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Research article

OPTIMIZATION OF A TWO-STEP CENTRIFUGATION PROTOCOL FOR BOVINE PLATELET-RICH PLASMA

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Platelet-rich plasma (PRP), an autologous platelet concentrated in plasma, is a source of diverse growth factors and is extensively utilized to promote tissue healing. Most of the clinical and laboratory investigations in veterinary medicine have focused on horses and dogs. Consequently, the types and detailed conditions of the PRP preparation method are based on those species. However, in bovine medicine, only a few studies have investigated the species-specific characteristics of bovine platelets. The aim of this study was to optimize the conditions of a two-step centrifugation method for bovine PRP. Whole blood samples were obtained from eight healthy lactating Holstein-Friesian cows. Eight running conditions for the first and second centrifugations each were evaluated based on the platelet recovery rate, the concentration of growth factors, and the adenosine diphosphate (ADP)-induced aggregation response, which reflects the premature platelet activation caused by the centrifugation process. The results of this study showed that for the first and second centrifugations, 900×g for 10 minutes and 1250×g for 15 minutes, respectively, were the most suitable conditions for the platelet recovery rate, and the concentration of growth factors was highest under these conditions without significant activation of premature platelet aggregation. Thus, we established an optimal two-step centrifugation protocol for bovine PRP that should provide a better understanding of bovine platelets.

Key words: centrifugal, growth factor, platelet rich plasma, premature activation, recovery rate

INTRODUCTION

Platelet-rich plasma (PRP) is a therapeutic hemo-component enriched with platelets (PLTs) and growth factors (GFs) with angiogenic, anti-inflammatory, mitogenic, and proliferative effects [1-3]. After platelets are activated by endogenous substances, such as collagen, or exogenous activators, several growth factors and cytokines stored in the

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alpha granules are released by degranulation [4]. These include Vascular Endothelial Growth Factor (VEGF), Platelet-Derived Growth Factor (PDGF), Transforming Growth Factor β (TGF-β), Epidermal Growth Factor (EGF), Basic Fibroblast Growth Factor (bFGF), and Insulin-like Growth Factor-I/II (IGF-I/II), etc., which promote regenerative processes such as angiogenesis, chemotaxis, proliferation and differentiation, deposition of proteins, and epithelial regeneration [5-8]. Recently, the antibacterial effect of platelet rich hemo-components has also been highlighted and several studies have demonstrated the antibacterial activity of platelets [9].

Since first described in the 1970's, PRP has been used predominantly in the musculoskeletal and wound healing fields [10-12]. Platelet-based therapeutic modalities are also used in other medical fields, including gynecology, plastic surgery, cardiac surgery, urology, dentistry, and ophthalmology [13]. In veterinary medicine, PRP is most commonly used for the treatment of joint diseases and tenodesmic lesions and for healing wounds in horses [14,15] and dogs [16]. In bovine medicine, the therapeutic effects of PRP on sole ulcers, mastitis, endometritis, and ovarian hypofunction have been described by a few studies [17-20].

In recent decades, various types of PRP preparations with different compositions have been devised in both human [2] and veterinary medicine [21]. Among the many ways to obtain PRP, the differential centrifugation method is mostly used in veterinary medicine due to its convenience and cost-effectiveness. The composition of PRP prepared by differential centrifugation varies with centrifugal force, spin time, and sample volume [22]. Furthermore, the centrifugation process can affect platelet functionality [23]. Bausset et al. confirmed that a higher centrifugal force induces premature activation of platelet aggregation, decreased the reactivity of platelets, and morphological changes [24]. If PRP is prematurely activated and not used within an adequate timeframe, its therapeutic efficacy is reduced, and growth factors are inactivated before its application [23-25].

Several studies have revealed species differences in the function, susceptibility to premature activation aggregation, and morphology of platelets, suggesting the need for individual studies on PRP for each species [26,27]. In veterinary medicine, the majority of species-specific PRP research has focused on horses and dogs. Unlike these animals, studies on bovine PRP are scarce. Only a few studies have described a double centrifugation method for concentrating bovine platelets, and these studies analyzed the effect of two anticoagulants on bovine PRP [27] and the differences in cellular and growth factor profiles depending on bovine PRP fractions or byproducts [28,29]. No previous study has optimized the centrifugation protocol for the preparation of bovine PRP.

Bovine PRP is mostly used for tissue regeneration or functional promotion. Therefore, growth factors and the platelets containing these growth factors are the key criteria to evaluate the therapeutic effect of bovine PRP. In the present study, we evaluated the impact of the centrifugal force and time on the platelet recovery rate, aggregation response, and concentration of growth factors to establish a species-specific centrifugation protocol for bovine PRP.

MATERIAL AND METHODS

Experimental animals

Eight clinically healthy lactating Holstein-Friesian cows between four and five years old were included in the present study. All animals had been free of any drugs known to affect platelet functions for 14 days. This study was approved by the Institutional Animal Care and Use Committee (Approval number: KU21104) of Konkuk University, and it was performed in accordance with the Guide for Laboratory Animal Care and Use. The owner of the cows was informed of the study objectives and procedures before providing consent to participate.

Blood collection

Blood was collected from the jugular vein of each cow using a 14-gauge intravenous catheter. The catheter was directly connected to a 50 mL syringe containing 5 mL of 3.2% w/v sodium citrate, and 45 mL of blood was obtained (the final dilution of blood:anticoagulant was 9:1). A total of 180 mL of blood was drawn from each cow. The syringes containing blood were maintained in continuous rolling motion and transported to the laboratory within 1 hour. After homogenization, the whole blood was divided into 10 mL aliquots in 15 mL conical tubes.

Preparation of platelet fractions

A two-step centrifugation protocol was utilized for all samples. For the first centrifugation, blood aliquots were centrifuged at one of 4 centrifugal forces (300, 600, 900, and 1200 g) for 5 and 10 minutes at room temperature for a total of 8 centrifugal force–time conditions. Eight replicates were conducted using blood collected from different animals. Each whole blood sample was tested three times under each centrifugation condition, and the mean of the three trials was used for analysis (n=8). The plasma fraction with the buffy coat was transferred to an empty 15 mL conical tube. This hemo-component was designated as the platelet concentrate (PC), and the platelet concentration and volume were measured to calculate the total number of platelets. The second centrifugation was performed and the PC prepared under the specific first spin condition showed the highest recovery rate of platelets. The PC was aliquoted into 15 mL conical tubes at 5 mL per tube and centrifuged at 1000, 1250, 1500, and 1750 g for 10 and 15 minutes at room temperature for a total of 8 centrifugal force–time conditions, with six replicates for each condition. Each PC sample was tested three times under each centrifugation condition, and the means of the three trials were used for analysis (n=6). The supernatant fraction was designated platelet-poor plasma (PPP). Platelet-rich plasma was obtained after removing 3.5 mL of the PPP and suspending the pellet in remnant PPP.

Hematological analysis

A complete blood count (CBC) test was performed on each the whole blood, PC, and PRP fraction using a flow cytometry hematology system (ProCyte Dx, IDEXX, ME, USA). The concentrations of platelets, red blood cells (RBCs), and white blood cells (WBCs) were analyzed, and the percentage of platelets recovered after the centrifugation procedures was measured to compare the recovery rate under each condition.

Aggregometry test

ADP-induced platelet aggregation responses were measured in each PC and PRP fraction using light transmission aggregometry (Chrono-Log Corporation, Model 490, PA, USA). PPP was used as a reference sample (100% light transmission). The final platelet count of PRP was adjusted to 200 x 10^9/L with autologous PPP. To perform this test, 500 µL of PPP and PRP were placed into the reference channel and prewarmed test channel, respectively. After incubating the PRP at 37 °C for 5 minutes, baselines were adjusted to 0%, and 5 µL of ADP was added to the 500 µL PRP sample for a final concentration of 10 µM to initiate aggregation. The test sample was maintained at 37 °C with continuous stirring by a magnetic stirrer. Light transmission was recorded for 10 minutes as a percentage (%) compared to the reference sample and was analyzed using a software program (Aggrolink 5.2; Chrono-Log Corporation, PA, USA).

Quantification of platelet-associated growth factors

The concentrations of platelet-associated growth factors, including TGF-ß1 and PDGF-BB, were measured to identify correlations with a two-step centrifugation protocol. The PC and PRP samples were activated by incubating at 37 °C for 1 hour in the presence of 120 mM $CaCl_2$, followed by 4 °C for 16 hours. Then, the samples were centrifuged for 15 minutes at 2989 g, and the supernatant was collected for growth factor measurement. TGF-ß1 and PDGF-BB were quantified using a bovine-specific enzyme-linked immunosorbent assay (ELISA) kit (MyBioSource, San Diego, CA, USA). The tests were performed according to the manufacturer's instructions. Each sample was measured in duplicate, and the average of the two measurements was used for analysis.

Statistical analysis

Statistical analysis was performed using SPSS (v25.0, SPSS Inc., IL, USA). Data are presented as the mean ± standard deviation and were analyzed using the Kruskal–Wallis test, followed by Dunn's post-hoc test for multiple comparisons. A P value < 0.05 was considered statistically significant.

RESULTS

Platelet concentrate: after the first centrifugation

As seen in Table 1, the impact of each centrifugation condition on the platelet fractions was measured and compared with each other. The platelet recovery rate increased as the centrifugal force or time increased; however, under 1200×g conditions, the platelet recovery rate decreased slightly as the centrifugation time increased from 5 to 10 minutes and 1200×g. The 10-minute condition resulted in a recover rate lower than that of the 900×g and 10-minute conditions. The highest mean platelet recovery was observed under Condition 6, which was significantly different from Conditions 1, 2, and 3. However, no significant differences were found between Condition 6 and Conditions 4, 5, 7, and 8. Considering these results, Condition 6 was concluded to be optimal for the first centrifugation. PC prepared under Condition 6 was used to find the optimal second centrifugation condition.

Tables 1. Platelet recovery rate and ADP-induced aggregation response of platelet concentrate after the first centrifugation and platelet-rich plasma after the second centrifugation

First centrifugaion			Second centrifugation			
Condition	Centrifugal force (× g)/time (minutes)	Recovery rate (%)	Condition	Centrifugal force (× g)/time (minutes)	Recovery rate (%)	Aggregation response (%)
1	300 / 5	21.54 ± 2.64*	1	1000 / 10	83.18 ± 1.84#	80.36 ± 7.56
2	300 / 10	27.91 ± 3.10*	2	1000 / 15	87.66 ± 2.11#	70.75 ± 3.59
3	600 / 5	30.72 ± 4.00	3	1250 / 10	90.93 ± 1.93	72.41 ± 6.77
4	600 / 10	53.70 ± 7.14	4	1250 / 15	96.06 ± 2.21	68.00 ± 6.81
5	900 / 5	44.63 ± 8.37	5	1500 / 10	91.90 ± 2.74	66.75 ± 7.31
6	900 / 10	63.71 ± 7.00	6	1500 / 15	93.33 ± 2.34	64.75 ± 6.84
7	1200 / 5	58.18 ± 8.86	7	1750 / 10	93.16 ± 3.40	61.83 ± 4.80†
8	1200 / 10	53.11 ± 6.67	8	1750 / 15	93.63 ± 2.60	56.11 ± 9.49†

Values are expressed as the mean ± standard deviation. Asterisk (*) indicates $p < 0.05$ compared with platelet concentrate (Condition 6) under which the highest mean platelet recovery rate was observed. A hash (#) indicates $p < 0.05$ compared with platelet-rich plasma (Condition 4), under which the highest mean platelet recovery rate is observed. Daggers (†) indicate there is a significant difference compared to platelet-rich plasma (Condition 1), which shows the highest mean ADP-induced aggregation response ($p<0.05$).

Platelet rich plasma: after the second centrifugation

The second centrifugation was conducted to further concentrate the platelets by dividing PCs into PRP and PPP. Five milliliter aliquots of PCs prepared by using the first centrifugation (Condition 6) were used for the optimization of the second centrifugation step. The percentage of platelets recovered from PCs, the concentration of growth factors in PRP, and platelet aggregation capacity were measured after the second centrifugation under the eight centrifugation conditions. Unlike the first centrifugation step, the tendency of the platelet recovery rate to increase as the centrifugal force or time increased was confirmed only under 1000×g and 1250×g conditions. Under conditions above 1250×g (Conditions 3, 4, 5, 6, 7, and 8), this tendency disappeared, and no statistically significant difference in recovery rate was found among centrifugation conditions. The second centrifugation under Condition 4 resulted in the highest recovery rate, which was significantly different from that of Conditions 1 and 2. Condition 1 showed the lowest recovery rate, which was significantly different from Conditions 4, 6, 7, and 8. The platelet yield after double centrifugation was calculated by dividing the platelet count of PRP by the platelet count of whole blood (Figure 1). The PRP under Condition 4 showed the highest mean platelet yield, which was significantly higher than that of Condition 3. ADP-induced platelet aggregability tended to decrease as centrifugal force or time increased, and the PRP obtained under Conditions 7 and 8 showed a significantly lower aggregation response compared to that of Condition 1. No significant differences were observed among the other conditions.

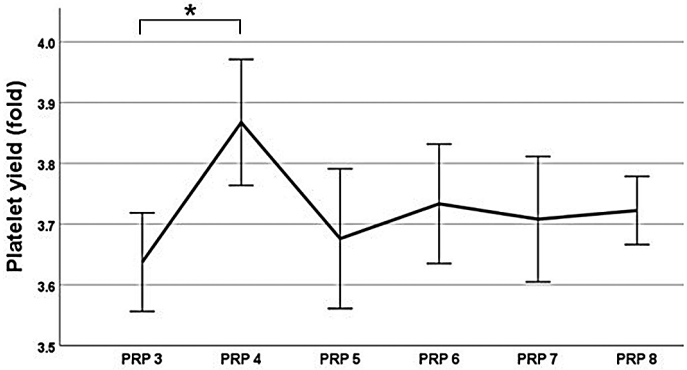

Figure 1. The platelet yield of platelet-rich plasma (PRP) is expressed as a fold increase relative to whole blood. The number following PRP indicates the number of centrifugation conditions. Data are presented as the mean ± standard deviation (n=8). Error bars represent standard errors. Asterisks (*) indicate P<.05 compared with Condition 4, under which the highest mean platelet yield is observed.

The concentrations of platelets, total WBCs, and RBCs in whole blood, PC (Condition 6), and PRP (Condition 4) are shown in Table 2. Approximately 61% of platelets were recovered from whole blood by the optimal double centrifugation protocol, in which the first and second centrifugation conditions were set to 900×g for 10 minutes and

1250×g for 15 minutes, respectively. PRP prepared using this protocol revealed a 68% platelet aggregation response to ADP.

Table 2. The values of complete blood count after double centrifugation.

Parameter	Whole blood	PC	PRP
PLT ($\times 10^3/\mu L$)	256.75±39.21	297.62±32.80	954.50±162.44*
WBC ($\times 10^3/\mu L$)	6.86±0.91	0.75±0.23*	1.82±0.45*
HCT (%)	24.45±1.29	0.0025±0.004*	0.0075±0.04*

Values are expressed as the mean ± standard deviation. Asterisks (*) indicate $p < 0.05$ compared with whole blood.
PC = platelet concentrate; PRP = platelet-rich plasma

Quantification of platelet-associated growth factor

The concentrations of growth factors, TGF-ß1 and PDGF-BB, were measured in the PC fraction with the highest mean platelet recovery rate (Condition 6) and in the various PRP fractions prepared under Conditions 3, 4, 5, 6, 7, and 8 (Figure 2). No significant difference was found in the concentration of PDGF-BB between all fractions. In contrast, the PRP fractions prepared under Conditions 4 and 5 showed a significantly higher concentration of TGF-ß1 compared to that of the PC fraction.

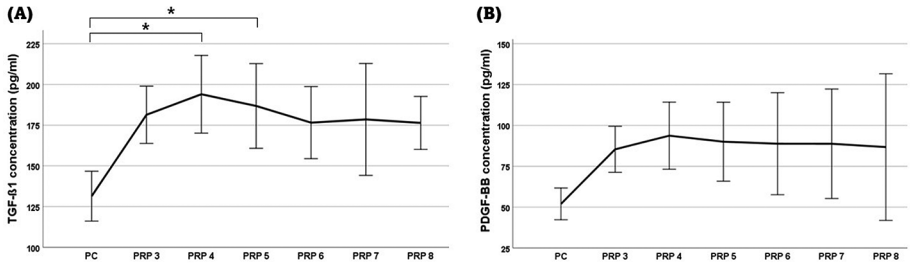

Figure 2. The concentrations of transforming growth factor beta-1 (TGF-β1) (A) and platelet-derived growth factor-BB (PDGF-BB) (B) in platelet concentrate (PC) and platelet-rich plasma (PRP). The enzyme-linked immunosorbent assay for growth factors was performed with the PC fraction with the highest mean platelet recovery rate and PRP fractions prepared under Conditions 3, 4, 5, 6, 7, and 8. Data are presented as the mean ± standard deviation (n=8). Error bars represent standard errors. Asterisk (*) indicates P<.05 compared with PC.

DISCUSSION

Several studies have reported that the centrifugation process prematurely activates platelets [23,24,30]. Platelet activation is a prerequisite for releasing therapeutic agents, such as platelet-derived growth factor (PDGF), transforming growth factor (TGF), vascular endothelial growth factor (VEGF), and epidermal growth factor (EGF). These growth factors have low stabilities and short half-lives after release (minutes

to a few hours) [31,32]. Avoiding premature activation assures that therapeutic agents will be present at the time of PRP application [31]. In addition, premature activation induces platelet aggregation, reducing the recovery rate and coagulability of platelets [23]. Accordingly, the therapeutic effects of PRP could be significantly affected by centrifugation conditions, resulting in the need for optimized centrifugation conditions [23,24].

Platelets show differences in species through many physiological aspects, including cellular morphology, the number and abundance of membrane glycoproteins, metabolic pathways, and activation aggregation responses to stimuli [25,26]. These physiological differences suggest that the effect on platelets and composition of PRP by centrifugation may be different for each species. A few studies have analyzed a double centrifugation tube protocol for concentrating bovine platelets and its effect on the properties of bovine platelets [27-29]. However, no study has analyzed the difference in the composition of bovine PRP according to the centrifugation conditions. This study aimed to establish an optimal double centrifugation protocol for preparing bovine PRP.

In this study, two centrifugation steps (the concentration of growth factors and the aggregation response to ADP) were evaluated based on the platelet recovery rate. The goal of the first centrifugation was to obtain as many platelets as possible from bovine whole blood. Our results revealed that Condition 6 (900×g, 10 minutes) obtained the highest platelet recovery, which was not significantly different from that of Conditions 4, 5, 7, and 8. Under the condition of 900×g or less, as the centrifugation time increased, more platelets were obtained. However, at 1200×g conditions, this trend disappeared with no significant difference between conditions (Conditions 7 and 8), indicating that at this specific centrifugal force, most of the platelets had settled at the bottom of the tube as a pellet in a short period of centrifugation time. The mean recovery rate under Condition 6 was higher than those of Conditions 7 and 8, showing that the centrifugal force of Conditions 7 and 8 (1200×g) might cause bovine platelets to excessively precipitate into the pellet, decreasing yields of the platelets that remained in the supernatant fraction (PC) after the first centrifugation. Since no RBCs were detected on the flow cytometry hematology system and no significant differences in the WBC recovery rate were found among centrifugation conditions, these factors were not considered in determining the optimal centrifugation protocol.

The purpose of the second centrifugation was to obtain the highest possible platelet count and growth factor concentration from the PC prepared by the first centrifugation while minimizing the activation of premature platelet aggregation. Condition 4 showed the highest mean platelet recovery rate, which was not significantly different from that of Conditions 3, 5, 6, 7, and 8. Under Conditions 5, 6, 7, and 8, the platelet pellets generated after the second centrifugation were occasionally not suspended normally in the PPP, resulting in floating particles of aggregated platelets. These results indicate that bovine platelets sufficiently settle into pellets at a centrifugal force of 1250×g or higher and excessively high centrifugal force, including 1500×g and 1750×g, could

induce the premature aggregation of platelets. The activation of premature platelet aggregation was quantified using light transmission aggregometry. The lowest mean aggregation in response to ADP was observed in the 1750×g conditions (Conditions 7 and 8), which was significantly different from the 1000×g, 10 minutes condition (Condition 1). Among the conditions below 1500×g, no significant differences were found. These results indicate that excessive centrifugal force could lead to the activation of premature platelet aggregation. The ELISA test for growth factors was performed to determine the effect of the centrifugation process on the concentration of growth factors, which is an important factor in the therapeutic effect of PRP along with the platelet recovery rate. In the TGF-ß1 test, the concentration of growth factor was observed to be higher at a statistically significant level in PRP prepared under Conditions 4 (1250×g, 15 minutes) and 5 (1500×g, 10 minutes) compared to that of PC. However, there was no significant difference between the groups in the PDGF-BB test.

To achieve the maximum therapeutic effect, the platelet recovery rate and the concentration of growth factors should be high, and the platelet function should not be severely deteriorated. As a result of a comprehensive analysis of the evaluation indicators of PRP, for the purpose of obtaining the maximum therapeutic effect, we concluded that the optimal double centrifugation conditions were 900×g for 10 minutes for the first centrifugation and 1250×g for 15 minutes for the second centrifugation.

Preparing PRP using differential centrifugation is inevitably accompanied by contamination by other cells, such as RBCs and WBCs. The effect of WBC contamination on the therapeutic efficacy of PRP is still controversial. While there are studies demonstrating that WBCs interfere with tissue regeneration and contribute to the expression of inflammatory cytokines, there are also studies showing that WBCs enhance the therapeutic effect by inducing the release of growth factors from platelets [33-36]. Currently, there are no controlled animal studies to demonstrate whether WBCs in PRP inhibit tissue healing and increase inflammation when applied topically.

In this study, the difference in WBC contamination level between groups was not confirmed; therefore, the difference was not considered when determining the optimal protocol. The degree of WBC contamination in the PRP obtained by optimized double centrifugation was lower than that reported in other studies, in which the double centrifugation tube method was utilized for canine PRP [37,38]. Since the clinical significance of WBCs in PRP was not proven, the effect of WBCs was not included in the analysis of the study results. In future research, it will be necessary to analyze the correlation between the therapeutic effect of bovine PRP and the degree of WBC contamination that allows the establishment of optimal centrifugation protocols.

This study has limitations in that premature platelet activation aggregation was indirectly estimated by measuring the platelet responsiveness to ADP. Therefore,

further studies should be performed to accurately evaluate the level of premature bovine platelet activation after double centrifugation.

The present study optimized the following double centrifugation protocol for the preparation of bovine PRP.

1. Blood samples were collected in 10 mL tubes containing 1 mL of 3.2% w/v sodium citrate with a blood:anticoagulant ratio of 9:1.
2. The tubes were centrifuged at 900×g for 10 minutes at room temperature.
3. The supernatants above the buffy coat (PC) were collected and aliquoted into 15 mL tubes at 5 mL per tube.
4. The tubes were centrifuged at 1250×g for 15 minutes at room temperature.
5. Then, 3.5 mL of supernatant (PPP) from the second tube was removed, and the remaining PPP was gently homogenized with platelet-containing pellets to prepare the PRP.

In conclusion, this study revealed that the cellular or molecular composition and physiology of bovine platelets could be affected by centrifugation conditions, including centrifugal force and time. To maximize the quality and therapeutic efficacy of bovine PRP, we tested various centrifugation conditions and optimized a simple, fast, and reproducible double centrifugation tube protocol for preparing bovine PRP. This study will form the basis for advanced comparative studies and clinical applications of bovine PRP.

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Authors' contributions

MYK and HJH contributed to the design and implementation of the research, to the analysis of the results and to the writing of the manuscript.

Declaration of conflicting interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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OPTIMIZACIJA PROTOKOLA DVOSTEPENOG CENTRIFUGIRANJA ZA GOVEĐU PLAZMU OBOGAĆENU TROMBOCITIMA

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Plazma obogaćena trombocitima (PRP-plazma), odnosno autologno koncentrisani trombociti plazme, predstavlja izvor brojnih faktora rasta i kao takva našla je široku primenu kao promoter zarastanja tkiva. Većina kliničkih i laboratorijskih istraživanja u veterinarskoj medicini bila je fokusirana na konje i pse. Shodno tome, tipovi i detaljni uslovi metode pripreme PRP zasnovani su na tim vrstama. Međutim, u medicini goveda, samo nekoliko studija se bavilo ispitivanjem specifičnih karakteristika goveđih trombocita. Cilj ovog istraživanja je bio da se optimizuju uslovi dvostepene metode centrifugiranja za PRP goveda. Uzorci pune krvi su uzeti od osam zdravih krava holštajn-frizijske vrste u laktaciji. Po osam radnih uslova za prvo i drugo centrifugiranje je procenjeno na osnovu brzine oporavka trombocita, koncentracije faktora rasta i agregacionog odgovora izazvanog adenozin difosfatom (ADP), koji odražava preranu aktivaciju trombocita uzrokovanu procesom centrifugiranja.

Rezultati ove studije su pokazali da su za prvo i drugo centrifugiranje, 900×g tokom 10 minuta i 1250×g tokom 15 minuta, bili najpogodniji uslovi za stopu oporavka trombocita, a koncentracija faktora rasta je najveća tokom centrifugovanja pri navedenim uslovima, a bez značajne aktivacije prerane agregacije trombocita. Tako smo uspostavili optimalni protokol centrifugiranja u dva koraka za goveđi PRP koji bi trebalo da obezbedi bolje razumevanje i poznavanje primene goveđih trombocita.