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Optimal double-spin method for maximizing the concentration of platelets in equine platelet-rich plasma

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This study optimized the double-spin conditions for preparing equine platelet-rich plasma (PRP): leukocyte-rich PRP (L-PRP) and leukocyte-poor PRP (P-PRP). Whole blood samples were centrifuged at various double-spin conditions. Both L-PRP and P-PRP were prepared at each stage, and complete blood counts and growth factor concentrations were compared. Samples centrifuged at 160 × 900 g, 160 × 2,000 g, and 400 × 2,000 g exhibited the highest platelet counts. P-PRP had significantly lower leukocyte and erythrocyte contents than L-PRP, especially at 400 × 2,000 g. No significant differences were observed in growth factor concentrations. Our data suggest that optimum L-PRP preparation should include centrifugation under the aforementioned conditions, whereas centrifugation at 400 × 2,000 g is optimal for P-PRP.

Key words: *double-spin, leukocyte, platelet, platelet-rich plasma*

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Platelet-rich plasma (PRP) therapy is currently widely used in human and veterinary medicine [1, 2, 7–12, 19, 26, 27]. PRP is prepared by centrifuging autologous whole blood and isolating the plasma portion containing concentrated platelets [7, 18]. When activated, these platelets release growth factors, including transforming growth factor-β1 (TGF-β1) and platelet-derived growth factor (PDGF), which promote cell migration and tissue healing [7, 18]. PRP can be prepared using commercial kits or through simple double centrifugation (double spin) [7, 22, 24, 28]. However, the cellular and cytokine compositions of the resulting PRP can vary depending on the preparation method, and numerous studies have discussed optimal methods for preparing PRP [5, 6, 17, 18, 21, 23]. When using a commercial kit, PRP can be aseptically and stably prepared with a consistent platelet content [22, 25]; however, the total platelet count is slightly lower than when using double-spin methods [8, 22, 24]. Since PRP with a high platelet content contains high levels of clinically relevant cytokines [7, 17, 18], a protocol, such as the double-spin method, is required for stably producing such PRP. However, few studies have compared various combinations of centrifugation conditions to determine the method for reliably obtaining a higher platelet content in PRP.

Numerous studies have investigated optimal methods for categorizing cellular components of PRP and the clinical effects of each PRP type [15, 17]. In particular, the need for leukocytes remains controversial. King *et al.* reported that leukocytes contain anti-inflammatory cytokines and that the use of PRP with a high leukocyte content is clinically beneficial [13]. However, leukocyte-rich PRP contains catabolic factors, which might negatively affect tissue regeneration [3, 14, 21, 28]. Therefore, we investigated the platelet content of two PRP types classified depending on their leukocyte content: leukocyte-rich PRP (L-PRP) and pure (leukocyte-poor) PRP (P-PRP), both prepared using the double-spin method [5]. W compared the effect of various double-spin conditions on the cell counts and growth factor concentrations of the resulting PRP isolates. Thereafter, the most appropriate method for reproducibly preparing PRP with the highest platelet content was examined for each PRP type based on the leukocyte content.

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Whole blood samples were obtained from five clinically healthy Thoroughbred horses (three males, two females, 4–8 years old, 411–541 kg). All experimental procedures were approved by the Animal Welfare and Ethics Committee of the Equine Research Institute of the Japan Racing Association (authorization number, 2019-7).

Equine whole blood (220 m*l*) was sampled from the jugular vein, using a syringe containing 10% sodium citrate anticoagulant (ACD-A injection, Terumo BCT Ltd., Tokyo, Japan). After determining the complete blood count (CBC) using an automated blood cell counter (thinka CB-1010, ARKRAY, Inc., Kyoto, Japan), 10 m*l* blood was dispensed into each of 22 conical polypropylene tubes before centrifugation (himac CR21G, Hitachi Koki Co., Ltd., Hitachinaka, Japan).

L-PRP and P-PRP were prepared using a combination of centrifugation methods (Fig. 1). We tested the optimal relative centrifugal force (RCF) for the initial centrifugation step by centrifuging samples at four different RCFs (160, 400, 900, and 2,000 g) for 7 min at 4°C. The tubes were divided into two groups per RCF value, and samples were aspirated and transferred into round-bottom polypropylene tubes; one group was comprised of aspirated fractions of plasma, buffy coat, and the top 3 mm of erythrocytes (prepared as L-PRP), and the other group was comprised of the aspirated plasma fraction only (prepared as P-PRP). One tube of every sample type prepared at each of the respective RCFs was used to determine the CBC.

Once suitable conditions for the first centrifugation were determined (based on cell counts for each preparation), we optimized the conditions for the second centrifugation step. Samples initially centrifuged at 160 or 400 g were subsequently centrifuged at one of the four different RCFs for 7 min at 4°C. The combinations for the double-spin method comprised the following RCFs for each type of PRP: 160 × 160 g, 160 × 400 g, 160 × 900 g, 160 × 2,000 g, 400 × 400 g, 400 × 900 g, and 400 × 2,000 g. The supernatant was aspirated until only 1 m*l* remained, and this 1 m*l* of remaining supernatant was used to resuspend the pellet for PRP preparation. The CBC in the final PRP was determined, and the leukocyte or platelet content was compared between the different PRP types and among RCF conditions.

To compare growth factor concentrations in optimized PRP samples, PDGF isoform BB (PDGF-BB) and TGF-β1 concentrations were determined using ELISA kits (Quantikine Human PDGF-BB ELISA DBB00, and Quantikine Human TGF-β1 ELISA DB100B, R&D Systems, Minneapolis, MN, U.S.A.) according to the manufacturer's instructions. Although these kits were designed for use on human samples, they have been validated for use in horses [22, 24]. To disrupt platelet membranes, all of the prepared PRP samples were frozen at −30°C overnight and thawed at room temperature. The samples were centrifuged at 10,000 g for 5 min at 4°C, and supernatants were collected. Growth factor concentrations in each supernatant were measured. To obtain baseline values, other autologous plasma samples

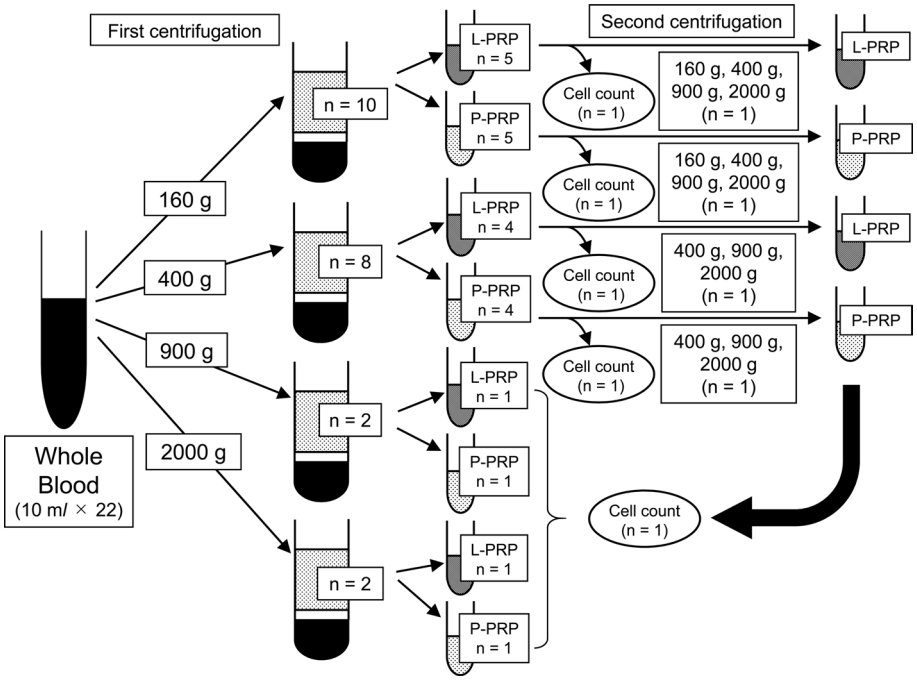

Fig. 1. Schematic representation of the double-spin protocol optimized in this study. PRP, platelet-rich plasma; L-PRP, leukocyte-rich PRP; P-PRP, pure (leukocyte-poor) PRP.

were prepared concurrently from centrifuged, citrate-anticoagulated blood and freeze-thawed, and growth factor concentrations were then determined.

Leukocyte and platelet contents and growth factor concentrations in PRP were compared via two-way repeated-measures analysis of variance (ANOVA), with *type of PRP* and *RCFs* as within-subject factors, followed by Tukey's test for post hoc analysis. Analyses were carried out using Microsoft Excel 2013 macro applications (Statcel4, OMS Publishing Inc., Saitama, Japan). Statistical significance was set at $P<0.05$.

All double-spin samples were produced approximately 2 hr after blood collection. The results from CBC analysis showed that after initial centrifugation, the erythrocyte content in L-PRP samples was markedly reduced in comparison with the whole blood, and erythrocytes were not detected at all in P-PRP samples (Table 1). The leukocyte content was significantly higher in L-PRP than in P-PRP, irrespective of the RCF value (Table 1, Fig. 2a). The leukocyte content in PRP centrifuged at 160 g was significantly higher than that processed at other RCFs, irrespective of the PRP type. The CBC results also showed that platelet content was significantly higher in L-PRP than in P-PRP, irrespective of the RCF value (Table 1, Fig. 2b), and that the platelet content was significantly higher in samples centrifuged at 160 and 400 g than in samples centrifuged at 900 and 2,000 g, irrespective of the PRP type.

Cell counts after the second centrifugation showed that the erythrocyte content in L-PRP was comparable to that of whole blood, whereas erythrocytes were barely detectable in P-PRP (Table 1). The leukocyte counts were significantly higher in L-PRP than in P-PRP, irrespective of the RCF combination (Table 1, Fig. 3a). Furthermore, the leukocyte content was higher at the initial RCF of 160 g compared with 400 g, irrespective of the PRP type. When the concentration ratio was calculated in relation to whole

Table 1. Summary of the complete blood count (CBC) of whole blood (WB), single centrifugation, and double centrifugation samples

Type of PRP	WB	Single centrifugation				Double centrifugation						
Cell components		160 g	400 g	900 g	2,000 g	160 × 160 g	160 × 400 g	160 × 900 g	160 × 2,000 g	400 × 400 g	400 × 900 g	400 × 2,000 g
L-PRP												
RBC ($\times10^{12}/l$)	8.4 ± 1.2	1.2 ± 0.3	1.0 ± 0.2	1.5 ± 0.3	1.7 ± 0.3	8.2 ± 2.2	5.8 ± 2.6	7.7 ± 1.6	7.7 ± 1.3	6.3 ± 1.6	7.8 ± 3.1	6.4 ± 1.5
WBC ($\times10^{9}/l$)	8.1 ± 1.9	9.1 ± 1.3	6.8 ± 2.9	6.0 ± 1.4	6.7 ± 2.7	46.9 ± 5.9	47.8 ± 9.8	46.3 ± 10.0	46.0 ± 16.9	32.7 ± 12.7	33.0 ± 9.3	39.7 ± 7.9
PLT ($\times10^{10}/l$)	14.3 ± 2.0	24.9 ± 5.3	21.4 ± 5.2	17.0 ± 2.7	15.2 ± 4.5	27.5 ± 9.7	55.8 ± 17.9	78.8 ± 23.4	110.5 ± 17.6	51.8 ± 7.2	70.5 ± 27.8	103.5 ± 13.6
P-PRP												
RBC ($\times10^{12}/l$)	8.4 ± 1.2	0	0	0	0	0	0.1	0.1	0.1	0	0	0
WBC ($\times10^{9}/l$)	8.1 ± 1.9	3.1 ± 0.4	0.4 ± 0.1	0.3 ± 0.6	0	14.8 ± 4.2	20.2 ± 9.0	20.0 ± 5.5	16.2 ± 6.4	2.0 ± 0.6	4.2 ± 2.2	4.2 ± 1.9
PLT ($\times10^{10}/l$)	14.3 ± 2.0	21.8 ± 6.6	17.4 ± 4.9	9.6 ± 3.1	4.2 ± 2.1	34.1 ± 9.9	54.2 ± 20.3	83.0 ± 35.1	87.6 ± 34.9	42.0 ± 11.7	59.7 ± 13.0	78.9 ± 19.3

Mean ± SD. L-PRP, leukocyte-rich platelet-rich plasma; P-PRP, pure (leukocyte-poor) platelet-rich plasma; RBC, red blood cell (erythrocyte); WBC, white blood cell (leukocyte); PLT, platelet.

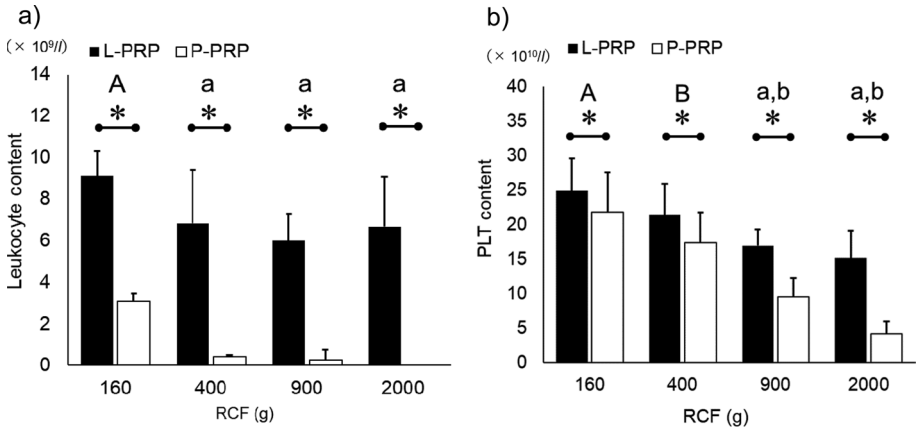

Fig. 2. (a) The leukocyte content and (b) platelet content of platelet-rich plasma (PRP) after the first centrifugation (mean ± 95% confidence interval (CI)). *Significant difference between leukocyte-rich PRP (L-PRP) and pure PRP (P-PRP). In the relative centrifugal force (RCF) comparisons, upper- and lowercase versions of the same letters indicate significant differences ($P<0.05$).

blood values, the ratios of leukocytes in P-PRP samples centrifuged at 400 × 400 g, 400 × 900 g, and 400 × 2,000 g were below 1 (Table 2). Irrespective of the PRP type, samples centrifuged at 160 × 900 g, 160 × 2,000 g, and 400 × 2,000 g had higher platelet contents than those centrifuged at other RCF combinations (Table 1, Fig. 3b). No significant differences among the PRP types were observed. When we compared the PDGF-BB and TGF-β1 concentrations in both PRP types prepared at the three optimal RCF combinations, no significant differences were observed among the RCF combinations or between PRP types (Fig. 4a, 4b). In contrast, the mean value of PDGF-BB ranged from 1,800 to 2,900 pg/m*l* and that of TGF-β1 ranged from 14,000 to 20,000 pg/m*l*, and these values were extremely high compared with the concentrations in plasma (PDGF-BB 56.8 ± 60.3 pg/m*l*; TGF-β1 2,423.6 ± 454.7 pg/m*l*; mean ± SD).

Tambella *et al.* [23] indicated that, in both humans and animals, the protocol for PRP preparation has not been optimized and that this has complicated the effects of PRP therapy on clinical outcomes. Therefore, it is necessary to investigate the optimal PRP preparation method preclinically.

In the double-spin method, the initial centrifugation step is used to separate whole blood into three fractions [5]. When L-PRP is required, the plasma fraction is aspirated together with the buffy coat and a small erythrocyte fraction and transferred into a fresh tube. For P-PRP, only the plasma fraction is isolated. During the second centrifugation, the

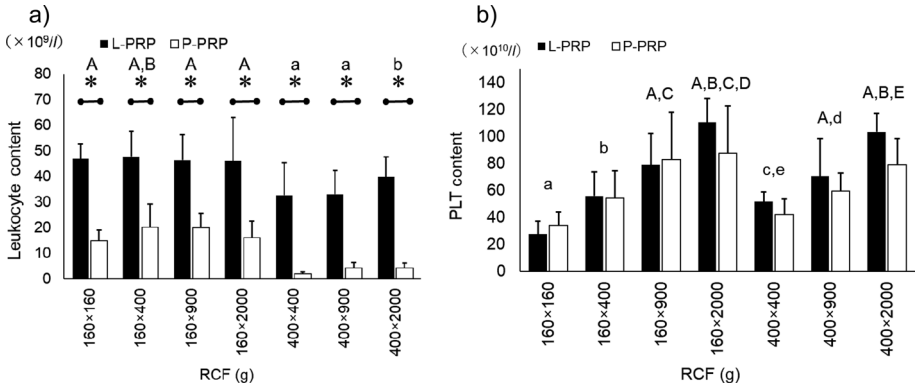

Fig. 3. (a) The leukocyte content and (b) platelet content of platelet-rich plasma (PRP) after double spin (mean ± 95% confidence interval (CI)). *Significant difference between leukocyte-rich PRP (L-PRP) and pure PRP (P-PRP). In the relative centrifugal force (RCF) comparisons, upper- and lowercase versions of the same letters indicate significant differences (P<0.05).

Table 2. The leukocyte and platelet concentration ratio in relation to whole blood values following the examined double-spin protocol

RCF	Type of PRP	Leukocyte		Platelet	
		Ratio	95%CI	Ratio	95%CI
160 × 160 g	L-PRP	6.0	4.9–7.1	1.9	1.3–2.5
	P-PRP	1.8	1.4–2.2	2.4	1.8–3.0
160 × 400 g	L-PRP	6.2	4.5–7.9	3.9	2.9–4.9
	P-PRP	2.4	1.8–3.0	3.8	2.4–5.2
160 × 900 g	L-PRP	5.8	4.8–6.8	5.6	4.1–7.1
	P-PRP	2.4	2.2–2.6	5.9	3.5–8.3
160 × 2,000 g	L-PRP	5.6	4.4–6.8	7.8	6.4–9.2
	P-PRP	2.0	1.4–2.6	6.1	4.3–7.9
400 × 400 g	L-PRP	4.0	3.2–4.8	3.7	3.1–4.3
	P-PRP	0.3	0.2–0.4	3.0	2.2–3.8
400 × 900 g	L-PRP	4.3	3.0–5.6	4.8	3.8–5.8
	P-PRP	0.5	0.3–0.7	4.2	3.3–5.1
400 × 2,000 g	L-PRP	5.2	3.7–6.7	7.3	6.6–8.0
	P-PRP	0.5	0.3–0.7	5.5	4.8–6.2

RCF, relative centrifugal force; L-PRP, leukocyte-rich platelet-rich plasma; P-PRP, pure (leukocyte-poor) platelet-rich plasma; 95%CI, 95% confidence interval.

use of a higher RCF is necessary to concentrate the cellular components of blood at the bottom of the tube [5]. The upper fraction of the sample is discarded, and the pellet is resuspended in the remaining plasma. The final product is thus a concentrated mixture of the cellular components of blood.

This study shows that more than 60% of leukocytes in whole blood were retained in L-PRP after initial centrifugation and that the leukocyte content was significantly higher in L-PRP than in P-PRP under the same RCF conditions. Furthermore, in P-PRP, the leukocyte content decreased with increasing RCF, and leukocytes were barely detectable at 900 g or more. These results suggest that most of the leukocytes in the centrifuged sample are present in the buffy coat and tend to accumulate in this fraction when the RCF is increased. Similar results were obtained for platelets. In particular, P-PRP centrifuged at 900 and 2,000 g contained fewer platelets than whole blood, probably because platelets contained in the plasma fraction sank into the layer below the buffy coat upon centrifugation at 900 g or more [20]. Therefore, we determined that an RCF of 160 or 400 g was appropriate for the initial centrifugation step, while the subsequent centrifugation was performed at an RCF equal to or higher than these values.

Our data showed that, irrespective of the PRP type, samples centrifuged at 160 × 900 g, 160 × 2,000 g, and 400 × 2,000 g had higher platelet contents than those centrifuged at other RCF values. These results suggest that these three double-spin conditions are appropriate for preparing PRP. The P-PRP samples had significantly lower leukocyte contents than the L-PRP samples, especially after centrifugation at 400 × 2,000 g, which reduced the leukocyte numbers to below the baseline values of whole blood. Despite these differences in cellular components, PRP prepared under the three selected conditions contained equally high concentrations of growth factors. Our results show that when leukocytes are required, centrifugation should be carried out at 160 × 900 g, 160 × 2,000 g, or 400 × 2,000 g to prepare L-PRP with high leukocyte and platelet contents. However, it should be noted that some studies have reported the risk of adverse effects caused by leukocytes contained in PRP [3, 14, 21, 28]. In addition to leukocytes, erythrocytes in PRP may also have deleterious effects on treated tissue [4]. A considerable number of erythrocytes are always found in L-PRP preparations, because aspiration of the neighboring erythrocyte layer is inevitable to achieve complete extraction of th buffy coat layer [16]. Considering these facts, when the best reduction of leukocytes and erythrocytes is needed, centrifugation at 400 × 2,000 g is optimal for P-PRP.

In this study, the centrifugation time for PRP production was unified to 7 min. In our pilot study, when the initial centrifugation time was set to 5 min, there were occasional cases where erythrocytes floated in the plasma fraction. However, when the time was set to 7 min, there were no erythrocytes floating in the plasma fraction. Thus, the centrifugation time was set to 7 min. As a limitation of the present study, it may be possible to shorten the second centrifugation time further. In addition, the platelet content of PRP is affected by various other factors, such as the breed and age of the horse, anticoagulants, blood sampling, and the technical skils of the clinician [5]. I this study, these factors were kept constant to minimize their effect on the results. In addition, the therapeutic effect of using PRP prepared by the optimized method was not investigated in this study. Tambella *et al.* [23] asserted that it is crucial to increase the number of preclinical studies on PRP applications, both *in vitro* and *in vivo*, and that the scientific community may then carry out systematic clinical trials in order to evaluate the

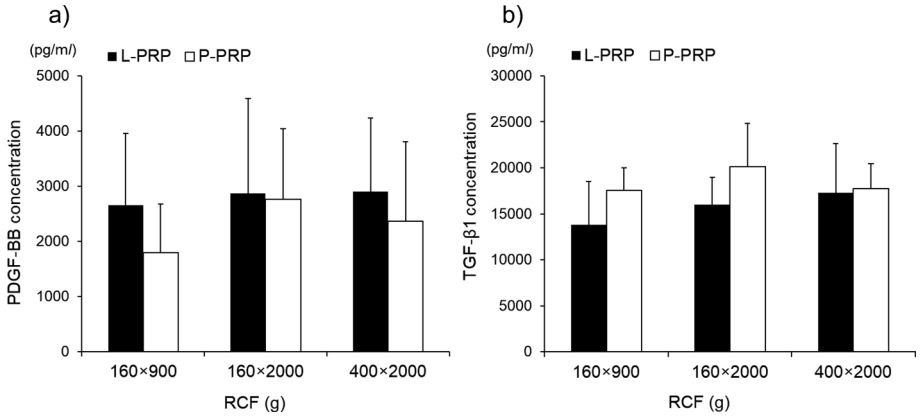

Fig. 4. The concentrations of (a) platelet-derived growth factor-BB (PDGF-BB) and (b) transforming growth factor-β1 (TGF-β1) in platelet-rich plasma (PRP) after double spin (mean ± SD). No significant differences were observed among the relative centrifugal force (RCF) combinations or the types of PRP.

safety and tolerability of PRP for clinical use. It is necessary to investigate the effect of PRP containing a high dose of growth factors and the effect of different leukocyte concentrations in PRP, prepared by the method considered to be optimal in this study, on the administered tissue according to the disease. The results of this study indicate that when preparing L-PRP, a double-spin protocol including centrifugation at 160 × 900 g, 160 × 2,000 g, or 400 × 2,000 g is optimal; however, when leukocyte- and erythrocyte-reduced PRP is required, a P-PRP protocol including centrifugation at 400 × 2,000 g is optimal.

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