lower than those of the control samples. However, the MMP-9 concentration was about 10 times higher in Biomet GPS (direct buffy coat extraction) than in the control. The MMP-9 concentration was strongly correlated with the WBC count while the IL-9 concentration was not, and the results indicate ng/ml-level concentrations of MMP-9 in Biomet and Prodizen and pg/ml-level concentrations in the SS, DS, and ACP preparations. (The latter were similar to or lower than those of the whole-blood samples.)

The effect of the activation depended on both the preparation method and on the type of cytokine. When calcium chloride was added exogenously to the PRP, a low level of thrombin formed endogenously and allowed a slower GF release over a longer period than exogenous thrombin. On the other hand, thrombin caused a rapid aggregation of platelets and an excessive condensing of the fibrin matrix with a rapid activation of the

platelets. A low dose of thrombin has been shown to increase the migration and the number of mesenchymal progenitor cells derived from bone marrow (Gruber et al., 2004) whereas high concentrations have been demonstrated to have limited effects on the proliferation of osteoblasts and alveolar bone cell, suggesting that the thrombin dose plays a role in the GF release kinetics of the PRPs (Choi et al., 2005).

The activation method or the thrombin dose must be considered when interpreting the results of this study. Ca-only activation had a significant effect on the DS PRP preparation (VEGF, FGF, and IL-1 concentrations) whereas Ca/thrombin activation had significant effects on the SS and DS PRP preparations (PDGF and VEGF concentrations sustainably and TGF and FGF concentrations shortly). These observations may be due to the fact that the biological activity of the platelets is sensitive to any kind of process-related stress and that more platelets are activated during the process with the DS method. In terms of the GFs, a low dose of thrombin caused sustainable increases in the PDGF and VEGF concentrations but with a rapid release and early depletion of the TGF and the FGF. On the other hand, Ca-only activation sustainably increased the TGF and the FGF.

These results are consistent with the findings reported by Mazzucco et al. (Mazzucco et al., 2009), who reported that the individual dynamics of the GF release depends exclusively on the type of GF rather than on the preparation method. They also demonstrated that TGF-B1 and b-FGF are promptly released within 24 h after exogenous activation whereas the GF release of the PDGF-BB and the VEGF are more dependent on the technique that is used. In terms of the catabolic cytokines, Ca or thrombin activation sustainably increased the IL-1 concentration to a level 10 times higher than that for the control but did not increase the MMP-9 release over time. These results suggest that the mechanisms underlying the synthesis, release, and/or degradation of IL-1b differ from those for MMP-9 and that the release of IL-1b may be a result of some de novo synthesis by leukocytes or platelets following PRP activation with a low concentration of Ca or thrombin (Frechette et al., 2005).

The limitations of this study are a result of the in vitro observations of cytokine release of the platelets. First, tissue healing and regeneration generally occur via a multiplicity of cellular interactions, but these processes were absent in the in

vitro assay in this study. In this regard, future studies should investigate the relationship between the GF kinetics and tissue healing/regeneration in vivo. Second, the subjects were relatively young, with a mean age of 34.6. The cytokine content is influenced mainly by inter-individual variability, which in turn may influence the release kinetics of the PRP GFs. Finally, although the separation and activation methods that were considered in this study are representative techniques, various other methods are also commercially available. However, not all PRP-kits could be considered due to patient recruitment and financial limitations.

PRP has distinct characteristics that reflect specific mixtures of bioactive molecules, and the regenerative potency of PRP may depend on the GF levels. GF content, which is a prior requirement for PRP effectiveness, was observed to be different between the SS and DS methods, and neither was superior in terms of the GF content. The PDGF and VEGF concentrations were higher in the DS methods than in the SS methods, but the TGF and FGF concentrations were higher in the SS methods despite their lower platelet count. A low dose of thrombin/calcium activation caused an overall increase in

cytokine content over a period of 7 days when compared to a calcium-only supplement or non-activation preparations, and the effect of the activation depended not only on the preparation method but also on the type of cytokine. Ca-only activation had a significant effect on DS PRP preparation while a low dose of thrombin with calcium activation had significant effects on both the SS and DS PRP preparations. In this regard, proper PRP components with specific platelet concentrations and activation methods should be considered in future studies by taking into account both the bio-molecular characteristics and the patient indications.

CONCLUSION

In conclusion, the double spin method generally leads to a higher concentration of PLT when compared to a single spin method, which had been considered to induce an optimal and efficient local cellular response. The cytokine content was not necessarily proportional to the cellular composition of the PRPs since a greater content was observed to have differences between the SS or DS method depending on the type of cytokine. In terms of the PRP activation, a low dose of thrombin/calcium activation increased the overall cytokine release of the PRP preparation over 7 days relative to those with calcium-only supplement or non-activation.

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국문 초록

서론: 혈소판 풍부 혈장은 다양한 방식으로 제조되고 활성화되어 사용되고 있는데, 이는 혈소판 풍부 혈장의 세포 구성이나 활성도에 상당한 차이를 초래하고 있다. 이번 연구는 혈소판 풍부 혈장의 제조 및 활성화 방법에 따른 세포 구성 및 사이토카인 방출 역학을 분석하고자 하였다.

방법: 14 명의 건강한 성인이 대상이 되었으며, 각각의 공여자에 대해 5 가지 방식으로 혈소판 풍부 혈장을 제조하였다: 단회전 (SS, 900 g – 5 min) 및 이회전 (DS, 900 g – 5 min and 1500 g – 15 min) 제조법과 세 종류의 상품화된 제품 (Arthrex ACP, Biomet GPS, and Prodizen Prosys) 을 평가하였다. 각각의 방법으로 제조된 혈소판 풍부 혈장은 4 개의 표본으로 나뉘어 1, 24, 72 시간 및 7 일간 배양되었다. 사이토카인 방출 역학을 평가하기 위해 platelet-derived growth factor (PDGF), transforming growth factor (TGF), vascular endothelial growth factor (VEGF), fibroblast growth factor (FGF), interleukin-1 (IL-1), and matrix metalloproteinase-9 (MMP-9) 농도를 bead-based sandwich immunoassay kits 을 이용하여 시간대 별로 측정하였다. 단회전 및 이회전으로 제조된 혈소판 풍부 혈장의 활성화에 따른 사이토카인 방출 양상을 평가하기 위해, 활성화 물질을 첨가하지 않

은 경우, 칼슘만 사용한 경우, 칼슘과 저농도 트롬빈을 사용한 경우를 비교하여 분석하였다.

결과: 이회전 혈소판 풍부 혈장은 단회전 혈소판 풍부 혈장보다 높은 혈소판 (4 배) 및 백혈구 (9 배) 농도를 보였다. 두 가지 제조 방법으로 만든 혈소판 풍부 혈장은 전혈에 비해 7 일의 기간 동안 지속적으로 높은 PDGF, TGF, VEGF, 및 FGF 의 방출을 보였고, IL-1 과 MMP-9 의 경우 전혈에 비해 낮은 방출을 보였다. 사이토카인 방출 역학은 사이토카인의 종류에 따라 뚜렷한 차이를 보였다. FGF 와 TGF 의 경우 빠르게 방출되어 1 시간 이내에 최고치에 도달하고 이후 지속적으로 감소하였다. PDGF 와 VEGF 는 7 일의 기간 동안 비교적 일정한 농도를 지속하였다. PDGF 와 VEGF 의 농도는 이회전 혈소판 풍부 혈장에서 높게 측정된 데 반해, TGF 와 FGF 의 농도는 단회전 혈소판 풍부 혈장에서 높게 측정되었다.

상품화된 제품의 경우, Arthrex ACP 의 사이토카인 방출 양상이 이번 실험의 단회전 혈소판 풍부 혈장과 비슷하였고, Prodizen Prosys 는 사이토카인 방출 양상이 이회전 혈소판 풍부 혈장과 유사했다. 세 제품 중, Biomet GPS 는 가장 높은 혈소판과 백혈구 농도를 보였는데, VEGF 와 MMP-9 이 가장 높았고, TGF 는 가장 낮게 방출되었다. Arthrex ACP 는 가장 높은 FGF 농도를 보인데 반해 가장 낮은 PDGF 농도를 보였다.

칼슘을 이용한 활성화는 이회전 혈소판 풍부 혈장의 사이토카인 방출에 주로 영향을 준데 반해, 칼슘과 트롬빈을 같이 사용한 경우는 단회전과 이회전 혈소판 풍부 혈장의 사이토카인 방출에 모두 영향을 주었다. IL-1 은 칼슘이나 트롬빈에 의해 방출이 증가한 데 반해 MMP-9 은 이들 영향을 받지 않았다.

결론: 혈소판 풍부 혈장은 제조법 및 활성화 방식에 따라 세포 구성이나 사이토카인 방출 양상에 상당한 차이를 보여주었다. 이회전 제조법이 단회전에 비해 보다 높은 혈소판 농도를 보였으며, 이는 혈소판 풍부 혈장의 효율적인 국소 세포 반응에 보다 적합한 조건으로 고려된다. 하지만 사이토카인의 양은 혈소판 풍부 혈장의 세포 조성에 반드시 비례하지 않았으며, 사이토카인 종류에 따라 보다 높게 측정되는 제조 방법이 달랐다. 혈소판 활성화의 경우 칼슘과 저농도 트롬빈을 같이 사용한 경우가 칼슘만을 사용하거나 활성화 물질을 넣지 않은 경우에 비해 대체로 보다 많은 사이토카인 방출을 유도하였다.

주요어 : 혈소판 풍부 혈장; 제조법; 활성화; 세포 구성; 사이토카인 ; 동역학

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