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Single-step production of autologous bovine platelet concentrate for clinical applications in cattle

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ABSTRACT

Platelet concentrate (PC) is an alternative therapy to treat mastitis in dairy cattle and is an alternative treatment for reproduction problems such as endometritis. Unfortunately, double-centrifugation processing methods described are time-consuming, require specialized laboratory equipment, and are usually done in a heterologous way, which risks herd health. To overcome this limitation, we evaluated single-step bovine PC processing methods readily applicable to a farm setting using an autologous conditioned plasma (ACP) production system. We investigated the hematologic findings, cytokines, and growth factors of the obtained PC samples. Autologous conditioned plasma was prepared using whole blood (WB) from 4 cows (group 1) using single-step centrifugation and 16 different processing methods. The 2 protocols that yielded the highest ratio of platelet to white blood cell (WBC) concentration were ACP-1 [720 × *g* (2,200 rpm), 5 min] and ACP-2 [929 × *g* (2,500 rpm), 3 min]. They were subsequently reproduced and compared using WB from 8 cows (group 2). Hematologic findings were quantified, IL-1β (cytokine) and growth factors [platelet-derived growth factor (PDGF), transforming growth factor (TGF)-β, bovine fibroblast growth factor (b-FGF)] were measured, and enrichment factors were compared between samples and processing methods. Hematological characteristics and platelet enrichment varied markedly among tested protocols and all were statistically different from WB. Protocol ACP-2 resulted in significantly greater platelet enrichment (mean 169% of WB) than ACP-1 (125% of WB). We found no significant difference between the 2 ACP preparation protocols with regard to leukocyte reduction (7.53–9.75% WBC compared with WB) or growth factor enrichment (124–125% PDGF, 95–100% TGF-β, 102–104% b-FGF, and 56–74% IL-1β compared with WB). In conclusion, both ACP protocols yielded a platelet concentration shown to promote healing for clinical applications in cattle, and the ACP-2 protocol resulted in a greater degree of platelet enrichment. Therefore, this protocol could be used for ACP production for clinical applications in cattle.

Key words: platelet-rich plasma (PRP), autologous conditioned plasma (ACP), platelet concentrate (PC), bovine, regenerative medicine

INTRODUCTION

Reproductive problems are frequently encountered in dairy operations and are an important cause of economic losses in the global dairy cattle industry. Decreased productivity from mastitis and reproductive problems generate economic losses from discarded milk during antimicrobial treatment, extended days open until conception, culling rate increase, death loss, veterinary costs, and labor costs (Cha et al., 2011; Liang 2013; Rollin et al., 2015). Regenerative therapies such as blood-derived products represent an alternative therapy and may be a cost-effective solution to reduce the losses caused by such diseases, including mastitis, endometritis, and repeat breeder syndrome (Zhao and Lacasse, 2008).

Research on the effect of platelet concentrates (**PC**) in cattle has surged in recent years, and increasing evidence has shown the positive role that PC plays in treating reproductive problems in cattle (Cremonesi et al., 2020; Duque-Madrid et al., 2021). Platelet-rich plasma (**PRP**) and autologous conditioned plasma (**ACP**) are PC blood products. The platelets and their cytokines are concentrated in a small plasma volume

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to reach supraphysiological concentrations. Platelets are non-nucleated cell fragments with a cytoplasm containing 3 different granule populations (α, dense, and lysosome) and storing multiple bioactive proteins that are released following cell activation. Platelet granules contain multiple proteins, including antimicrobial peptides (Drago et al., 2013), fibronectin and vitronectin (Urman et al., 2019), numerous growth factors (**GF**) such as platelet-derived growth factor (**PDGF**), epidermal growth factor, fibroblast growth factor (**FGF**), hepatocyte growth factor, transforming growth factor-β (**TGF-β**), insulin-like growth factor, and vascular endothelial growth factor (Böck et al., 2002), and cytokines comprising anti-inflammatory and proinflammatory interleukins (Frantz et al., 2020). Upon platelet activation in the event of an injury or inflammation, these proteins are secreted and delivered to the damaged tissues, thus participating in the healing process through chemotaxis and immune response and in cell proliferation and growth, angiogenesis, and collagen synthesis (Foster et al., 2009; Redler et al., 2011; Maleki-Hajiagha et al., 2020). With a local infusion of PC, the increased concentration of platelets yields an increase in GF concentration, thus acting directly on the local healing environment and enhancing the ability of diseased tissues to generate a repair response (Marx, 2004). In the context of reproductive applications, PDGF, TGF-β, and bovine (b-)FGF are particularly important because they regulate the inflammatory process and are essential for endometrial progenitor cell activity, with PDGF also being an isoform promoting endometrial stromal cell proliferation, migration, and contractility (van der Meijden and Heemskerk, 2019; Melo et al., 2020).

Lange-Consiglio et al. (2014) were the first to evaluate the effect of PC on the control of clinical acute and chronic cases of bovine mastitis. The authors showed that intramammary PC that yields 1×10^9 platelets/mL could quickly reduce the inflammatory response from infection, played a role in limiting the tissue damage to the mammary gland parenchyma, and allowed reduction of mastitis recurrence rates (Lange-Consiglio et al., 2014). Recently, intramammary PC administration was shown to be as effective as an antimicrobial agent to treat and prevent the recurrence of subclinical mastitis in dairy cattle (Dal et al., 2019). Similarly, PC has been investigated in treating bovine reproductive problems, including endometritis, repeat breeder syndrome, and ovarian hypofunction. Intrauterine PC administration has the potential to decrease bovine endometrial inflammation (Marini et al., 2016) and increase the percentage of pregnant cows from 33 to 70% from a repeat breeder cohort (Lange-Consiglio et al., 2015). Furthermore, intraovarian PC administration by ultrasound-guided injection using a transvaginal needle pushed through the fornix to enter the ovarian stroma to treat bovine ovarian failure (anestrus) improves ovarian function after 2 mo and allows fertility restoration by reducing follicular atresia (Cremonesi et al., 2020). Platelet concentrate was also shown to be effective in treating induced skin wounds and sole defects in cattle and has a beneficial effect on bovine articular cartilage and ligament healing (Tsuzuki et al., 2012; Petrera et al., 2013; Xie et al., 2015; Tambella et al., 2018).

Most PC preparations involve drawing venous blood followed by 1 or 2 centrifugations to obtain a plasma fraction of autologous blood with a platelet concentration above the baseline before centrifugation (Marx, 2001). Protocols that require only one centrifugation step during preparation have an advantage in speed of execution. In cattle, single centrifugation of citrated whole blood (**WB**) at a slow speed for a prolonged period (10 to 20 min) has only been described to produce bovine PC for transfusion purposes (Divers, 2005). Double-centrifugation processing methods have been described for PC production from bovine WB intended for clinical applications, but they are time consuming and require specialized laboratory equipment (Lange-Consiglio et al., 2014, 2015; Marini et al., 2016; Cremonesi et al., 2020). Many systems to derive PC from human WB using single centrifugation are commercially available, and the use of such systems has been reported in dogs and horses (Redler et al., 2011; Stief et al., 2011; Hessel et al., 2015; Franklin and Birdwhistell, 2018). Despite the growing evidence for use of PC in dairy cattle, a processing method using a single centrifugation technique easily feasible in a clinical or farm setting has not yet been validated for bovine WB. Because of these limiting factors for PC preparation using double-centrifugation methods and to have the prescribed amount of PC immediately available as soon as mastitis or another relevant clinical problem is diagnosed, PC is usually obtained from WB donor cow and used for heterologous treatment (Lange-Consiglio et al., 2014). The use of heterologous PC represents an inherent risk for herd health because it could lead to widespread hematogenous transmission of diseases such as bovine leukemia virus and bovine anaplasmosis, which are associated with significant economic losses (Bartlett et al., 2014; Zabel and Agusto, 2018).

Although standard protocols have been described for ACP in other species, we cannot extrapolate them to cattle because of differences in the sedimentation rate of bovine platelets (Clemmons et al., 1983). Indeed, sedimentation depends on relative difference between platelet and red blood cell (**RBC**) volumes, and bovine platelets are known to be small compared with those of dogs and horses. (Roland et al., 2014). Therefore,

the objectives of the present study were (1) to assess the feasibility of bovine autologous PC production, yielding a platelet concentration with healing modulation capabilities for clinical applications (defined as at least 1-fold enrichment or 1×10^9 platelet/mL) using a simple single-centrifugation technique with a commercially available preparation system, and (2) to compare cytokine and GF concentrations of bovine ACP obtained from 2 different single-centrifugation processing methods (varying in speed and time) that yielded the highest platelet-to-white blood cell (**WBC**) concentration ratio. We hypothesized that production of bovine ACP would enrich platelets at least 100% compared with WB or achieve a final platelet count of at least 1×10^9 platelets/mL optimized at a lower centrifugation gravitational force for the same amount of time than current protocols used for WB of horses and dogs and the same commercial system.

MATERIALS AND METHODS

Patients and Overview

The study group was composed of 12 nonlactating healthy intact Holstein female cows, with a mean age of 6.5 yr (range: 2.5–10 yr) and mean BW, estimated using a measuring tape, of 684 kg (range: 500–910 kg). The cows were randomly assigned to the pilot study group (n = 4 cows, 8 ACP protocols tested per cow) or the main study group (n = 8 cows). The research ethics committee approved the study at the institution at which the study was conducted (Université de Montréal, St-Hyacinthe, Québec, Canada). The study was performed according to the institutional guidelines for the care and use of animals (approval number 16-Rech_1827). The experiment was conducted in a farm and clinical setting and took part in 2 periods: (1) a pilot study to determine the best processing methods (speed and time of centrifugation) to obtain an ACP with at least 1-fold or 1×10^9 platelets/mL and with the highest platelet-to-WBC concentration ratio; and (2) the main study to compare cytokine and GF concentrations found in selected ACP. Sixteen processing methods were tested in the pilot study (n = 4 cows) and the results are available in the Appendix. The 2 processing methods chosen for the main study (n = 8 cows) were ACP-1 [$720 \times g$ (2,200 rpm; Rotofix 32A, Andreas Hettich GmbH) for 5 min] and ACP-2 [$929 \times g$ (2,500 rpm; Rotofix 32A, Andreas Hettich GmbH) for 3 min]. The sample size included in the main study (n = 8 per group) was based on power calculation using the mean platelet fold change in ACP compared with the WB baseline obtained during the pilot study. Based on a comparison of the standardized expected mean between the 2 processing methods chosen for the main study (1.65 and 1.92 for ACP-1 and ACP-2, respectively, with an anticipated SD of 10%, being 0.19), the sample size needed to compare 2 means for a 2-tailed test, using a power of 0.8 and a type I error rate of 0.05, was 8 per group.

Sampling and Processing

The main study took part on 5 days over 2 wk. Eight cows were enrolled to test the 2 processing methods determined in the pilot study. Forty-five milliliters of blood was drawn by venipuncture of the jugular vein with a 14-gauge over-the-needle type catheter attached to a 16-cm extension set using standard aseptic preparation and technique. All processing methods (centrifugations) were performed within 30 min following blood collection, and all samples were processed and analyzed within 4 h. Blood was drawn over 3 min (not faster than 1 mL/2 s) and collected into glass tubes or syringes suitable for further processing depending on the procedures performed.

WB and ACP Preparation

For WB analysis, 20 mL of the blood drawn was collected into glass tubes containing EDTA. Fifteen milliliters of blood was collected for both ACP samples to be produced using a commercial system (Arthrex Autologous Conditioned Plasma Double Syringe System; Arthrex). The syringes were prefilled with 1.5 mL of acid citrate dextrose-A (**ACDA**) solution (citrate-to-blood ratio of 1:10) and centrifuged at room temperature with a benchtop (Rotofix 32A; Andreas Hettich GmbH) and centrifuge system (Centrifuge 5810 R; Eppendorf) recommended by the manufacturer. The 2 processing methods with the highest platelet-to-WBC ratio from the pilot study (see Appendix) and used during the main study were ACP-1 [$720 \times g$ (2,200 rpm; Rotofix 32A, Andreas Hettich GmbH); 5 min] and ACP-2 [$929 \times g$ (2,500 rpm; Rotofix 32A, Andreas Hettich GmbH); 3 min]. Following ACP syringe centrifugation and separation of red blood cells from the platelet-containing plasma solution, the supernatant was carefully drawn using the smaller inner syringe. Because of the thick buffy coat and poor separation of plasma and RBC (Figure 1), the plunger of the smaller syringe was pulled until a flash of blood was obtained, usually corresponding to when the plunger was within 1 to 2 mL away from the buffy coat layer. All ACP samples were placed on an oscillating blood mixer for $\geq$5 and <60 min to ensure adequate and even suspension of the platelets within the plasma before further analysis.

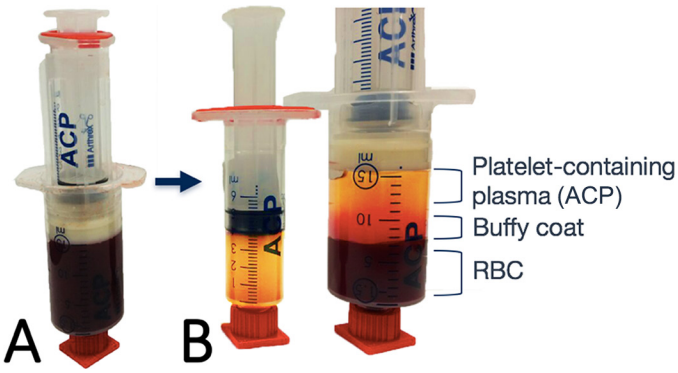

Figure 1. Autologous conditioned plasma (ACP) production from bovine whole blood using a commercial double-syringe system (Arthrex Autologous Conditioned Plasma Double Syringe System; Arthrex) and a single-step centrifugation procedure. (A) Whole blood is collected with acid citrate dextrose-A (ACDA) solution as an anticoagulant (citrate to blood ratio of 1:10) and centrifuged at 929 × *g* (2,500 rpm; Rotofix 32A, Andreas Hettich GmbH) for 3 min. (B) Three layers were obtained: red blood cells (RBC), a buffy coat layer, and platelet-containing plasma (ACP). In most samples, the buffy coat was reddish and had greater volume compared with the ACP layer.

Hematologic Evaluation and Efficiency of Platelet Collection

Platelet, RBC, and WBC counts were measured in 3.5 mL of WB from each cow as baseline values and in each ACP end-product (0.5-mL samples) immediately following their preparation using an optical scatter analyzer (Advia 120; Siemens Healthcare Diagnostics). Accurate readings were confirmed by blood smear by a diplomate of the American College of Veterinary Pathologists (C. G.-B.). The analyses were performed at the Service de Diagnostic at the Faculty of Veterinary Medicine of the Université de Montréal. Manual counts of platelet concentration were performed on all ACP samples using the Thrombo-TIC system (Thrombo-TIC, Bioanalytic GmbH) following the manufacturer's instructions for use with PRP. All counts were performed blindly by the same individual (C. C.). An average of manually counted platelets from both counting chambers at 400× magnification was calculated and used for analysis. Because of several aberrant decreased platelet counts from automated platelet counts in ACP samples compared with manual counts (mean decrease of 56%), platelet counts were provided for statistical analysis from the automated cell analyzer for WB and from the Thrombo-TIC for ACP. The efficiency of platelet collection was calculated for each ACP produced. The following formula was used to calculate efficiency: platelet concentration in ACP samples times the volume of ACP obtained, divided by the product of the platelet concentration in WB by the volume of WB used (fixed at 15 mL; Weibrich et al., 2005).

GF and Cytokine Concentration

One milliliter of WB was used for baseline measurements, and 0.5 mL of each ACP produced was cryopreserved at −80°C for later analysis. Concentrations of PDGF, TGF-β, b-FGF, and IL-1β were determined after 1 freeze-thaw cycle for platelet GF release (Textor and Tablin, 2012). Frozen samples were thawed at 37°C before analysis, and concentrations of GF and cytokines were determined using a competitive ELISA following the manufacturer's instructions (ELISA kits; Neobiolab). Samples were measured in duplicate and appropriately diluted to fit the respective calibration curves. The analyte concentration was determined from its absorbance value, plotted on the vertical y-axis (1/absorbance), and the concentration of the standards, plotted on the horizontal x-axis. All kits were validated for bovine blood and plasma and, therefore, ACP contents. The ELISA plates were read on an absorbance reader (Synergy HT Absorbance reader; Biotek) and analyzed using Gen5 software (version 1.11; Biotek).

Statistical Analysis

All statistical analyses were conducted using SAS software (version 9.2; SAS Institute Inc.). Descriptive statistics and measurements of central tendency and dispersion were provided. Appropriate transformation (logarithm) was performed for raw leukocyte counts, mononuclear counts, and IL-1β concentration to achieve more homogeneity before further assessment. Linear mixed-effects models were used to evaluate the significance of association between the studied factors (platelets, RBC, WBC, GF, cytokine) and different samples (WB, ACP-1, ACP-2). In every model, the level of primary interest was fixed as the type of the sample considering the random variance of the donor cow (subject effect). Post hoc multiple comparisons were performed following linear modeling using the adjusted Tukey test, with $P < 0.05$ considered significant.

RESULTS

Hematologic Evaluation and Enrichment Factors

The complete cellular concentrations and enrichment factors are given in Table 1 and Figure 2. Both processing methods yielded platelet enrichment >1-fold and platelet count >1 × 10^9 platelets /mL. Mean platelet count increased with both processing methods, with counts >1 × 10^9 platelets /mL. Their enrichment factor was significantly increased (more than 100% compared with baseline) in both ACP samples compared with WB ($P = 0.034$). The second ACP processing method

Table 1. Cellular concentrations (mean ± SD) in whole blood (WB) and autologous conditioned plasma (ACP) obtained and prepared using a commercially available system[1]

Analyte	Baseline (WB)	ACP-1	ACP-2	*P*-value[2]
Platelets ($\times 10^9$ cells/L)	252.25 ± 127.34*	383.56 ± 139.87*	422.63 ± 84.70*	0.42
% change relative to WB	—	124.5 ± 0.09	168.6 ± 0.09	0.001
RBC ($\times 10^9$ cells/L)	6.34 ± 0.59*	0.08 ± 0.03*	0.11 ± 0.03*	0.18
% change relative to WB	—	1.25 ± 0.01*	1.66 ± 0.00*	0.0001
WBC ($\times 10^9$ cells/L)	7.01 ± 2.90*	0.54 ± 0.28*	0.69 ± 0.37*	0.83
% change relative to WB	—	7.53 ± 0.02	9.75 ± 0.04	0.08
Mononuclear cells ($\times 10^9$ cells/L)	3.96 ± 1.46	0.51 ± 0.27	0.69 ± 0.36	0.09
Percentage of all WBCs	—	12.04 ± 0.04	15.91 ± 0.06	0.41
Efficiency platelet concentration	—	29.6 ± 0.04	41.9 ± 0.04	0.07
End-product volume (mL)	—	4.6 ± 0.2	4.2 ± 0.3	0.61

[1]The ACP processing methods were 720 × *g* (2,200 rpm; Rotofix 32A, Andreas Hettich GmbH) for 5 min for ACP-1 and 929 × *g* (2,500 rpm) for 3 min for ACP-2. RBC = red blood cells; WBC = white blood cells.

[2]*P*-values represent comparisons between the 2 processing methods.

*$P < 0.05$: significant difference between WB and ACP samples.

(ACP-2: 929 × *g* for 3 min) yielded a significantly ($P < 0.001$) greater degree of platelet enrichment (169% vs. 125%) than did ACP-1 (720 × *g* for 5 min). The efficiency of platelet concentration did not differ ($P = 0.07$) between the 2 processing methods (41.9% vs. 29.6%). Both ACP samples showed a decrease in absolute WBC counts (0.54 and 0.69 × 10^9 cells/L for ACP-1 and ACP-2, respectively) compared with WB (7.01 × 10^9 cells/L). Neutrophil, lymphocyte, and monocyte counts were reduced similarly. Both ACP samples showed a decrease in RBC counts (0.08 and 0.11 × 10^9 cells/L for ACP-1 and ACP-2, respectively) compared with WB (6.34 × 10^9 cells/L). The decreases in RBC and WBC counts were significant ($P < 0.001$). The ACP-2 protocol had lower ($P = 0.002$) RBC enrichment than the ACP-1 protocol (1.25% vs. 1.66%)

GF and Cytokine Concentrations

Growth factors and cytokine concentrations with their enrichment factors are presented in Table 2 and Figure 3. After processing, the concentration of PDGF was higher ($P = 0.01$) in ACP than in WB and enriched (124–125%); neither b-FGF nor TGF-β was enriched in ACP samples, and their concentrations were similar to those of baseline WB ($P = 0.54$ and 0.48). Concentrations of GF following the ACP protocols were not different (PDGF, $P = 0.92$; b-FGF, $P = 0.78$; TGF-β, $P = 0.32$). Interleukin-1β was marginally enriched (<8% increase) in ACP, and its concentration was significantly higher in ACP-2 than at baseline ($P = 0.04$).

DISCUSSION

This study presents novel and complementary information regarding the production of PC intended as a potential treatment for mastitis and reproduction problems in cattle. We explored a fast and reliable way to produce ACP from bovine WB using a single centrifugation technique intended for clinical applications. The tested production protocols were successful and feasible in a clinical or farm setting. Both autologous ACP protocols tested were suitable for clinical applications because they produced a PC with at least 1 × 10^9 platelet/mL and decreased WBC. The second ACP protocol (929 × *g* for 3 min) may be the more suitable method because it yielded a higher platelet count. In contrast to our hypothesis, higher centrifugation gravitational forces than are typically used in other animal species were required.

The protocols established in our study required only one centrifugation step during PC preparation and held the advantage of speed applicable to a farm or clinical setting (Kisiday et al., 2012). Additionally, no outsourced processing was needed, and the required modifications to make an optimal product for clinical application were limited and easy to perform. No special training was required to carry out sampling or perform the procedures. Animal technicians or personnel could easily be taught to perform the technique, and the small overall dimensions of the centrifuge facilitate production entirely at the farm. Although the blood sampling for ACP production should be done under good hygienic conditions, the immediate use of the produced ACP without storage may make it a safer biological product for farm use because the risk of bacterial growth in case of sample contamination is extremely unlikely. If done in a clinical setting, the use of the proposed commercial system and centrifuge can be attractive to practitioners working in a mixed animal practice, as the system was previously validated for ACP production from equine and canine blood (Stief et al., 2011; Hessel et al., 2015; Franklin and Birdwhistell,

Table 2. Mean ± SD concentration (pg/mL) of different growth factors and cytokines[1] in whole blood (WB) and autologous conditioned plasma (ACP) obtained and prepared using a commercially available system after platelet (PLT) activation by use of the freeze-thaw method[2]

Analyte	Baseline (WB)	ACP-1	ACP-2	*P*-value
PDGF (pg/mL)	45.197 ± 10.236*	57.184 ± 19.778*	57.251 ± 17.975*	0.99
% change relative to WB	—	125 ± 24	124 ± 17	0.93
Concentration normalized to PLT count	0.218 ± 0.118	0.158 ± 0.057	0.142 ± 0.058	0.97
TGF-β (pg/mL)	154.614 ± 58.517	163.298 ± 66.220	164.383 ± 67.443	0.94
% change relative to WB	—	100 ± 17	95 ± 36	0.32
Concentration normalized to PLT count	0.699 ± 0.468	0.464 ± 0.233	0.413 ± 0.202	0.95
b-FGF (pg/mL)	128.806 ± 26.701	119.548 ± 54.307	133.149 ± 39.209	0.77
% change relative to WB	—	104 ± 12	102 ± 15	0.78
Concentration normalized to PLT count	0.612 ± 0.268	0.342 ± 0.203	0.339 ± 0.183	1.00
IL-1β (pg/mL)	51.008 ± 17.311*	66.068 ± 35.185	80.474 ± 55.772*	0.36
% change relative to WB	—	74 ± 42	56 ± 51	0.96
Concentration normalized to PLT count	0.008 ± 0.004	0.005 ± 0.002	0.005 ± 0.001	0.95

[1]PDGF = platelet-derived growth factor; TGF-β = transforming growth factor-β; b-FGF = bovine fibroblast growth factor.

[2]The ACP processing methods were 720 × *g* (2,200 rpm; Rotofix 32A, Andreas Hettich GmbH) for 5 min for ACP-1 and 929 × *g* (2,500 rpm) for 3 min for ACP-2. RBC = red blood cells; WBC = white blood cells.

[3]*P*-values represent comparisons between the 2 processing methods.

*$P < 0.05$: significant difference between WB and ACP samples.

2018). Additionally, the new PC protocols in this study mean that the prescribed amount of autologous PC is immediately available for use as soon as mastitis or other clinically relevant problems are diagnosed. This negates the need to use previously processed and stored heterologous PC obtained from lengthy double-centrifugation technique using WB from a donor cow (Lange-Consiglio et al., 2014), which has inherent risks such as hematogenous diseases transmission or unwanted inflammatory reaction (Bartlett et al., 2014; Zabel and Agusto, 2018). In addition to being slower to perform, double-centrifugation techniques require specialized equipment, including a larger centrifuge compared with the countertop model used in this study.

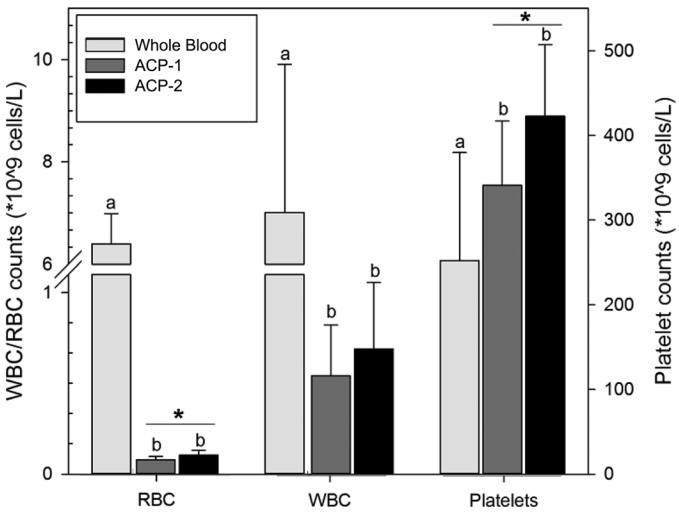

Figure 2. Concentrations of red blood cells (RBC), white blood cells (WBC), and platelets in whole blood and autologous conditioned plasma (ACP) obtained and prepared during the main study (n = 8 cows) using a double syringe system. The ACP processing methods were 720 × *g* (2,200 rpm; Rotofix 32A, Andreas Hettich GmbH) for 5 min for ACP-1 (protocol #13 in the pilot study) and 929 × *g* (2,500 rpm) for 3 min for ACP-2 (protocol #14 in the pilot study). Bars represent the mean ± SD. Letters (a, b) indicate a significant difference between the nature of the sample; * indicates a significant difference between the 2 processing methods in enrichment of the respective cellular component in ACP samples ($P < 0.05$; mixed-model linear regression).

For the same centrifugation time, the ideal centrifugation speed was almost twice that currently recommended by the manufacturer for ACP production from canine (1,300 rpm; Rotofix 32A, Andreas Hettich GmbH) and equine (1,100 rpm; Rotofix 32A, Andreas Hettich GmbH) WB for platelet enrichment. Size and density differences in cellular blood elements necessitate specific separation procedures. Bovine platelet aggregation generally requires longer centrifugation times and lower gravitational forces than that needed for other species (Clemmons et al., 1983). Despite this, the lower speeds in this study resulted in suspension of a large quantity of RBC in the plasma portion. To ensure production of a good ACP end-product, the platelets need to be concentrated but the RBC concentration should also be decreased (Magalon et al., 2016). A PC is considered very pure if the percentage of platelets in the PC is more than 90% compared with RBCs and leukocytes (Magalon et al., 2016). Blood with a higher erythrocyte sedimentation rate requires longer for the WBC and other components to leave the plasma and form a sediment in response to centrifugal force. Consequently, bovine blood requires greater centrifugation force to separate its blood components compared with equine or canine blood.

Platelet concentration (384×10^9 to 423×10^9/L) and collection efficiency (30 to 42%) were superior to those achieved with double-centrifugation protocols

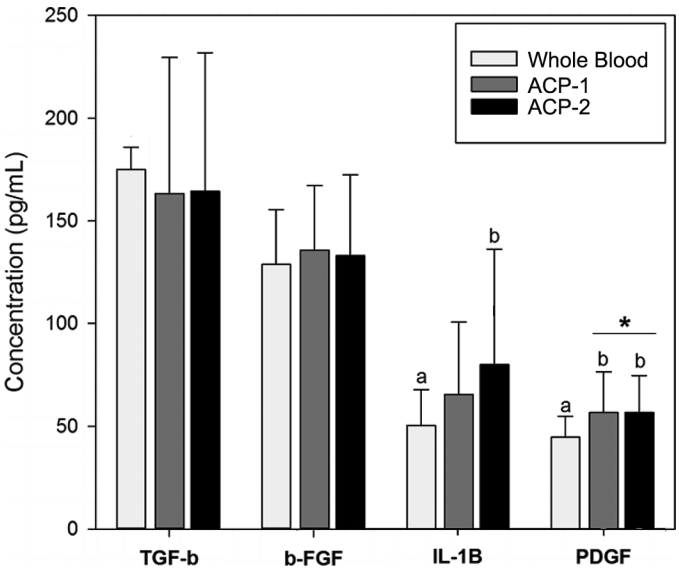

Figure 3. Concentration of platelet-derived growth factor (PDGF), transforming growth factor-β (TGF-β), bovine fibroblast growth factor (b-FGF), and IL-1β in whole blood and autologous conditioned plasma (ACP) obtained and prepared during the main study (n = 8 cows) using a double syringe system after platelet degradation by use of the freeze-thaw method. The ACP processing methods were 702 × *g* (2,200 rpm; Rotofix 32A, Andreas Hettich GmbH) for 5 min for ACP-1 (protocol #11 in the pilot study) and 929 × g (2,500 rpm) for 3 min for ACP-2 (protocol #14 in the pilot study). Bars represent the mean ± SD. Letters (a, b) indicate a significant difference between the nature of the sample; * indicates a significant difference between the 2 processing methods in enrichment of the respective cellular component in ACP samples ($P < 0.05$; mixed-model linear regression).

previously used in cattle with successful clinical applications (Lange-Consiglio et al., 2014, 2015; Marini et al., 2016; Cremonesi et al., 2020). The bovine ACP produced here had a similar platelet enrichment factor (125 to 169%) to that reported in humans (199%), dogs (100%), and horses (130%) using the same commercial system (Redler et al., 2011; Stief et al., 2011; Hessel et al., 2015; Franklin and Birdwhistell, 2018). Although a lack of consensus might exist concerning the therapeutic platelet concentration across clinical applications and species, PRP can be defined as "an autologous liquid clot with an injectable consistency, which has platelets above the baseline value of whole blood and supraphysiological doses of growth factors and cytokines" (Arora and Arora, 2021). According to this definition, the clinically useful product is a concentration of platelets in a small plasma volume, which implies that all ACP samples produced during this study met the definition for PRP. In addition, processing methods resulting in platelet concentrations similar to baseline and reduction of other blood components showed positive healing in vivo in clinical bovine patients (Lange-Consiglio et al., 2014, 2015; Marini et al., 2016; Cremonesi et al., 2020). The volume of ACP produced with one syringe system (4.6 to 4.2 mL) was slightly below the previously used PC volume (5 mL) for in vivo treatment in cattle (Lange-Consiglio et al., 2014). However, the higher platelet concentration in the ACP produced in this study would allow for an increased absolute number of platelets to be delivered and should remain therapeutic. Nevertheless, the ease of ACP production with the tested protocols allows the use of multiple syringe systems from the same blood draw and centrifugation of up to 4 syringes simultaneously, thus producing more than the required amount of ACP for a single treatment without significantly increasing production time.

The main therapeutic properties of ACP come from the GF released by activated platelets. The ACP produced in the current study yielded a moderate increase in PDGF (125%) compared with WB. The effects of PDGF on healing mainly result from its ability to induce selective mitosis of multiple cell types such as mesenchymal cells, osteoblasts, fibroblasts, and smooth muscle cells; regulate collagenase secretion and collagen synthesis; and affect chemotaxis of neutrophils and macrophages (Gonçalves et al., 2020). For reproductive problems, the local application of PDGF via ACP treatment can play a significant role in the healing of uterine tissue as it is essential for endometrial progenitor cell activity and is an isoform promoting endometrial stromal cellular proliferation, migration, and contractility (Matsumoto et al., 2005; Gargett et al., 2008; Aghajanova et al., 2018). Although only mild to no changes in TGF-β and b-FGF concentrations compared with WB baseline were found, local delivery may still be sufficient to allow positive healing and promote an anti-inflammatory response (Foster et al., 2009; Redler et al., 2011; Maleki-Hajiagha et al., 2020). More specifically, the relevant effect of TGF-β and b-FGF on tissue regeneration includes the proliferation and differentiation of different cell lineages and mitogenic cellular regulation (Gonçalves et al., 2020). Nevertheless, the manufacturer reports that enrichment of GF in humans can reach 25 times baseline values. Therefore, enrichment of GF of bovine ACP was lower than expected. Bias in ELISA testing performance, different GF release kinetics in bovine blood, or early platelet activation during processing may explain the unexpectedly low GF enrichment. However, similar results were observed in studies with the same syringe system using canine and equine WB (Hessel et al., 2015; Mageed et al., 2015; Sawyere et al., 2016). The enrichment factor of TGF-β of bovine ACP was lower than that reported in dogs in a different study using the same syringe system (Franklin and Birdwhistell, 2018). Growth factor enrichment from ACP compared with other PRP protocols using human WB also shows a tendency toward

lower enrichment (Oudelaar et al., 2019). In this study, deep freezing was used for intracellular platelet GF release but this may have been suboptimal compared with other activation techniques (Pesonen et al., 1989). Canine ACP intentionally activated with $CaCl_2$ or thrombin has a significant effect on TGF-β1, resulting in higher concentrations than obtained in samples that are not activated (Franklin and Birdwhistell, 2018). Despite in vitro GF results in ACP similar to those of blood, in vivo GF concentrations could be sufficient for healing modulation. Following contact of platelets with tissue collagen, activation and degranulation occur, followed by GF release in the surrounding environment and further stimulation of platelet aggregation (Pesonen et al., 1989).

Interleukin-1β was mildly increased in ACP compared with baseline. This is an expected finding when preparing autologous plasma with a low WBC concentration. Canine ACP shows similar IL-1β concentrations between ACP and serum (Sawyere et al., 2016). Interleukin-1β is a potent proinflammatory cytokine produced by activated macrophages. Its purpose in the study protocol was to serve as a positive inflammatory control, thus differentiating platelet-related and leukocyte enrichment. These results should be interpreted cautiously because activated platelets may contribute to in vivo expression of various inflammatory mediators, such as IL-1β, on endothelial cells (Cha et al., 2000).

Among the limitations of this study, the use of different anticoagulants (EDTA for WB, ACDA for PC) may have affected platelet counts and aggregation (Stokol and Erb, 2007). The ability to adequately enumerate platelets within the prepared ACP products was not established in cattle. The use of different methods of platelet enumeration for WB (automated cell counter) compared with ACP (Thrombo-TIC) represents the main variability of the study (O'Shea et al., 2015). The use of the Thrombo-TIC system was based on aberrant cell counts in ACP from the automated cell counter, which might be explained by the small size of cattle platelets or their inadequate sphering (O'Shea et al., 2015). The ACP protocol proposed in this study was solely harvesting platelets from WB to create a portion of the plasma fraction that has a platelet concentration above baseline. This aspect is vital because the presence of platelets in the end-product does not necessarily imply that the platelets will become activated in vivo and release their stored polypeptides (GF and cytokines). Exogenous platelet activation of ACP products for clinical application can be achieved by adding thrombin, $CaCl_2$, or endogenous type I collagen to the solution (DeLong et al., 2012). However, activation of the end-product was not recommended by the manufacturer when this study was conducted and was therefore not performed.

CONCLUSIONS

The most efficient processing method to produce ACP using a commercially available system using bovine WB is 929 × *g* for 3 min. This fast and straightforward processing method produced ACP with moderate platelet enrichment, sufficient for clinical application, and significant WBC dilution to overcome the limitations from double-centrifugation methods requiring specialized laboratory equipment. Platelet concentrate could become more widely available in clinical or farm settings to treat mastitis and reproductive problems and ameliorate the negative economic impact of these diseases in dairy cattle. In addition, this method can help create treatment options for some common cattle diseases for which platelet concentrate has been proven useful.

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APPENDIX: PILOT STUDY

Material and Methods

During the pilot study, each cow (n = 4) was handled 2 times at 24-h intervals to obtain WB for producing 8 ACP samples from different processing methods. A total of 80 mL of blood was drawn by venipuncture from the jugular vein as described in the main text. Preparation of WB and ACP was performed as described in the main text. The 16 processing methods used during the pilot study are described in Table A1. Hematologic evaluation (platelet, RBC, and WBC counts) and platelet collection efficiency were assessed as described in the main text; GF and cytokine concentrations were not measured during the pilot study.

Results

Not all processing methods tested achieved increased platelet concentrations compared with their WB baseline. The complete cellular concentrations from the pilot study are reported in Table A2 and Table A3. The ACP production protocol that yielded the highest platelet:WBC ratio in cattle was 720 × *g* (2,200 rpm, based on the maximum radius of the rotor and carrier; Rotofix 32A, Andreas Hettich GmbH) for 5 min (protocol #11). A faster protocol, 929 × *g* (2,500 rpm, based on the maximum radius of the rotor and carrier) for 3 min (protocol #14), yielded the highest efficiency of platelet collection. These 2 processing methods were chosen for the second part of the study and were identified as ACP-1 and ACP-2, respectively.

Table A1. Processing methods (centrifugation speed and time using Rotofix 32A, Andreas Hettich GmbH) performed during the pilot study (n = 4 cows) to produce autologous conditioned plasma (ACP) using a commercially available system[1]

Protocol no.	Speed (rpm)	Speed (× *g*)	Time (min)
1	500	37	10
2	700	73	5
3	1,000	150	5
4	1,300	250	10
5	1,500	335	5
6	1,500	335	10
7	1,700	430	5
8	1,700	430	10
9	2,000	595	4
10	2,000	595	5
11	2,200	720	5
12	2,300	790	3
13	2,500	929	2
14	2,500	929	3
15	2,500	929	5
16	3,000	1,340	2

[1]Autologous Conditioned Plasma Double Syringe System (Arthrex).

Table A2. Cellular concentrations (mean ± SD) of standard plasma and autologous conditioned plasma (ACP) processing methods (pilot study; 4 cows, 16 processing methods for a total of 32 samples) compared with whole blood (WB) baseline using a commercially available system[1]

	Cellular concentration ($\times 10^9$/L)		
Item	Platelets	White blood cells	Red blood cells
Baseline (WB)	240 ± 50	7.25 ± 1.3	6.7 ± 0.6
Control plasma	38 ± 7	0.008 ± 0.005	0.01 ± 0.005
ACP samples	292 ± 105	2.17 ± 2.1	1.9 ± 2.1

[1]Autologous Conditioned Plasma Double Syringe System (Arthrex).

Table A3. Comparison of different tested autologous conditioned plasma processing methods performed during the pilot study (n = 4 cows) using a commercially available system[1,2]

Protocol no.	Cellular ratio			Efficiency of platelet collection
	PLT	WBC	[PLT]:[WBC]	
1	0.94	0.83	34	0.00
2	0.82	0.89	28	0.00
3	0.82	0.79	32	0.00
4	1.20	0.11	439	0.26
5	1.22	0.45	86	0.26
6	1.23	0.10	459	0.36
7	0.89	0.09	376	0.25
8	1.00	0.07	499	0.27
9	1.31	0.27	144	0.35
10	1.16	0.17	174	0.36
11[3]	1.65	0.09	644	0.46
12	1.55	0.21	209	0.41
13	1.65	0.26	243	0.29
14[3]	1.92	0.19	380	0.51
15	0.93	0.06	765	0.36
16	1.20	0.14	250	0.33

[1]Autologous Conditioned Plasma Double Syringe System (Arthrex).

[2]Fold change in platelet (PLT) and white blood cells (WBC) in platelet concentrate compared with baseline (WB). Efficiency of platelet collection refers to the platelet concentration in ACP samples multiplied by the volume of ACP obtained, divided by the product of the platelet concentration in WB by the volume of WB used (fixed at 15 mL). [PLT]:[WBC] refers to the concentration ratio of PLT to WBC.

[3]Protocols were subsequently reproduced and compared using WB collected from 8 cows during the main study.