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Optimal activation methods for maximizing the concentrations of platelet-derived growth factor-BB and transforming growth factor-β1 in equine platelet-rich plasma

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ABSTRACT. Platelet-rich plasma (PRP) therapy has been widely applied in various medical fields including humans and horses. This study aimed to establish an optimal activation method to stably and reproducibly maximize the concentrations of platelet-derived growth factor-BB (PDGF-BB) and transforming growth factor-β1 (TGF-β1) contained in equine PRP. Autologous PRP was prepared from 11 Thoroughbreds. For the activation test, PRP was activated by either a single freeze-thaw cycle (*Fr*) or adding calcium and autologous serum containing thrombin (*Ca*). PDGF-BB and TGF-β1 concentrations in *Fr*, *Ca*, nonactivated (*No*), and platelet-poor plasma (PPP) samples were determined using ELISA and compared. For repetitive freeze-thaw test, PRP was subjected to single (*Fr1*), double (*Fr2*), triple (*Fr3*), or quadruple *(Fr4*) freeze-thaw cycles and the concentrations of both growth factors in samples were compared similarly. The PDGF-BB concentration in *Ca* was significantly higher than that in other preparations. The TGF-β1 concentrations in *Fr* and *Ca* were significantly higher than those in PPP and *No*, with no significant differences between *Fr* and *Ca*. The concentrations of both factors were significantly increased in PRP treated with multiple cycles of freeze-thaw compared with that in PRP treated with a single cycle. No significant differences were noted among *Fr2*, *Fr3*, and *Fr4*. Our findings suggest that activation by adding calcium and autologous serum is optimal for instant use of PRP and that double freeze-thawing is an easier and optimal activation method for cryopreserved PRP.

KEY WORDS: equine platelet-rich plasma, freeze-thawing, platelet activation, platelet-derived growth factor-BB (PDGF-BB), transforming growth factor-β1 (TGF-β1)

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Platelet-rich plasma (PRP) therapy has been widely applied to both human and veterinary medicine [1, 7, 15, 16, 23, 33] since the innovative report of therapeutic efficacy of PRP by Marx *et al* [20, 21]. Derived from autologous whole blood, PRP is a concentrated platelet solution in plasma. Platelets in PRP contain various therapeutic ingredients, including platelet-derived growth factor (PDGF), transforming growth factor-β1 (TGF-β1), vascular endothelial growth factor (VEGF), epidermal growth factor (EGF), and fibroblast growth factor (FGF) [7]. Upon activation, these growth factors are released from concentrated platelets and enhance various healing processes around the site of administration [7, 20]. In addition, the use of PRP therapy in humans has been reported for a variety of orthopedic conditions such as osteoarthritis and injuries in tendons, muscles, and ligaments [10, 12, 23]. Currently, it is used to treat similar disorders in veterinary medicine [13, 15, 16, 32, 33].

One of the reasons for the rapid and wide acceptance of PRP therapy is the simplicity of preparing PRP through the double centrifugation method [7, 28, 31, 34]. However, Sundman *et al.* [27] showed that cellular and cytokine compositions in the resulting PRP varied depending on the preparation method and suggested that it influences the biologic effects of PRP. Hence, the cellular composition of PRP that should be prepared according to the conditions is disputable [6, 17, 19, 20, 27].

The concentration of growth factors in PRP depends not only on its composition but also on its activation method [18, 19]. Platelets in PRP are activated by physical or chemical stimuli that cause the release of numerous growth factors, such as those mentioned above [7, 20]. In equine veterinary practice, two methods are widely used for the activation of PRP: freeze-thawing

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(*Fr*) to physically disrupt platelet membranes or adding calcium and autologous thrombin-containing serum to induce physiological activation of platelets (*Ca*) [3, 5]. Since platelets are activated only by contact with collagen fibers within the extra-cellular matrix *in situ* [25], it has been thought that activation of PRP is not required before administration. However, Textor *et al.* showed that administration of resting (nonactivated) PRP results in the release of only a fraction of the growth factors contained within the platelets [31]. In clinical use, a high dose of growth factors is frequently required to immediately enhance the healing process [13]. However, few studies have reported an optimal method of platelet activation to stably and reproducibly maximize growth factor concentrations in PRP, and it is considered essential to standardize the activation method to obtain such PRP preclinically [29].

In this study, we investigated the concentrations of PDGF isoform BB (PDGF-BB) and TGF-β1 in equine PRP, which are representative growth factors contained in platelets. In brief, PRP was activated by each of the above mentioned methods or by the repetitive freeze-thawing method. Thereafter, their concentrations in the resulting PRP were determined using ELISA and compared.

MATERIALS AND METHODS

Animals

Eleven healthy Thoroughbreds (seven male and four female, 5.0 ± 1.5 years old, 496 ± 26 kg, mean ± SD) were used in this study. All experimental procedures were approved by the Animal Welfare and Ethics Committee of the Equine Research Institute of the Japan Racing Association (authorization numbers 16-7 and 17-8).

Preparation of PRP

Autologous PRP was prepared using a double-spin method as previously described [8]. One hundred m*l* of equine whole blood was collected from the jugular vein of each of the 11 Thoroughbred horses using a disposable plastic syringe containing 10% sodium citrate anticoagulant (ACD-A injection, Terumo BCT Ltd., Tokyo, Japan). Equal amounts of blood were dispensed into ten polypropylene tubes and centrifuged at 400 × *g* for 7 min at 4°C. The plasma fraction of each tube was transferred into another polypropylene tube and centrifuged at 2,000 × *g* for 7 min at 4°C. The supernatant was removed and kept as platelet-poor plasma (PPP). One m*l* of supernatant was left in the bottom of each tube and used to resuspend the pellet for the preparation of PRP. The concentrations of platelets and leukocytes in the PRP and PPP were determined using an automated blood cell counter (Sysmex K-4500, Sysmex Corp., Kobe, Japan). The PRP samples were randomly divided into two groups and used for an activation test (n=6) or a repetitive freeze-thaw test (n=5). Concurrently, autologous serum was prepared for the activation of PRP by centrifuging peripheral blood in a blood collection tube (BD Vacutainer SST™ II Advance Tube, BD Limited, Plymouth, UK) at 2,000 × *g* for 10 min at 4°C.

PRP activation test

Two different activation methods were conducted. In the *Fr* method, the prepared PRP was frozen at −80°C for 2 hr and completely thawed at room temperature for 30 min. In the *Ca* method, a mixture of 10% $CaCl_2$ solution and autologous serum at a ratio of 1:3 was mixed in a 1:10 ratio with PRP in a microtube. The mixed PRP was allowed to gel by incubation at 37°C for 5 min. The gelled PRP was centrifuged at 10,000 × *g* for 5 min at 4°C before collection of the supernatant. Similarly, *Fr* and nonactivated (*No*) samples were centrifuged under the same conditions before collection of the supernatant. *Ca*, *Fr*, *No*, and PPP supernatants were preserved at 4°C for the analysis of growth factors.

The concentrations of PDGF-BB and TGF-β1 in samples were determined in duplicate using ELISA kits (Quantikine Human PDGF-BB ELISA DBB00 and Quantikine Human TGF-β1 ELISA DB100B, R&D Systems, Minneapolis, MN, USA) according to the manufacturer's instructions. These kits were designed for testing human samples but have also been validated for use in horses [3, 11, 28, 30, 31]. All procedures were performed within 6 hr of preparation to ensure the stability of growth factors [11].

To observe morphological changes in platelets and leukocytes, smears of PPP, *No*, *Fr*, and *Ca* supernatants were stained using a commercial kit (Diff-Quick staining kit, Sysmex Corp.) and examined microscopically.

PRP repetitive freeze-thaw test

PRP samples were frozen for 1 month and were subjected to single (*Fr1*), double (*Fr2*), triple (*Fr3*), or quadruple *(Fr4*) freeze-thaw cycles, with repeated overnight freezing at −30°C and thawing at room temperature. All samples were divided into two aliquots after each freeze-thaw cycle (Fig. 1). One aliquot was centrifuged at 10,000 × *g* for 5 min at 4°C before collection of the supernatant. The remaining precipitate was dissolved and incubated for 30 min at room temperature with 0.5% Triton™ X-100 (SIGMA-ALDRICH, Co., St. Louis, MO, USA) PBS solution containing a protease inhibitor (cOmplete™ ULTRA Tablets, Roche Ltd., Basel, Switzerland). To dissolve platelet membranes and determine the total growth factor content in PRP samples, another aliquot was incubated for 30 min at room temperature using the Triton buffer described above (washed *Fr*). The concentrations of PDGF-BB and TGF-β1 in the supernatant, dissolved precipitate, and washed *Fr* (total concentration) were determined in the same manner as described previously and compared.

One half of the supernatant was frozen at −30°C overnight and thawed at room temperature for 30 min (*treated supernatant*) to determine the effect of the freeze-thaw treatment itself on growth factor concentration. The concentrations of PDGF-BB and TGF-β1 in these samples were also determined and compared to the concentrations in the pre-treated supernatant samples. Additionally, to clarify the amount of growth factors accumulated during freeze-thawing, the concentrations of growth factors

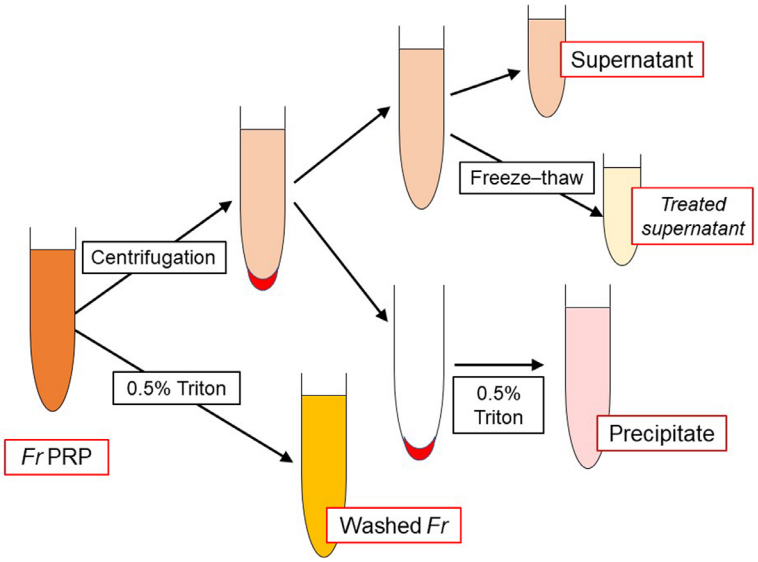

Fig. 1. Schematic representation of the platelet-rich plasma (PRP) repetitive freeze-thaw test. *Fr* PRP: freeze-thawing PRP.

in supernatants collected after freeze-thawing PRP were compared with those in freeze-thawing PRP supernatant (*treated supernatant*). Smear examination was also performed for each *Fr* sample as mentioned above.

Statistical analysis

Growth factor concentrations were compared using one-way ANOVA, and *post-hoc* analyses were performed using Tukey's test. Concentration ratios were compared using the Kruskal-Wallis test, and the subsequent *post-hoc* analysis was performed using the Steel-Dwass test. Concentrations of the freeze-thawing supernatants were compared using paired *t*-tests. Analyses were performed using Microsoft Excel 2013 Macro applications (Excel TOKEI ver. 7.0, ESUMI Co., Ltd., Tokyo, Japan). Statistical significance was set at $P<0.05$.

RESULTS

Platelet and leukocyte counts in whole blood and PRP are shown in Table 1. Microscopic examination of smear samples revealed that leukocytes in PRP predominantly comprised lymphocytes (approximately >90%). Neither leukocytes nor platelets were observed in PPP or the supernatants of *Ca* gel. Platelets appeared to maintain their morphology, whereas leukocytes were not observed in *Fr1, Fr2*, and *Fr3* (Fig. 2).

PRP activation test

The concentration of PDGF-BB in PPP and *No* was below the linear range of measurement (<31.2 *pg*/m*l*). The concentration of PDGF-BB in *Ca* (5,222 ± 3,957 *pg*/m*l,* mean ± SD) was significantly higher than that in other preparations (*Fr*, 1,369 ± 1,138 *pg*/m*l*, Fig. 3A). The concentrations of TGF-β1 in *Fr* (7,235 ± 2,842 *pg*/m*l*) and *Ca* (8,084 ± 2,257 *pg*/m*l*) were significantly higher than those in PPP (1,779 ± 461 *pg*/m*l*) and *No* (1,994 ± 442 *pg*/m*l*), with no significant differences between *Fr* and *Ca* (Fig. 3B).

PRP repetitive freeze-thaw test

The total PDGF-BB concentrations in *Fr,1 Fr2, Fr3,* and *Fr4* samples (9,486 ± 553 *pg*/m*l*, 11,545 ± 1,873 *pg*/m*l*, 12,295 ± 1,709 *pg*/m*l*, and 11,288 ± 1,591 *pg*/m*l,* respectively) were significantly higher than those in the respective supernatants (2,698 ± 1,510 *pg*/m*l*, 5,224 ± 913 *pg*/m*l*, 6,294 ± 1,254 *pg*/m*l*, and 6,351 ± 1,419 *pg*/m*l,* respectively) and precipitates (3,512 ± 2,045 *pg*/m*l*, 2,906 ± 654 *pg*/m*l*, 2,236 ± 344 *pg*/m*l*, and 2,069 ± 572 *pg*/m*l,* respectively) (Fig. 4A). No significant difference was observed between the *Fr1* supernatant and precipitate. However, in the *Fr2*, *Fr3*, and *Fr4* samples, the concentration of PDGF-BB in supernatants was significantly higher than that in precipitates. The concentrations of PDGF-BB in the *Fr1* and *Fr2* supernatants were significantly decreased by freeze-thawing with concentrations in the *Fr1* and *Fr2 treated supernatants* of 2,154 ± 1,188 *pg*/m*l* and 4,218 ± 852 *pg*/m*l*, respectively, which were significantly lower than those in freeze-thawing PRP supernatant (Fig. 4B). The concentrations of PDGF-BB in the *Fr2*, *Fr3*, and *Fr4* supernatants were significantly higher than that in the *Fr1* supernatant, whereas no significant differences in PDGF-BB concentration were noted among the *Fr2*, *Fr3*, and *Fr4* supernatants (Fig. 4C). There were no significant differences in precipitate or total PDGF-BB concentrations among the four groups.

There were no significant differences in TGF-β1 concentrations within samples of the same freeze-thaw cycle number, except in total concentration of the *Fr4* (55,506 ± 22,461 *pg*/m*l*), which was significantly higher than that of the precipitate of the *Fr4* (15,166 ± 3,210 *pg*/m*l*) (Fig. 4D). The concentration of TGF-β1 in the *Fr3* supernatant (40,386 ± 14,338 *pg*/m*l*) was significantly

Table 1. The summary of obtained platelet-rich plasma (PRP) (n=11)

	Leukocyte ($\times 10^6$/m*l*)	Platelet ($\times 10^8$/m*l*)
Whole blood	6.7 ± 1.2	1.2 ± 0.2
PRP	3.7 ± 1.6	9.2 ± 1.6
Concentration ratio	0.6 ± 0.3	7.4 ± 0.9

Mean ± SD. Concentration ratio is the value of PRP/whole blood.

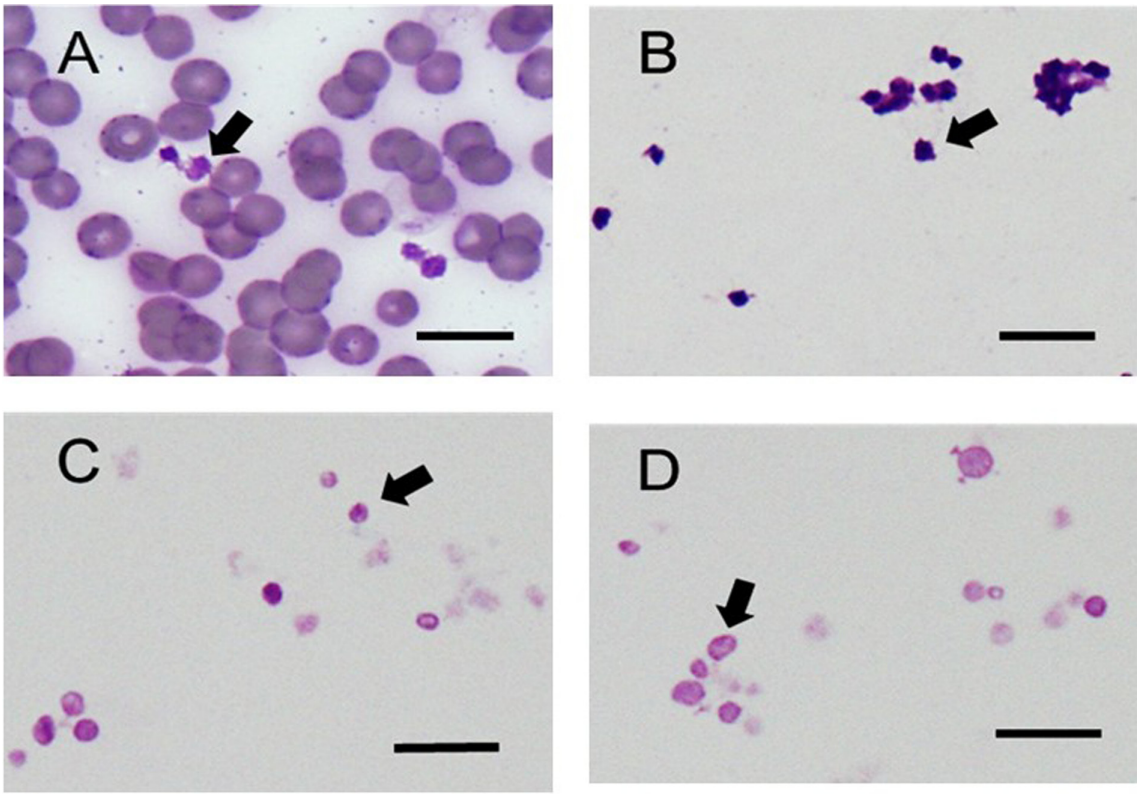

Fig. 2. Micrographs of Diff-Quick-stained whole blood smears (A), fresh platelet-rich plasma (PRP) (B), single freeze-thawing PRP (C), and triple freeze-thawing PRP (D). Platelets maintaining native morphology are indicated with black arrows. Bars=10 μm.

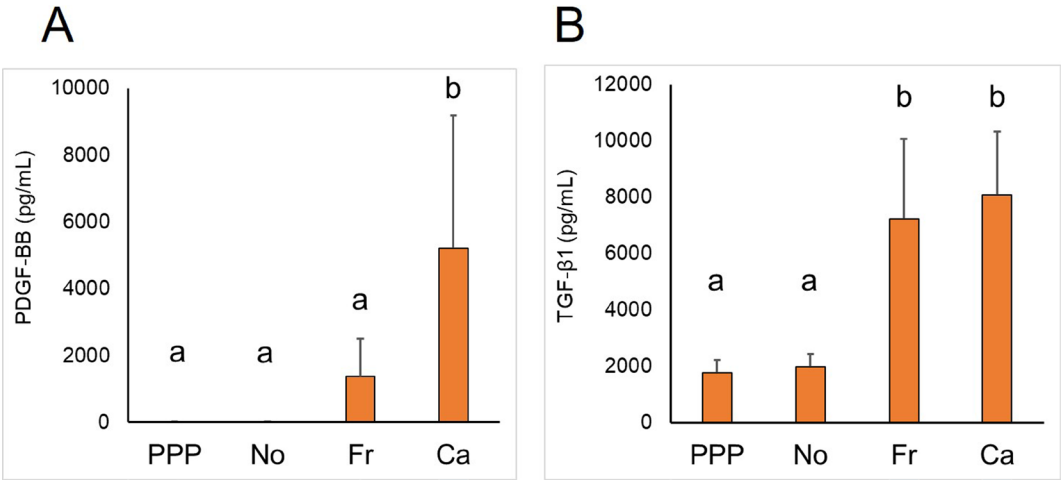

Fig. 3. The concentrations of platelet-derived growth factor-BB (PDGF-BB) (A) and transforming growth factor-β1 (TGF-β1) (B) in platelet-poor plasma (PPP), nonactivated platelet-rich plasma (PRP) (*No*), freeze-thawing PRP (*Fr*), and PRP to which calcium and autologous serum were added (*Ca*) (mean ± SD, n=6). Different letters indicate significant differences among groups (P<0.05).

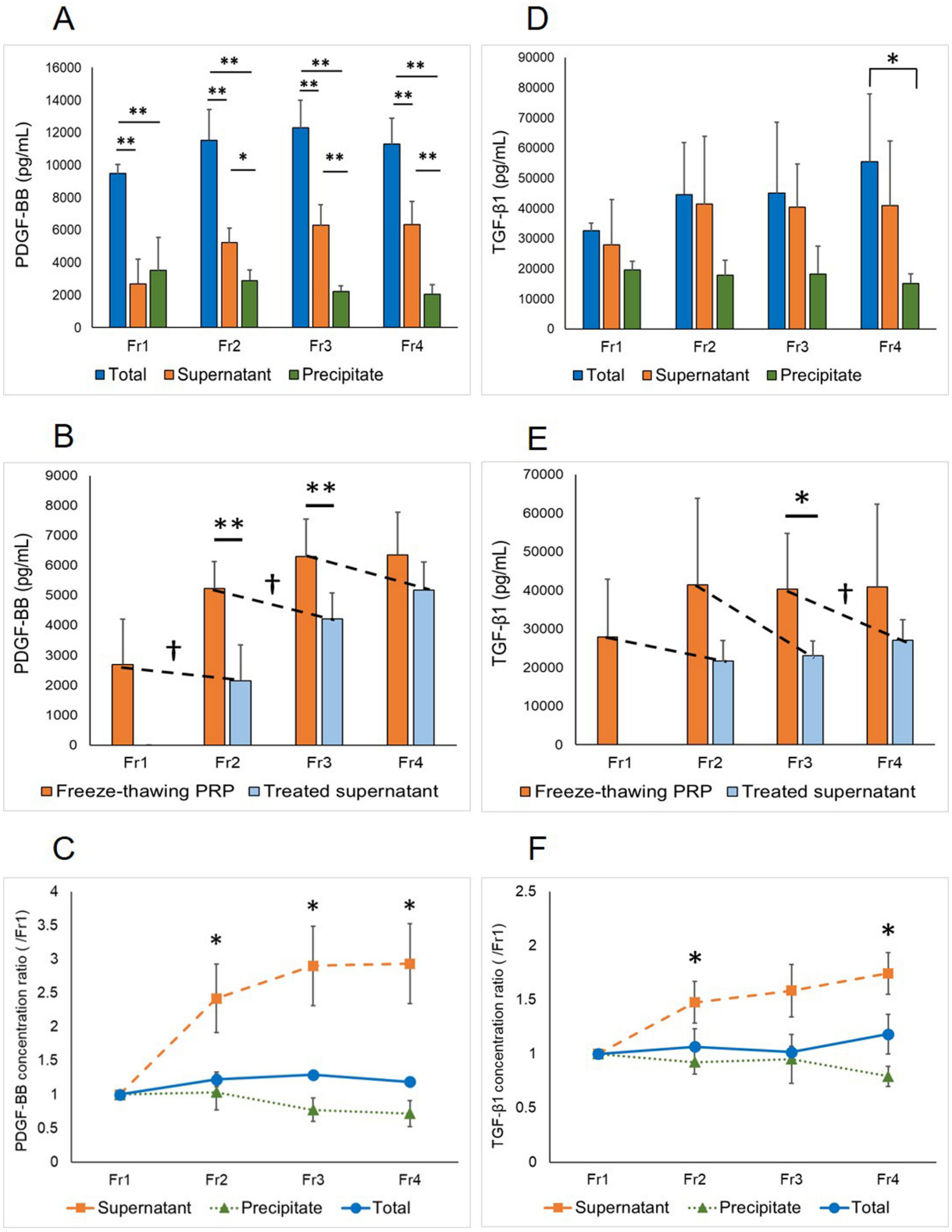

Fig. 4. Platelet-derived growth factor-BB (PDGF-BB) (A) and transforming growth factor-β1 (TGF-β1) (D) concentrations in single (*Fr1*), double (*Fr2*), triple (*Fr3*), and quadruple (*Fr4*) freeze-thawing platelet-rich plasma (PRP) (mean ± SD, n=5). Comparisons among total concentration, concentration in the supernatant, and concentration in the precipitate obtained after centrifugation (**P*<0.05, ***P*<0.01). PDGF-BB (B) and TGF-β1 (E) concentration in freeze-thawing PRP supernatant (mean ± SD). Freeze-thawing PRP in (B) and (E)=supernatant in (A) and (D), respectively. Dashed lines indicate comparisons of the supernatant concentrations before and after freeze-thawing (*, †*P*<0.05; **, ††*P*<0.01). PDGF-BB (C) or TGF-β1 (F) concentration ratios compared to *Fr1* (mean ± SE). (C) The concentrations in the *Fr2*, *Fr3*, and *Fr4* supernatants were significantly higher than that in the *Fr1* supernatant. (F) The concentrations in the *Fr2* and *Fr4* supernatants were significantly higher than that in the *Fr1* supernatant. No significant differences were observed among the *Fr2*, *Fr3*, and *Fr4* supernatants (**P*<0.05).

decreased by freeze-thawing with concentrations in the *Fr3* treated supernatants of 27,074 ± 5,336 *pg*/m*l* (Fig. 4E). The *Fr3* supernatant contained significantly higher levels of TGF-β1 than the freeze-thawing *Fr2* supernatant (23,044 ± 3,887 *pg*/m*l*). The concentrations of TGF-β1 in the *Fr2* and *Fr4* supernatants were significantly higher than that in the *Fr1* supernatant, whereas no significant differences in TGF-β1 concentration were noted among the *Fr2, Fr3,* and *Fr4* supernatants (Fig. 4F). There were no significant differences in precipitate or total TGF-β1 concentrations among the four groups.

DISCUSSION

There are many methods for preparing PRP with various cellular and molecular components. Furthermore, the methods of PRP activation are diverse [29]. Consequently, although PRP therapy is widely applied in human and equine orthopedic surgery, the clinical outcomes vary [4, 24, 29]. Because instantly effective ingredients are present in the supernatant of activated PRP, we examined an activation method to stably maximize the growth factor concentration in PRP supernatant. In addition, we posited that the variation in outcomes can be minimized by using such PRP for treatment.

Upon injury, PDGF stimulates mitogenicity and chemotaxis of leukocytes, fibroblasts, and smooth muscle cells to the wound site, and also enhances angiogenesis via upregulation of *VEGF* gene expression [2]. TGF-β1 also plays a crucial role in angiogenesis and connective tissue regeneration in wound healing. Additionally, TGF-β1 is a potent inhibitor of metalloproteinase preventing collagen breakdown [2]. In humans, because the levels of these growth factors are decreased in chronic wounds, recombinant human variants of PDGF-BB have been successfully administered for such lesions [2]. In equine practice, because autologous growth factor therapy may be considered a safer application than xenogeneic therapy [30], it seems clinically important to use PRP with high concentrations of these growth factors for treatment.

PRP activation tests revealed that activated PRP contains a high concentration of growth factors regardless of the activation method. Activation of PRP by the *Ca* method resulted in the highest PDGF-BB concentrations. In contrast, no significant differences in TGF-β1 concentrations between the *Fr* and *Ca* methods were observed. Similar conclusions have been reported by Textor *et al* [30].

Our results suggest that single freeze-thawing PRP contains an abundance of PDGF-BB in the precipitate, similar to that in the supernatant. Therefore, it is likely that the single freeze-thaw method is insufficient to entirely release PDGF-BB from platelets in PRP. Compared with the addition of calcium and serum, this method results in lower levels of PDGF-BB. However, there was no difference in the TGF-β1 concentration between the *Fr* and *Ca* methods. There was a large variation in the TGF-β1 concentrations between individual PRP supernatants activated by the single freeze-thaw method. It was previously reported that TGF-β1 from disrupted leukocytes contributes to its concentration in PRP [35]. In this study, although we attempted to prepare PRP in such a manner that the presence of leukocytes was kept as low as possible, the contamination of low-dose leukocytes was inevitable. We considered the possibility that the variation in TGF-β1 concentration among individual samples was due to a non-negligible amount of TGF-β1 released from leukocytes, as has been previously reported in humans [35]. Consequently, we did not observe differences in TGF-β1 concentrations corresponding to the activation method. Our results show that a method involving the addition of calcium and autologous serum may be superior for activation of PRP for instant use. However, the concentrations of growth factors in *Ca* supernatants showed high variability. Therefore, it was considered important to establish an activation method capable of more stably attaining high growth factor concentrations.

Our results also suggested that repeated freeze-thaw cycles result in higher growth factor concentrations in the supernatant of PRP than that obtained by a single freeze-thaw cycle. In humans, it was reported that double freeze-thawing results in higher PDGF concentrations in PRP supernatant than that obtained by single freeze-thawing [35]. It was also reported that the concentrations of PDGF-BB, TGF-β1, EGF, and FGF increased by repetitive freeze-thawing and plateaued at 3 and 5 cycles [26]. We found that the supernatants of PRP that were freeze-thawed multiple times contained significantly higher concentrations of growth factors than those subjected to single freeze-thawing. On the other hand, no significant differences were noted among PRP that was freeze-thawed two or more times. We hypothesized that concentrations of both PDGF-BB and TGF-β1 would increase in PRP supernatants because the number of freeze-thaw cycles should cause increased disruption of platelets and growth factor release. However, we found that the concentration of neither growth factor was increased with increasing cycles of freeze-thaw. We speculate that PDGF-BB is considerably vulnerable to degradation upon freeze-thawing. Conversely, the increase in growth factor concentrations in freeze-thawing PRP is caused by de-novo release of growth factors from disrupted platelets remaining in the precipitate. Therefore, it is reasonable to hypothesize that additional cycles do not significantly affect growth factor concentration because amounts lost and produced are in equilibrium. Conversely, TGF-β1 is highly tolerant to degradation upon freeze-thawing, as has been reported in humans [9, 14]. Therefore, freeze-thawing of PRP resulted in the accumulation of newly released TGF-β1. In contrast, even after repeated freeze-thawing, there was no clear difference in TGF-β1 concentrations among the total, supernatant, and precipitate samples. It is reasonable to assume that the TGF-β1 concentration in PRP depends not only on platelets but also on other components, such as leukocytes [35]. Hence, no significant differences were observed among the *Fr2*, *Fr3*, and *Fr4* supernatants. Based on our findings, the optimal growth factor concentration in PRP supernatant may be obtained by freezing and thawing at least twice.

Growth factor concentrations in *Fr1* samples were higher than those in *Fr* samples in the activation test, although these were similar supernatants activated by the single freeze-thaw method. McClain and McCarrel indicated that the growth factor content in equine PRP varies with long-term cryopreservation [22]. In our activation test, it was necessary to determine the concentrations of growth factors in PRP preparations within 6 hr in order to measure stable concentrations consistently between all sample types

[11]. In contrast, frozen PRP stored for at least 1 month was thawed and measured in the repetitive freeze-thaw test, which may be the cause of the difference in values obtained. We found that high concentrations of growth factors can be maintained even after 1 month of PRP cryopreservation. Furthermore, 2.4-fold higher PDGF-BB and 1.5-fold higher TGF-β1 concentrations were obtained using double freeze-thawing for activation than by using single freeze-thawing. This indicates that double freeze-thawing can result in growth factor concentrations and stability that are comparable to calcium and serum addition. Consequently, PRP activated by the *Ca* method can be applied quickly and appears to be suitable for instant use. However, growth factors once extracted into the supernatant are prone to inactivation. Hence, if activated PRP is intended for repeated long-term use, it is easier to prepare PRP in large cryopreserved batches by dispensing it in small quantities and double freeze-thawing when used.

In this study, we investigated only two growth factors and did not investigate other components in PRP. On the other hand, as mentioned above, it is known that these two growth factors, which are abundant in platelets, are essential contributors to the wound healing process. Therefore, when considering the clinical effects of PRP therapy, these growth factors must be focused on. However, further studies are needed to determine the appropriate application method of equine PRP optimally prepared and activated for clinical use [29]. In conclusion, our findings suggest that activation by adding calcium and autologous serum is optimal for instant use of PRP and that double freeze-thawing is an easier and optimal activation method for cryopreserved PRP.

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