Platelet-Rich Plasma Differs According to Preparation Method and Human Variability

Augustus D. Mazzocca, MS, MD, Mary Beth R. McCarthy, BS, David M. Chowaniec, BS, Mark P. Cote, DPT,
Anthony A. Romeo, MD, James P. Bradley, MD, Robert A. Arciero, MD, and Knut Beitzel, MD

Investigation performed at the Department of Orthopaedic Surgery, University of Connecticut Health Center, Farmington, Connecticut

Background: Varying concentrations of blood components in platelet-rich plasma preparations may contribute to the variable results seen in recently published clinical studies. The purposes of this investigation were (1) to quantify the level of platelets, growth factors, red blood cells, and white blood cells in so-called one-step (clinically used commercial devices) and two-step separation systems and (2) to determine the influence of three separate blood draws on the resulting components of platelet-rich plasma.

Methods: Three different platelet-rich plasma (PRP) separation methods (on blood samples from eight subjects with a mean age [and standard deviation] of 31.6 ± 10.9 years) were used: two single-spin processes (PRP_{LP} and PRP_{HP}) and a double-spin process (PRP_{DS}) were evaluated for concentrations of platelets, red and white blood cells, and growth factors. Additionally, the effect of three repetitive blood draws on platelet-rich plasma components was evaluated.

Results: The content and concentrations of platelets, white blood cells, and growth factors for each method of separation differed significantly. All separation techniques resulted in a significant increase in platelet concentration compared with native blood. Platelet and white blood-cell concentrations of the PRP_{HP} procedure were significantly higher than platelet and white blood-cell concentrations produced by the so-called single-step PRP_{LP} and the so-called two-step PRP_{DS} procedures, although significant differences between PRP_{LP} and PRP_{DS} were not observed. Comparing the results of the three blood draws with regard to the reliability of platelet number and cell counts, wide variations of intra-individual numbers were observed.

Conclusions: Single-step procedures are capable of producing sufficient amounts of platelets for clinical usage. Within the evaluated procedures, platelet numbers and numbers of white blood cells differ significantly. The intra-individual results of platelet-rich plasma separations showed wide variations in platelet and cell numbers as well as levels of growth factors regardless of separation method.

Clinical Relevance: The variability of components and its effects on dosage should be considered in single or consecutive treatments of platelet-rich plasma. Significant differences in components were observed in different separation methods and may have specific results on treated tissue.

Over the past decade, platelet-rich plasma has gained increased attention in orthopaedic sports medicine. Several investigators have advocated the use of platelet-rich plasma in the management of bone, muscle, tendon, and cartilage injury[1-3]. Platelets contain growth factors in their alpha-granules, such as transforming growth factor-beta (TGF-β), fibroblast growth factor-2 (FGF-2), platelet-derived growth factors (PDGF-AB), insulin-like growth factor-1 (IGF-1), epi-dermal growth factor (EGF), hepatocyte growth factor (HGF), and vascular endothelial growth factor (VEGF-A), which are thought to produce beneficial effects on the healing process. The ultimate goal of platelet-rich plasma treatment is to concentrate these growth factors and reintroduce them to a site of injury[1].

Despite the growing popularity of platelet-rich plasma treatment, little is known regarding the specifics of plasma preparations or the devices used in their production[1-3]. Platelet-

30. D'Elia CO, Uchoa MR, Bitar AC, Tatsui N, Pecora JR, Hernandez AJ, Camanho GL. A comparative study of the use of platelet-rich plasma and autologous iliac crest as graft sources in opening wedge high tibial osteotomy. Presented at the 7th Biannual Meeting of the International Society of Arthroscopy, Knee Surgery and Orthopaedic Sports Medicine; 2009 Apr 5-9; Osaka, Japan. E-poster no.1002.

31. Dallari D, Savarino L, Stagni C, Cenni E, Cenacchi A, Fornasari PM, Albisinni U, Rimondi E, Baldini N, Giunti A. Enhanced tibial osteotomy healing with use of bone grafts supplemented with platelet gel or platelet gel and bone marrow stromal cells. J Bone Joint Surg Am. 2007;89:2413-20.

32. Savarino L, Cenni E, Tarabusi C, Dallari D, Stagni C, Cenacchi A, Fornasari PM, Giunti A, Baldini N. Evaluation of bone healing enhancement by lyophilized bone grafts supplemented with platelet gel: a standardized methodology in patients with tibial osteotomy for genu varus. J Biomed Mater Res B Appl Biomater. 2006;76:364-72.

33. Lee TG, Ahmad TS. Intralesional autologous blood injection compared to corticosteroid injection for treatment of chronic plantar fasciitis. A prospective, randomized, controlled trial. Foot Ankle Int. 2007;28:984-90.

34. Kiter E, Celikbas E, Akkaya S, Demirkan F, Kiliç BA. Comparison of injection modalities in the treatment of plantar heel pain: a randomized controlled trial. J Am Podiatr Med Assoc. 2006;96:293-6.

35. Castricini R, Longo UG, De Benedetto M, Panfoli N, Pirani P, Zini R, Maffulli N, Denaro V. Platelet-rich plasma augmentation for arthroscopic rotator cuff repair: a randomized controlled trial. Am J Sports Med. 2011;39:258-65.

36. Randelli P, Arrigoni P, Ragone V, Aliprandi A, Cabitza P. Platelet rich plasma in arthroscopic rotator cuff repair: a prospective RCT study, 2-year follow-up. J Shoulder Elbow Surg. 2011;20:518-28.

37. Horstmann WG, Slappendel R, van Hellemondt GG, Wymenga AW, Jack N, Everts PA. Autologous platelet gel in total knee arthroplasty: a prospective randomized study. Knee Surg Sports Traumatol Arthrosc. 2010 July 18. [Epub ahead of print].

38. Peerbooms JC, Sluimer J, Bruijn DJ, Gosens T. Positive effect of an autologous platelet concentrate in lateral epicondylitis in a double-blind randomized controlled trial: platelet-rich plasma versus corticosteroid injection with a 1-year follow-up. Am J Sports Med. 2010;38:255-62.

39. Schepull T, Kvist J, Norrman H, Trinks M, Berlin G, Aspenberg P. Autologous platelets have no effect on the healing of human Achilles tendon ruptures: a randomized single-blind study. Am J Sports Med. 2011;39:38-47.

40. Peerbooms JC, de Wolf GS, Colaris JW, Bruijn DJ, Verhaar JA. No positive effect of autologous platelet gel after total knee arthroplasty. Acta Orthop. 2009;80:557-62.

41. Zavadil DP, Satterlee CC, Costigan JM, Holt DW, Shostrom VK. Autologous platelet gel and platelet-poor plasma reduce pain with total shoulder arthroplasty. J Extra Corpor Technol. 2007;39:177-82.

42. Thomas W, Tafuro L, Thomas S. Osteoinductive gel in cementless hip joint replacement: a randomized prospective study. Curr Orthop Prac. 2009;20:655-9.

43. Calori GM, Tagliabue L, Gala L, d'Imporzano M, Peretti G, Albisetti W. Application of rhBMP-7 and platelet-rich plasma in the treatment of long bone non-unions: a prospective randomised clinical study on 120 patients. Injury. 2008;39:1391-402.

44. Everts PA, Devilee RJ, Brown Mahoney C, van Erp A, Oosterbos CJ, Stellenboom M, Knape JT, van Zundert A. Exogenous application of platelet-leukocyte gel during open subacromial decompression contributes to improved patient outcome. A prospective randomized double-blind study. Eur Surg Res. 2008;40:203-10.

45. Figueroa D, Melean P, Calvo R, Vaisman A, Zilleruelo N, Figueroa F, Villalón I. Magnetic resonance imaging evaluation of the integration and maturation of semitendinosus-gracilis graft in anterior cruciate ligament reconstruction using autologous platelet concentrate. Arthroscopy. 2010;26:1318-25.

46. Radice F, Yánez R, Gutiérrez V, Rosales J, Pinedo M, Coda S. Comparison of magnetic resonance imaging findings in anterior cruciate ligament grafts with and without autologous platelet-derived growth factors. Arthroscopy. 2010;26:50-7.

47. Silva A, Sampaio R. Anatomic ACL reconstruction: does the platelet-rich plasma accelerate tendon healing? Knee Surg Sports Traumatol Arthrosc. 2009;17:676-82.

48. Hartmann EK, Heintel T, Morrison RH, Weckbach A. Influence of platelet-rich plasma on the anterior fusion in spinal injuries: a qualitative and quantitative analysis using computer tomography. Arch Orthop Trauma Surg. 2010;130:909-14.

49. Jenis LG, Banco RJ, Kwon B. A prospective study of Autologous Growth Factors (AGF) in lumbar interbody fusion. Spine J. 2006;6:14-20.

50. Jo CH, Kim JE, Yoon KS, Lee JH, Kang SB, Lee JH, Han HS, Rhee SH, Shin S. Does platelet-rich plasma accelerate recovery after rotator cuff repair? A prospective cohort study. Am J Sports Med. 2011 Jul 7 [Epub ahead of print].

51. Mishra A, Pavelko T. Treatment of chronic elbow tendinosis with buffered platelet-rich plasma. Am J Sports Med. 2006;34:1774-8.

52. Everts PA, Devilee RJ, Oosterbos CJ, Mahoney CB, Schattenkerk ME, Knape JT, van Zundert A. Autologous platelet gel and fibrin sealant enhance the efficacy of total knee arthroplasty: improved range of motion, decreased length of stay and a reduced incidence of arthrofibrosis. Knee Surg Sports Traumatol Arthrosc. 2007;15:888-94.

53. Filardo G, Kon E, Della Villa S, Vincentelli F, Fornasari PM, Marcacci M. Use of platelet-rich plasma for the treatment of refractory jumper's knee. Int Orthop. 2010;34:909-15. 2009 Jul 31 [Epub ahead of print].

54. McCormack HM, Horne DJ, Sheather S. Clinical applications of visual analogue scales: a critical review. Psychol Med. 1988;18:1007-19.

55. Creaney L, Wallace A, Curtis M, Connell D. Growth factor-based therapies provide additional benefit beyond physical therapy in resistant elbow tendinopathy: a prospective, single-blind, randomised trial of autologous blood injections versus platelet-rich plasma injections. Br J Sports Med. 2011 Mar 15. [Epub ahead of print].

56. de Vos RJ, van Veldhoven PL, Moen MH, Weir A, Tol JL, Maffulli N. Autologous growth factor injections in chronic tendinopathy: a systematic review. Br Med Bull. 2010;95:63-77.

57. Griffin XL, Smith CM, Costa ML. The clinical use of platelet-rich plasma in the promotion of bone healing: a systematic review. Injury. 2009;40:158-62.

58. Bhandari M, Richards RR, Sprague S, Schemitsch EH. The quality of reporting of randomized trials in the Journal of Bone and Joint Surgery from 1988 through 2000. J Bone Joint Surg Am. 2002;84:388-96.

59. Poolman RW, Struijs PA, Krips R, Sierevelt IN, Lutz KH, Bhandari M. Does a "Level I Evidence" rating imply high quality of reporting in orthopaedic randomised controlled trials? BMC Med Res Methodol. 2006;6:44.

60. Poolman RW, Struijs PA, Krips R, Sierevelt IN, Marti RK, Farrokhyar F, Bhandari M. Reporting of outcomes in orthopaedic randomized trials: does blinding of outcome assessors matter? J Bone Joint Surg Am. 2007;89:550-8.

61. Hulley SB, Cummings SR, Browner WS, Grady D, Newman TB. Designing clinical research. 3rd ed. Philadelphia: Lippincott Williams and Wilkins; 2006.

62. Castillo TN, Pouliot MA, Kim HJ, Dragoo JL. Comparison of growth factor and platelet concentration from commercial platelet-rich plasma separation systems. Am J Sports Med. 2011;39:266-71.

63. de Mos M, van der Windt AE, Jahr H, van Schie HT, Weinans H, Verhaar JA, van Osch GJ. Can platelet-rich plasma enhance tendon repair? A cell culture study. Am J Sports Med. 2008;36:1171-8.

64. Virchenko O, Aspenberg P. How can one platelet injection after tendon injury lead to a stronger tendon after 4 weeks? Interplay between early regeneration and mechanical stimulation. Acta Orthop. 2006;77:806-12.

65. McCarrel T, Fortier L. Temporal growth factor release from platelet-rich plasma, trehalose lyophilized platelets, and bone marrow aspirate and their effect on tendon and ligament gene expression. J Orthop Res. 2009;27:1033-42.

rich plasma is a generic term referring to any sample of autologous plasma with platelet concentrations above baseline blood values[2]. Defining platelet-rich plasma preparations according to platelet count can be difficult, as consensus on adequate concentration is lacking. Platelet concentrations of 200×10^3 platelets/μL up to 1000×10^3 platelets/μL are considered therapeutic for tissue-healing, whereas much higher counts appear to be biologically unfavorable[1,4-6]. Describing platelet-rich plasma according to the method of production may be more appropriate as either one or two-step centrifugation processes are used to fraction whole blood and concentrate the platelets[1,3,7].

Currently, there are several commercial devices available for the preparation of platelet-rich plasma. While their common purpose is to fraction whole blood into its composite parts to allow isolation of plasma with elevated levels of platelets, each device functions differently. Specifically, these devices and their protocols differ in their method of isolation (one or two-step centrifugation), type and operation of the collecting tube, speed of the centrifuge, and other processes of production, which result in plasma preparations with varying volumes, platelet numbers, quantities of growth factors, and concentrations of residual white and red blood cells[1,3,7,8].

Variability in the cellular composition of platelet-rich plasma preparations can create methodological challenges for investigators[1,3,7]. Results obtained from both in vitro and in vivo studies utilizing platelet-rich plasma can be difficult to interpret if cell type and quantity of the plasma preparations are inconsistent. Despite its importance, research to quantify differences in separation methods is limited. Recently, Castillo et al. compared cell concentrations in platelet-rich plasma preparations from three different single-step separation methods (MTF Cascade, Arteriocyte Magellan, and Biomet GPS III)[9]. While concentrations of platelets, red blood cells, fibrinogen levels, and active TGF-β1 remained consistent, substantial differences in the amount of VEGF-A and PDGFs as well as the amount and concentration of white blood cells were observed[9]. Considering the concentration-dependent function of white blood cells to be either beneficial or harmful in platelet-rich plasma treatments, it is critical to define the differences in the cellular characteristics of platelet preparations[10-12].

To elucidate differences between methods of procurement of platelet-rich plasma, laboratory investigation conducted in a clinically applicable manner is needed. Historically, basic-science investigation has utilized automatic cell separation to produce platelet-rich plasma[1]. While valid, this method does not reflect a clinical environment in which centrifuge devices are used and platelet-rich plasma treatment is sometimes administered in repeated doses over a given period of time. These conditions raise questions over the consistency of platelet preparations with regard to methods of separation and within-individual consistency of preparations with repeated blood draws[1].

Our hypotheses were that (1) despite variability in specific methods, so-called one-step separation methods will result in comparable preparations of platelet-rich plasma with regard to platelet, growth factor, and red and white blood-cell concentrations, and (2) inconsistent blood cell counts and concentrations will be seen with each system following repeated blood draws. Two major study goals developed for these hypotheses were (1) to quantify the level of platelets, growth factors, red blood cells, and white blood cells in one-step (clinically used commercial devices) and two-step separation systems (literature-based platelet-rich plasma method) and (2) to determine the influence of three separate blood draws in terms of within-subject differences on the resulting components of platelet-rich plasma.

Materials and Methods

Subjects

Blood samples were obtained from eight healthy subjects (two female and six male subjects with a mean age [and standard deviation] of 31.6 ± 10.9 years) as part of an investigative study examining various properties of platelet-rich plasma. Institutional review board approval was obtained. Inclusion criteria included healthy subjects between the ages of eighteen and sixty-five years without known blood dyscrasia. Exclusion criteria included a medical history of blood-derived illness or any medication known to affect platelet or bone marrow function or concentration for a minimum of two weeks prior to testing.

Platelet-Rich Plasma Preparation

Approximately 125 mL of peripheral blood was drawn from each subject at three different time points (zero, fourteen, and thirty days) to allow sufficient platelet recovery. A 60-mL syringe prefilled with 5 mL of acid citrate dextrose (ACD-A) was used for the standardized blood draw. ACD-A binds calcium and prevents blood clotting with no known interference to platelet function. Blood was then transferred directly to each of the three different separation systems. As separation methods, a single-spin method that would be expected to result in platelet-rich plasma (PRP) with a lower platelet and white blood-cell number (PRP_{LP}), an alternative system expected to result in a high amount of platelets and high number of white blood cells (PRP_{HP}), and a double-spin method (PRP_{DS}) were chosen to represent an overall survey of the techniques clinically available.

PRP_{LP}

The Arthrex ACP Double Syringe (Arthrex, Naples, Florida) was used for production of autologous conditioned plasma. Ten milliliters of blood was filled into the double syringe to produce 3 mL of PRP_{LP}. Syringes were centrifuged at 1500 rpm for five minutes. This separated the erythrocytes from the remaining plasma components. The top portion of plasma was drawn up with use of the inner syringe without disruption of the erythrocyte layer.

PRP_{HP}

The GPS III Platelet Concentrate System (Biomet, Warsaw, Indiana) was used to produce approximately 3 mL of PRP_{HP} out of 27 mL of blood. The tubes were centrifuged for fifteen minutes at 3200 rpm according to the manufacturer's protocol. With the specific construction of the tubes, it was possible to draw the portion of platelet-rich plasma into a 3-mL syringe according to the manufacturer's instructions.

PRP_{DS}

A literature-based double-spin method was utilized to fractionate whole blood[13]. After a first centrifugation of 1500 rpm for five minutes, the top layer of plasma was separated and centrifuged a second time (twenty minutes at 6300 rpm). Finally, half of the superficial plasma layer was removed, and the platelet pellet was suspended in the remaining half of the plasma volume.

Platelet Concentration and Number of Blood Cells

A 1-mL sample of each platelet-rich plasma preparation and each native blood specimen were analyzed by the clinical core laboratory at the University of Connecticut Health Center. The platelet concentration, number of red blood cells,

TABLE I Concentration of White Blood Cells*

	Lymphocytes $(10^3/\mu L)$	Neutrophils $(10^3/\mu L)$	Eosinophils $(10^3/\mu L)$	Basophils $(10^3/\mu L)$	Monocytes $(10^3/\mu L)$
Blood	1.77 ± 0.55	3.19 ± 1.2	0.14 ± 0.07	0.02 ± 0.01	0.35 ± 0.08
PRP_{LP}	0.5 ± 0.3	0.06 ± 0.03	0.01 ± 0.00	0.01 ± 0.0	0.06 ± 0.04
PRP_{HP}	8.29 ± 3.54	8.71 ± 3.86	0.15 ± 0.18	0.90 ± 2.94	1.66 ± 0.97
PRP_{DS}	0.48 ± 0.26	0.06 ± 0.03	0.00 ± 0.00	0.01 ± 0.01	0.05 ± 0.04

*The values are given as the mean and the standard deviation. PRP_{LP} = platelet-rich plasma prepared with single-spin method resulting in lower number of white blood cells and platelets, PRP_{HP} = alternative method resulting in a high amount of white blood cells and platelets, and PRP_{DS} = double-spin method.

and white blood-cell differentiation was determined by blood cell count (Gen-S System 2 Hematology Analyzer; Coulter, Miami, Florida)[14]. Linearity is $10 - 1000 \times 10^3/\mu L$ for platelet count, $0.3 - 7.0 \times 10^6/\mu L$ for red blood-cell count, and $0.1 - 100 \times 10^3/\mu L$ for white blood-cell count, respectively (data provided by the University of Connecticut Health Center blood laboratory).

Growth Factor Concentration
Enzyme-linked immunosorbent assay (ELISA) in duplicate aliquots with the Quantikine Human Immunoassay kits (R&D Systems, Minneapolis, Minnesota) were used to quantify the growth factor concentration of each

platelet-rich plasma preparation and native blood sample. Samples for each were immediately frozen ($-20°$) to preserve growth factor integrity, stored for less than twenty-eight days, and thawed on ice (one freeze-thaw cycle) before the ELISA assays were performed. Due to the high costs of the multiple ELISA assays, only quantification for all products of all subjects at the time point of the first blood draw was feasible. All assays had in common the employment of the quantitative sandwich enzyme immunoassay technique.

The growth factors EGF, FGF-2, HGF, IGF-1, PDGF-AB, TGF-β1, and VEGF-A were chosen for analysis because of their specific roles in tissue-healing
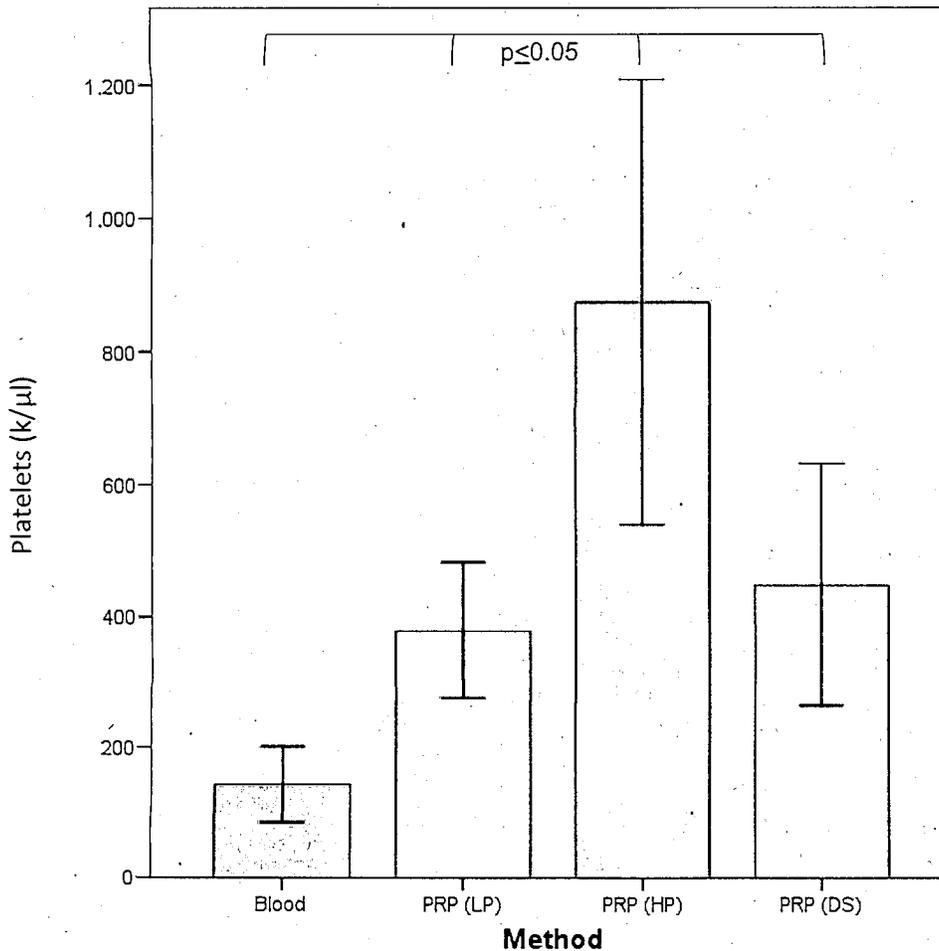

Fig. 1
Platelet concentration according to separation method. The values are given as the mean and the standard deviation. PRP(LP) = platelet-rich plasma prepared with single-spin method resulting in lower number of white blood cells and platelets, PRP(HP) = alternative method resulting in a high amount of white blood cells and platelets, and PRP(DS) = double-spin method.

TABLE II Reliability for Repetitive Blood Draws

Sex, Age of Subjects (yr)	Blood* (10^3/μL)	PRP_{LP}* (10^3/μL)	PRP_{HP}* (10^3/μL)	PRP_{DS}* (10^3/μL)
F, 22	77.0 ± 20.55	265.0 ± 34.60	649.3 ± 340.74	403.7 ± 52.77
M, 22	99.4 ± 28.14	275.7 ± 41.97	578.3 ± 137.25	397.7 ± 209.55
M, 23	133.8 ± 40.00	432.3 ± 31.47	1004.3 ± 319.06	328.0 ± 260.86
M, 24	144.8 ± 34.12	439.7 ± 20.31	607.0 ± 64.09	474.0 ± 165.95
M, 30	128.7 ± 53.40	296.0 ± 95.57	733.3 ± 59.68	380.7 ± 116.10
M, 33	193.6 ± 89.31	376.0 ± 142.79	1077.7 ± 531.14	472.7 ± 290.15
M, 48	178.6 ± 16.91	500.7 ± 43.66	1283.7 ± 138.16	580.7 ± 172.15
F, 50	185.5 ± 72.78	441.3 ± 58.77	1056.7 ± 72.45	544.3 ± 202.46
Overall	142.68 ± 44.40	378.3 ± 58.64	873.8 ± 207.82	447.7 ± 183.75
α	0.75	0.44	−0.84	0.10

*The values area given as the mean platelet concentration (and standard deviation) of three repetitive blood draws (zero, fourteen, and thirty days) for each subject and overall (n = 8). Overall intrasubject variability was calculated with Cronbach α for standardized items for each product. PRP_{LP} = platelet-rich plasma prepared with single-spin method resulting in lower number of white blood cells and platelets, PRP_{HP} = alternative method resulting in a high amount of white blood cells and platelets, and PRP_{DS} = double-spin method.

and regeneration[1,7]. Human EGF was measured with a 3.5 to 4.5-hour solid phase ELISA containing *Escherichia coli*-derived recombinant human EGF and antibodies raised against the recombinant factor. The FGF basic (FGF-2) kit contained recombinant human FGF-2 and antibodies raised against the recombinant factor (4.5-hour solid phase). The 4.25 to 4.5-hour HGF ELISA contains *Sf* 21-expressed recombinant human pro-HGF and antibodies raised
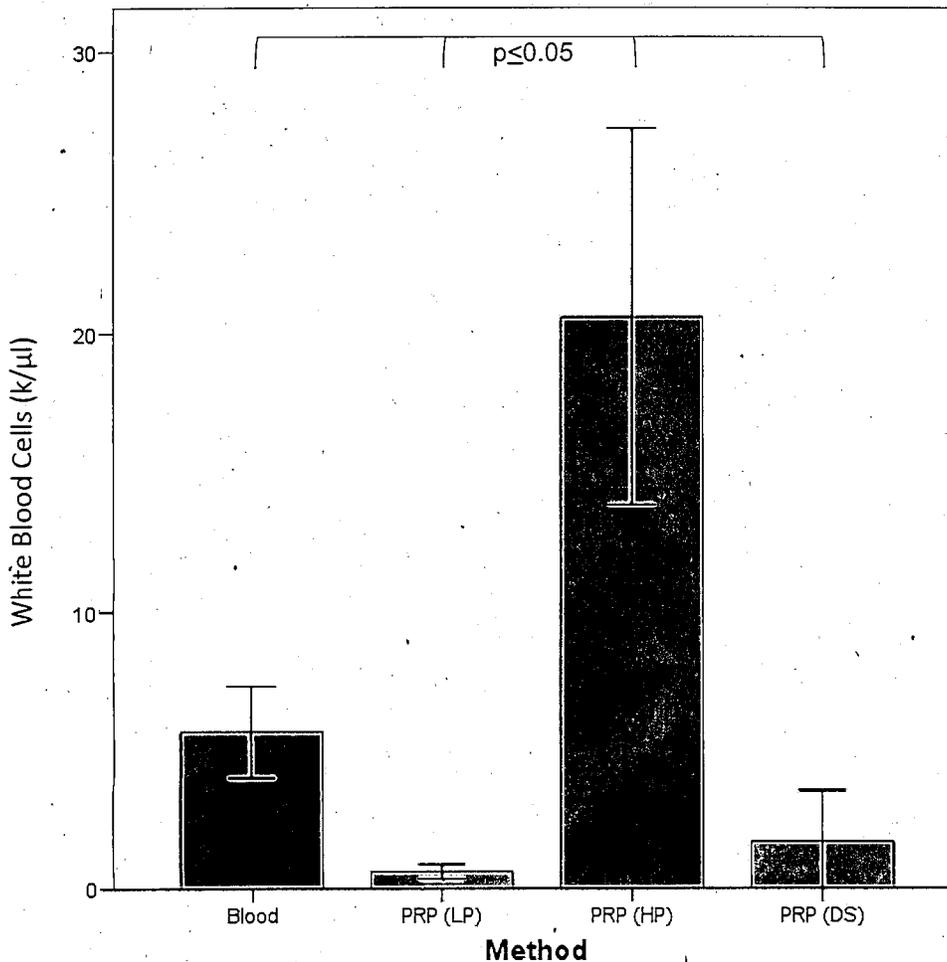

Fig. 2
White blood-cell concentration according to separation method. The values are given as the mean and the standard deviation. PRP(LP) = platelet-rich plasma prepared with single-spin method resulting in lower number of white blood cells and platelets, PRP(HP) = alternative method resulting in a high amount of white blood cells and platelets, and PRP(DS) = double-spin method.
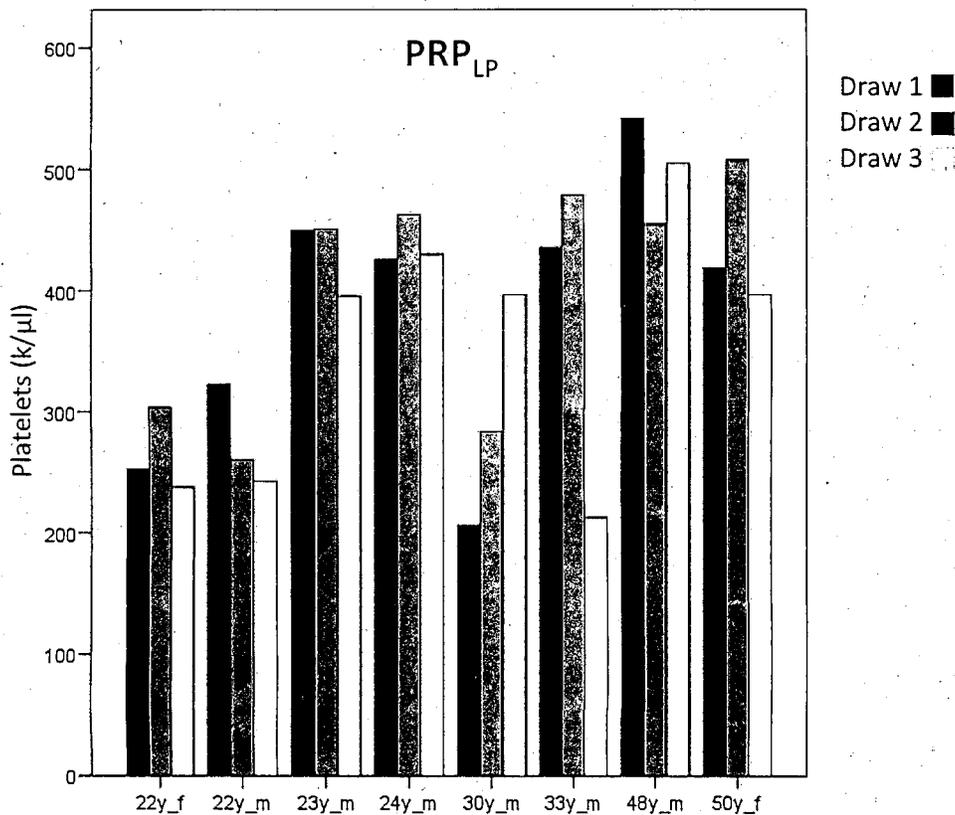
Fig. 3-A

Fig. 3-B

The concentration of platelets after repetitive blood draws according to subject and group, including PRP$_{LP}$ (Fig. 3-A), PRP$_{HP}$ (Fig. 3-B), and PRP$_{DS}$ (Fig. 3-C). PRP$_{LP}$ = platelet-rich plasma prepared with single-spin method resulting in lower number of white blood cells and platelets, PRP$_{HP}$ = alternative method resulting in a high amount of white blood cells and platelets, and PRP$_{DS}$ = double-spin method.
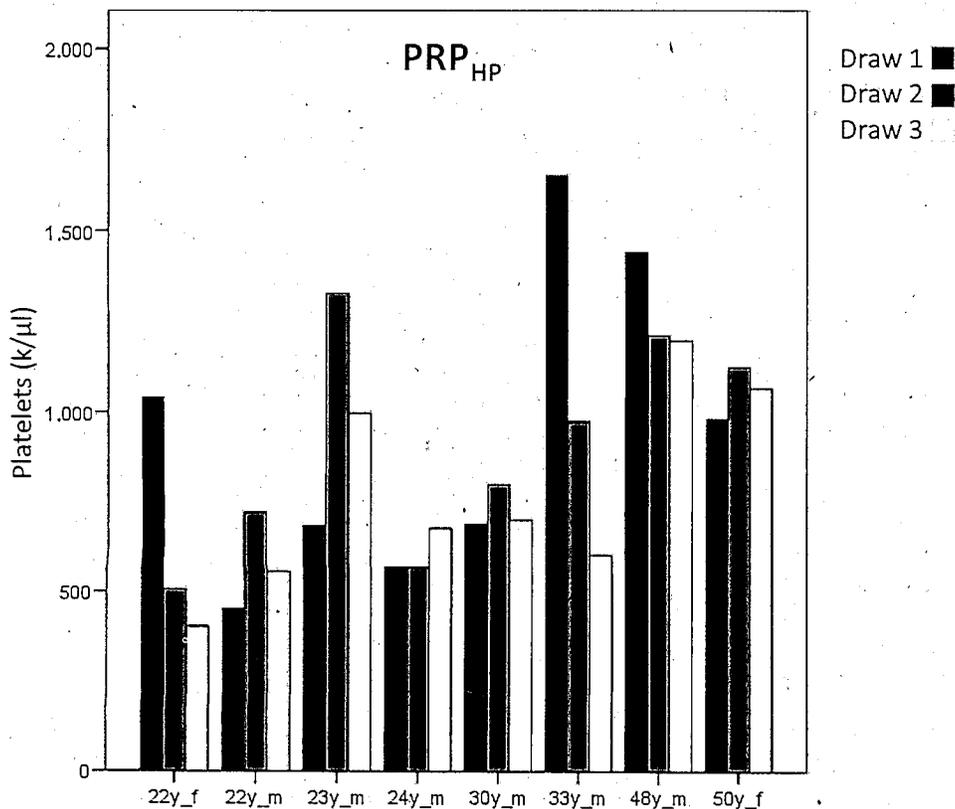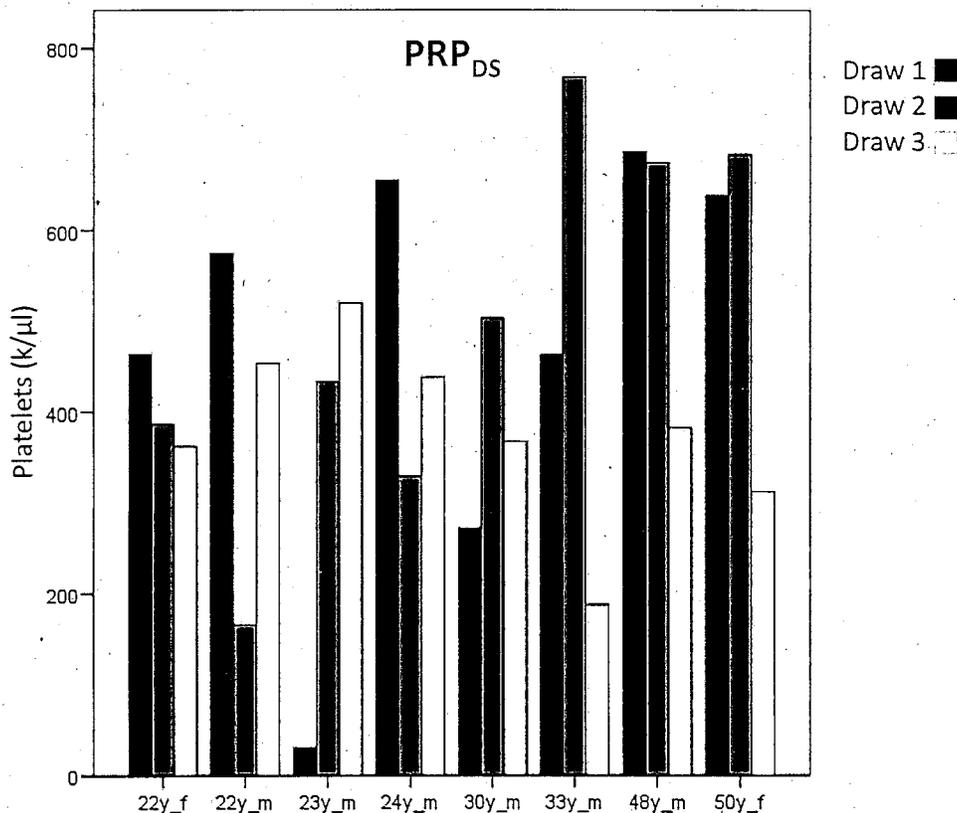
Fig. 3-C

against the recombinant factor. The Quantikine Human IGF-1 Immunoassay is a 3.5-hour solid-phase ELISA containing *Escherichia coli*-expressed recombinant human IGF-1. PDGF-AB was determined by a 4.5-hour solid-phase ELISA containing *Escherichia coli*-expressed recombinant human PDGF-AB. The TGF-β1 assay contained recombinant human TGF-β1 expressed by CHO (Chinese hamster ovary) cells and used a 4.5-hour solid-phase ELISA. Finally, the human VEGF kit contained *Sf* 21-expressed recombinant human $VEGF_{165}$ and antibodies raised against the recombinant protein to measure $VEGF_{165}$ in a 4.5-hour solid-phase ELISA.

Statistical Analysis

Data were analyzed with SPSS software (version 15.0; SPSS, Chicago, Illinois). The Kolmogorov-Smirnov and Shapiro-Wilk tests were performed for each variable to identify non-normal distributions. Since none of the variables showed normative distribution ($p \leq 0.05$), the nonparametric Kruskal-Wallis test was used to compare group means. Comparisons with a significant difference in means were followed by post hoc tests (Tamhane T2). A p value of ≤ 0.05 was used to determine significance. For comparison of the variability of repetitive blood draws, means and standard deviations were determined for each subject and overall. Additionally, Cronbach α was calculated as a measure of reliability with the intraclass correlation measurement for two-way random average measurements ($\alpha \geq 0.7$ was regarded as reliable). This was used as a measure of the internal consistency and/or reliability of a repetitive measurement in multiple subjects.

Results

Platelet Concentration

With regard to the total number of platelets, all separation systems produced a significantly increased platelet number compared with native blood ($142.7 \pm 44.40 \times 10^3/\mu L$). The PRP_{HP} ($873.8 \pm 207.82 \times 10^3/\mu L$) also showed a significantly higher number of platelets compared with PRP_{LP} ($378.3 \pm 58.64 \times 10^3/\mu L$) or PRP_{DS} ($447.7 \pm 183.7 \times 10^3/\mu L$) ($p \leq 0.05$). No significant difference in platelet number was seen when PRP_{LP} was compared with PRP_{DS} ($p = 0.52$) (Fig. 1).

Red Blood-Cell Concentration

Overall, the highest level of red blood cells was in native blood ($4.1 \pm 0.4 \times 10^6/\mu L$). This was significantly different from all of the other separations. The PRP_{LP} ($0.2 \pm 0.1 \times 10^6/\mu L$) and the PRP_{DS} ($0.02 \pm 0.04 \times 10^6/\mu L$) were not significantly different compared with each other. However, both the PRP_{LP} and the PRP_{DS} group had significantly fewer red blood cells compared with the PRP_{HP} group ($1.0 \pm 1.4 \times 10^6/\mu L$).

White Blood-Cell Concentration

There were significantly different amounts of white blood cells in all four separations compared with each other ($p \leq 0.05$). The PRP_{HP} ($20.5 \pm 6.7 \times 10^3/\mu L$) showed the highest amount, whereas the PRP_{LP} ($0.6 \pm 0.3 \times 10^3/\mu L$) system showed the fewest amounts of white blood cells. The PRP_{DS} contained

TABLE III Growth Factor Concentration Compared Between Separation Methods

Growth Factor*	PRP$_{LP}$† (pg/mL)	PRP$_{HP}$† (pg/mL)	PRP$_{DS}$† (pg/mL)
EGF	659.8 ± 35.9	2639.5 ± 197.7	670.7 ± 185.1
FGF-2	15.6 ± 2.4	75.2 ± 21.4	15.2 ± 3.4
HGF	645.2 ± 72.1	4277.3 ± 1508.2	581.7 ± 43.2
IGF	64.8 ± 55.4	672.9 ± 378.4	45.1 ± 60.7
PDGF	16,668.1 ± 5512.3	42,273.9 ± 2902.4	12,263.7 ± 3632.7
TGF-β	66,246.2 ± 7620.4	141,286.9 ± 12,576.1	83,011.7 ± 14,129.8
VEGF	138.7 ± 11.2	142.9 ± 12.5	138.7 ± 9.1

*EGF = epidermal growth factor, FGF-2 = fibroblast growth factor, HGF = hepatocyte growth factor, IGF = insulin-like growth factor, TGF-β = transforming growth factor-beta, and VEGF = vascular endothelial growth factor. †The values are given as the mean and the standard deviation. PRP$_{LP}$ = platelet-rich plasma prepared with single-spin method resulting in lower number of white blood cells and platelets, PRP$_{HP}$ = alternative method resulting in a high amount of white blood cells and platelets, and PRP$_{DS}$ = double-spin method.

fewer white blood cells ($1.7 \pm 1.8 \times 10^3/\mu L$) than native blood ($5.6 \pm 1.7 \times 10^3/\mu L$) (Fig. 2).

White Blood-Cell Distribution
The distribution of the white blood cells according to separation methods is shown in Table I. All cell types showed significant differences compared with each other ($p \leq 0.05$), except for PRP$_{LP}$ and PRP$_{DS}$, which showed no significant difference between any cell types. No significant differences were observed when numbers of eosinophils isolated from the PRP$_{HP}$ were compared with whole blood.

Influence of Draw Repetition on Platelet Count
Table II and Figures 3-A, 3-B, and 3-C show the intrasubject and intersubject variability of platelet concentrations according to different blood draws.

Growth Factors
Results for each growth factor are shown in Table III. The single-spin method (PRP$_{LP}$) had a significantly higher concentration of the factors HGF, IGF-1 and PDGF-AB in comparison with the double-spin method (PRP$_{DS}$), whereas the PRP$_{HP}$ separation produced significantly more growth factors compared with the other separations, with the exception of VEGF-A ($p \leq 0.05$). The PRP$_{LP}$ separation method released significantly more HGF, IGF-1, and PDGF-AB compared with the PRP$_{DS}$ separation ($p \leq 0.05$).

Discussion
This study was designed to explore differences between methods of procurement and administration for platelet-rich plasma. We observed differing amounts of platelets, white blood cells, and growth factors with each method of separation as well as wide intrasubject variation in cell type and count with repetitive blood draws. However, as anticipated, all separation techniques resulted in a significant increase in platelet concentration compared with native blood[1,2]. Platelet concentrations in the PRP$_{HP}$ procedure were significantly higher than platelet concentrations in the single-step PRP$_{LP}$ and the two-step PRP$_{DS}$

procedures; however, significant differences between PRP$_{LP}$ and PRP$_{DS}$ were not observed. While these data assist in determining the extent to which devices and repetitive blood draws influence the type and number of cells in platelet-rich plasma preparation, it does not provide proof of clinical efficacy. In this regard, the results observed in the present study between methods of procurement and repetitive blood draws are discussed with respect to previously published laboratory research on plasma preparations.

Previous investigators have defined platelet-rich plasma according to platelet concentration. Marx required plasma preparations to have a platelet concentration of $1000 \times 10^3/\mu L$ to be considered therapeutic platelet-rich plasma[4]. In the present study, the PRP$_{HP}$ resulted in the highest amounts of platelets ($873.8 \pm 207.82 \times 10^3/\mu L$), which approximately meets this criterion. Other investigators have reported lower platelet concentrations for therapeutic platelet-rich plasma. On the basis of the current use of centrifuges and plasma isolation procedures and the undetermined and unclear effect of platelets on targeted tissue, Mazzucco et al., in 2009, defined platelet numbers of $>200 \times 10^3/\mu L$ as sufficient for a therapeutic effect[6]. This definition seems reasonable, as platelet concentrations of approximately 2.5 times greater than native blood ($142.7 \pm 57.9 \times 10^3/\mu L$) have positive effects on osteoblasts and fibroblasts in vitro. Additionally, adverse events have been observed at higher dosages (>3.5 times platelet concentration of native blood)[15]. While the other evaluated methods, PRP$_{LP}$ ($378.3 \pm 58.64 \times 10^3/\mu L$) and PRP$_{DS}$ ($447.7 \pm 183.7 \times 10^3/\mu L$), resulted in significantly lower platelet numbers than the PRP$_{HP}$, all methods exceeded concentrations of $>300 \times 10^3/\mu L$, thereby satisfying this criterion of $>200 \times 10^3/\mu L$.

Investigators have reported differences between one and two-step procedures[16]. In the present study, comparison of the two-step procedure (PRP$_{DS}$) did not result in significantly higher levels of platelet separation than the one-step procedures (PRP$_{HP}$ and PRP$_{LP}$). These results support the effectiveness of a one-step procedure to produce comparable amounts of platelets for therapeutic application. These data may be useful to clinicians as the

increase in time required for two-step procedures may not be needed to produce therapeutic platelet-rich plasma preparations[16].

Our findings differ from the recent work of Castillo et al. who reported no significant difference regarding platelet concentration among the methods evaluated (MTF Cascade, Arteriocyte Magellan, and Biomet GPS III)[9]. Mean values of platelet concentrations for the Biomet GPS III (PRP_{HP}) in our study ($873.8 \pm 207.82 \times 10^3/\mu L$) were much higher than the results reported by Castillo et al. ($566.2 \pm 292.6 \times 10^3/\mu L$)[9]. We utilized three repeated measurements to establish mean values, which resulted in concentrations closely matching the manufacturer's information (platelet numbers that were nine times that of native blood) and other published data[3]. Interestingly, both studies demonstrated high standard deviations, indicating significant variability in the data. While each used subjects with comparable ages, the difference in results may be due to variability within individuals in each study as well as to the number of subjects.

The quantity of white blood cells in plasma preparations is of particular interest. Amounts of white blood cells and their effect in platelet-rich plasma application have been widely discussed. Some investigators have recommended avoiding tissue exposure to white blood cells as an inflammatory reaction may occur[3,17]. Other investigators have reported a beneficial effect of white blood cells in terms of increased antibacterial and immunological resistance[18,19]. Additionally, white blood cells have been reported to correlate with increased growth factor release[20]. Our results demonstrate significant differences in white blood cells by the production method, which is consistent with other investigators who have reported similar differences between production methods[9].

The differentiation of white blood-cell types may permit estimation of their respective effects. We evaluated the type of white blood cells present in each plasma preparation. As expected, the overall number of each type of white blood cells was significantly higher with the PRP_{HP} separation method. Quantities of lymphocytes were more than quadrupled and neutrophils were more than doubled compared with native blood with this technique. While these cell types have been reported to produce negative effects on tissue-healing because of their capacity to promote inflammatory effects[11,19], there is no clinical evidence to support their effect on cells. Rather, these data are intended to provide clinicians with data to assist in choosing a separation method to match the intended use of platelet-rich plasma.

Growth factor analysis was also performed in the present study for all platelet-rich plasma preparations in all subjects at the time point of the first blood draw. The single-spin method (PRP_{LP}) had a significantly higher concentration of the factors HGF, IGF-1, and PDGF-AB in comparison with the double-spin method (PRP_{DS}), whereas the PRP_{HP} separation produced significantly more growth factors compared with the other separations, with the exception of VEGF-A. This increase may be due in part to higher amounts of white blood cells and/or platelets, which correlate with variable levels of growth factors in different methods of platelet-rich plasma preparations[12,21]. Considering the results of the variability in platelet concentrations observed

for repetitive blood draws, high variability in growth factor content might also be present for platelet-rich plasma preparations separated from repetitive blood draws. Additionally, quantifying growth factors can be performed through the activation of platelets with the application of calcium chloride or thrombin to stimulate complete release, which has been shown to substantially increase the locally available growth factor content[22]. In the present study, platelet activation was not performed to evaluate the physiologic growth factor levels.

Comparison of our results with the data of Castillo et al. revealed that different levels of growth factors were observed for the same separation systems[9]. Considering their results were higher than other reported data, contributing factors, including mechanical forces induced during the handling process, were discussed as potential causes of increased growth factors released[9]. According to the manufacturer's data (Quantikine ELISA kits; R&D Systems), incomplete removal of platelets and blood cells may cause variable results for assays of factors contained in platelets, although data within studies are comparable. Because of red blood-cell concentration, ELISA data should only be compared within studies, and comparison with other studies can only be performed with limitations.

We observed significant variation in platelets and white blood cells with repetitive blood draws from the same individual at different times for all methods of platelet-rich plasma separation. In an effort to quantify the degree to which blood draws produced consistent amounts of blood cells, Cronbach α was calculated for all individuals. The cell counts in the native blood of all included subjects were consistent between the three blood draws (zero, fourteen, and thirty days). However, the platelet concentration of all three methods showed a high variability within the subjects for the three time points. Considering platelet-rich plasma application methods tend toward a repetitive treatment, the lack of consistency at the level of the individual is important, as reliable platelet-rich plasma dosages may be needed to produce consistent results[1].

There are several limitations of this study. The sample size (eight subjects), although consistent with other platelet-rich plasma-related reports, may have impacted the study results[1,3,7]. Variability due to different subjects and possible alterations in production methods impact the ability to compare our results with previously published data. We attempted to evaluate representative separation methods; however, since there are multiple methods commercially available, a study comparing all of the different available platelet-rich plasma kits would be financially prohibitive. As a result, we examined the higher (PRP_{HP}) and lower (PRP_{LP}) spectrum of routine platelet-rich plasma separation methods. ■

Augustus D. Mazzocca, MS, MD
Mary Beth R. McCarthy, BS
David M. Chowaniec, BS
Mark P. Cote, DPT

PLATELET-RICH PLASMA DIFFERS ACCORDING TO
PREPARATION METHOD AND HUMAN VARIABILITY

Robert A. Arciero, MD
Knut Beitzel, MD
Department of Orthopaedic Surgery,
University of Connecticut, Farmington, CT 06033.
E-mail address for A.D. Mazzocca: mazzocca@uchc.edu

Anthony A. Romeo, MD
Rush University Medical Center, Rush University,

1161 West Harrison Avenue, Suite 300,
Chicago, IL 60612

James P. Bradley, MD
Center for Sports Medicine,
University of Pittsburgh Medical Center,
200 Delafield Road, Suite 4010,
Pittsburgh, PA 15215

References

1. Foster TE, Puskas BL, Mandelbaum BR, Gerhardt MB, Rodeo SA. Platelet-rich plasma: from basic science to clinical applications. Am J Sports Med. 2009;37: 2259-72.

2. Pauly S, Klatte F, Strobel C, Schmidmaier G, Greiner S, Scheibel M, Wildemann B. Characterization of tendon cell cultures of the human rotator cuff. Eur Cell Mater. 2010;20:84-97.

3. Lopez-Vidriero E, Goulding KA, Simon DA, Sanchez M, Johnson DH. The use of platelet-rich plasma in arthroscopy and sports medicine: optimizing the healing environment. Arthroscopy. 2010;26:269-78.

4. Marx RE. Platelet-rich plasma (PRP): what is PRP and what is not PRP? Implant Dent. 2001;10:225-8.

5. Nikolidakis D, Jansen JA. The biology of platelet-rich plasma and its application in oral surgery: literature review. Tissue Eng Part B Rev. 2008;14:249-58.

6. Mazzucco L, Balbo V, Cattana E, Guaschino R, Borzini P. Not every PRP-gel is born equal. Evaluation of growth factor availability for tissues through four PRP-gel preparations: Fibrinet, RegenPRP-Kit, Plateltex and one manual procedure. Vox Sang. 2009;97:110-8.

7. Alsousou J, Thompson M, Hulley P, Noble A, Willett K. The biology of platelet-rich plasma and its application in trauma and orthopaedic surgery: a review of the literature. J Bone Joint Surg Br. 2009;91:987-96.

8. Spang JT, Tischer T, Salzmann GM, Winkler T, Burgkart R, Wexel G, Imhoff AB. Platelet concentrate vs. saline in a rat patellar tendon healing model. Knee Surg Sports Traumatol Arthrosc. 2010 Oct 15 [Epub ahead of print].

9. Castillo TN, Pouliot MA, Kim HJ, Dragoo JL. Comparison of growth factor and platelet concentration from commercial platelet-rich plasma separation systems. Am J Sports Med. 20 10 Nov 4 [Epub ahead of print].

10. Pourfathollah AA, Shaiegan M, Namiri M, Babae GR. Effect of gamma irradiation on lymphocyte proliferation and IL-8 production by lymphocytes isolated from platelet concentrates. Arch Med Res. 2008;39:590-3.

11. Zimmermann R, Reske S, Metzler P, Schlegel A, Ringwald J, Eckstein R. Preparation of highly concentrated and white cell-poor platelet-rich plasma by plateletpheresis. Vox Sang. 2008;95:20-5.

12. Zimmermann R, Arnold D, Strasser E, Ringwald J, Schlegel A, Wiltfang J, Eckstein R. Sample preparation technique and white cell content influence the detectable levels of growth factors in platelet concentrates. Vox Sang. 2003;85: 283-9.

13. de Mos M, van der Windt AE, Jahr H, van Schie HT, Weinans H, Verhaar JA, van Osch GJ. Can platelet-rich plasma enhance tendon repair? A cell culture study. Am J Sports Med. 2008;36:1171-8.

14. Picard F, Gicquel C, Marnet L, Guesnu M, Levy JP. Preliminary evaluation of the new hematology analyzer COULTER GEN-S in a university hospital. Clin Chem Lab Med. 1999;37:681-6.

15. Graziani F, Ivanovski S, Cei S, Ducci F, Tonetti M, Gabriele M. The in vitro effect of different PRP concentrations on osteoblasts and fibroblasts. Clin Oral Implants Res. 2006;17:212-9.

16. Nagata MJ, Messora MR, Furlaneto FA, Fucini SE, Bosco AF, Garcia VG, Deliberador TM, de Melo LG. Effectiveness of two methods for preparation of autologous platelet-rich plasma: an experimental study in rabbits. Eur J Dent. 2010;4:395-402.

17. Tidball JG. Inflammatory cell response to acute muscle injury. Med Sci Sports Exerc. 1995;27:1022-32.

18. Moojen DJ, Everts PA, Schure RM, Overdevest EP, van Zundert A, Knape JT, Castelein RM, Creemers LB, Dhert WJ. Antimicrobial activity of platelet-leukocyte gel against Staphylococcus aureus. J Orthop Res. 2008;26:404-10.

19. Dohan Ehrenfest DM, Rasmusson L, Albrektsson T. Classification of platelet concentrates: from pure platelet-rich plasma (P-PRP) to leucocyte- and platelet-rich fibrin (L-PRF). Trends Biotechnol. 2009;27:158-67.

20. Zimmermann R, Jakubietz R, Jakubietz M, Strasser E, Schlegel A, Wiltfang J, Eckstein R. Transfusion. Different preparation methods to obtain platelet components as a source of growth factors for local application. 2001;41:1217-24.

21. Weibrich G, Kleis WK, Hafner G, Hitzler WE, Wagner W. Comparison of platelet, leukocyte, and growth factor levels in point-of-care platelet-enriched plasma, prepared using a modified Curasan kit, with preparations received from a local blood bank. Clin Oral Implants Res. 2003;14:357-62.

22. Everts PA, Brown Mahoney C, Hoffmann JJ, Schönberger JP, Box HA, van Zundert A, Knape JT. Platelet-rich plasma preparation using three devices: implications for platelet activation and platelet growth factor release. Growth Factors. 2006;24:165-71.