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Influence of haematological parameters on size of the advanced platelet-rich fibrin+ (A-PRF+) in the horse

Montano Chiara [a], de Chiara Mariaelena [a,*], Crisci Alessandro [b], De Biase Davide [c], Ciuca Lavinia [d], Maurelli Maria Paola [d], Lamagna Barbara [a], Del Prete Chiara [e], Fabiana Flagiello [f], Pasolini Maria Pia [a]

[a] Department of Veterinary Medicine and Animal Production, Unit of Surgery, University of Naples "Federico II", Via Federico Delpino 1, 80137 Naples, Italy
[b] Unit of Dermosurgery, Cutaneous Transplantation and Hard-To-Heal Wound, "Villa Fiorita" Private Hospital, Via Filippo Saporito, 24, 81031 Aversa (CE), Italy
[c] Department of Pharmacy University of Salerno, Via Giovanni Paolo II 132, 84084 Fisciano (SA), Italy
[d] Department of Veterinary Medicine and Animal Production, University of Napoles Federico II, CREMOPAR, WHO Collaborating Centre ITA-116, Naples, Italy
[e] Department of Veterinary Medicine and Animal Production, Unit of Animal Reproduction, University of Naples "Federico II", Via Federico Delpino 1, 80137 Naples, Italy
[f] Analysis Laboratory "Villa Fiorita" Private Hospital, Aversa (CE), Italy

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ABSTRACT

The advanced-PRF+ (A-PRF+) is a platelet concentrate, showing a higher concentration of growth factors, an increased number of cells and looser structure of the fibrin clot than leukocyte-PRF. A high variability in the size of PRF associated with patients, haematological features and centrifugation protocols was reported. The aims of this study were to evaluate the feasibility of A-PRF+ production in the field and the correlation between haematological parameters, macroscopic and microscopic features in equine A-PRF+. Samples from twenty Standardbred horses (3–7 years) were harvested with glass tubes without anticoagulants, previously heated at 37 °C. Blood samples were centrifugated at 1300 rpm for 8 min with a fixed-angle centrifuge and a horizontal centrifuge in the field, at a temperature of 15–17 °C. Clots were measured and placed on the Wound Box® for a 2-min compression. Membranes were measured and fixed in 10% formalin for histological examination. Clot and membrane surface did not differ between sex and centrifuge. Haematological parameters did not show a significant correlation to clot and membrane size. Membranes obtained from both centrifugation protocols showed a loose fibrin structure and cells evenly distributed throughout the clot. Tubes' warming was effective to obtain A-PRF+ clots from all samples, regardless the environmental temperature. Further studies are needed to evaluate the influence of other blood molecules on the A-PRF+ structure and size.

1. Introduction

Platelet-rich fibrin (PRF) is a second-generation platelet-derived product developed by Choukroun and colleagues in 2001. Whole blood is centrifuged without anticoagulants, to obtain a fibrin clot rich in growth factors (GFs) such as platelet-derived growth factor (PDGF), transforming growth factor β1 (TGF- β1), vascular endothelial growth factor (VEGF), endothelial growth factor (EGF), and leukocytes. These factors modulate the inflammatory response and enhance the healing processes (Masuki et al., 2016; Naik et al., 2013). The three-dimensional fibrin matrix allows a gradual and slow release of GFs (Kobayashi et al., 2016), promotes haemostasis and increases cell migration and proliferation, while supporting the immune system (Crisci et al., 2019a, 2019b).

Due to the widespread use of PRF in human medicine, new platelet concentrates based on different production processes, such as the advanced-PRF (A-PRF), have been developed. The A-PRF is produced from centrifugation of whole blood at 1500 rpm for 14 min and exhibits a higher concentration of GFs, an increased number of cells, and a looser structure with wider interfiber space compared to L-PRF (Crisci et al., 2020; Jayadevan et al., 2021). A further increase of the concentration of GFs, leukocytes and platelets is present in A-PRF+, developed by Ghanaati et al. (2014), changing the centrifugation protocol, at 1300 rpm for 8 min (Ghanaati et al., 2014).

* Corresponding author.
E-mail address: mariaelena.dechiara@unina.it (C. Mariaelena).

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The A-PRF and A-PRF+ are commonly used in human medicine for the treatment of diabetic foot ulcers (Crisci et al., 2019a, 2019b), in oral surgery (Liu et al., 2022; Nowak et al., 2021; Selahi et al., 2022) and in plastic surgery (Kovacevic et al., 2021).

Wounds are very common in equine patients (Caston, 2012), and, among many therapeutic options, autologous products have demonstrated to modulate tissue healing in horses (Harman et al., 2023). To date, the ability of A-PRF+ to promote the tissue healing in horses has not been studied. Given that equine wounds often involve a significant amount of traumatized tissue (Caston, 2012), understanding the final dimensions of the fibrin clot is useful to ensuring that the A-PRF+ membrane adequately covers the entire affected area. Therefore, gaining insights into parameters that can predict the final dimensions of A-PRF+ may be interesting.

Few studies have reported a high variability in morphology and size of PRF clots and membranes, linked to various variables. These include patients' gender, age, centrifuge characteristics and centrifugation protocols and whole blood features (Dohan Ehrenfest et al., 2018; Miron et al., 2019a). In authors knowledge, there are no published studies concerning correlations between A-PRF+ size and multiple parameters in horses.

For these reasons, the aim of this study was to evaluate the correlation between macroscopic and microscopic features of A-PRF+, patients' sex and haematological features in equine A-PRF+, obtained with two different centrifuges. Moreover, we evaluate the feasibility of A-PRF+ membrane production in the field.

2. Materials and methods

2.1. Animals

The study was conducted on 20 sport horses, housed at the Hippodrome of Agnano (Naples, Campania, Italy). The clinical and vaccine histories of the animals were thoroughly assessed and all horses underwent a general clinical examination.

Informed consent was obtained from each owner. The study was conducted in compliance with the protocol, reviewed and approved by the Ethical Animal Care and Use Committee of the University of Naples Federico II (OPBA - protocol number PG/2022/0119337).

2.2. Sample collection, centrifugation protocol and A-PRF preparation

The research was carried out in November 2022. The environmental temperature was assessed with a digital thermometer (TP-65, iTronics, GmbH, Wilich, Germany). If the temperature was lower than 21 °C, the tubes for blood samples were heated at 37 °C for 15 min in a thermostat (MINI ASAL BATT MOD. 805, Cernusco sul Naviglio (MI) - Italy). Whole blood was sampled from the left jugular vein of all the horses using a vacutainer system (BD Vacutainer Safety-Lok-Blood Collection Set 21Gx3/3"x7", Becton Dickinson and Company) and collected in two 10-ml sterile glass tubes without anticoagulants (A-PRF tubes Process for PRF™, Nice, France; Mectron, Cologne, Germany) and in two 9 ml K3E 5.4 mg EDTA tube (VacuMed). The EDTA tubes were stored for the blood count (CBC) and PCR for detection of equine piroplasmosis, while each glass tube was centrifuged within 2 min of sampling using either a fixed-angle centrifuge (FC) (Duo centrifuge, Process for PRF™, Nice, France; Mectron, Cologne, Germany) or a horizontal centrifuge (HC) (5702 Eppendorf, Hamburg, Germany). Tubes were centrifuged at 1300 rpm for 8 min for production of A-PRF+, as previously described (Fujioka-Kobayashi et al., 2017). At the end of the centrifugation process, three distinct layers were visible in the tube: a red clot, comprising red blood cells at the bottom, the platelet-free plasma at the top, and the fibrin clot in the middle. Subsequently, all clots were carefully extracted from the tubes and separated from the red blood cell portion using sterile tweezers and scissors. A-PRF+ clots were weighed in grams using an electronic scale (TP-500, DIPSE, Germany) and height, width, and length were measured in millimetres using a digital caliper (Newaner, Shenzhen, China). The clots were then placed on the Wound Box® for a 2-min compression, after which the weight and dimensions of the resulting membrane were recorded. The surface areas (mm^2) of both clots and the membranes were measured with the Calcderm measurement software, developed by Crisci and colleagues (Crisci et al., 2014). Following the measurements, the membranes were fixed in 10% neutral buffered formalin. All procedures were consistently performed by the same experienced practitioner to minimize interpersonal variations.

2.3. Blood analysis

The blood samples in EDTA underwent a CBC, where the following parameters were evaluated: total red blood cells (RBCs), packed cell volume (PCV), haemoglobin (Hb), mean corpuscular volume (MCV), mean corpuscular haemoglobin concentration (MCHC), red cell distribution width (RDW), white blood cells (WBCs), total neutrophiles and percentage, total lymphocytes and percentage, total monocytes and percentage, total eosinophils and percentage, total basophiles and percentage, platelet count (PC), mean platelet volume (MPV) and plateletcrit (PCT). The CBCs were performed using a ProCyte Dx® Haematology Analyser (IDEXX Laboratories, Inc.) based on laser flow cytometry.

2.4. DNA extraction and PCR amplification for diagnosis of equine piroplasmosis

For the diagnosis of equine piroplasmosis, caused by *Theileria equi* and *Babesia caballi,* blood samples in EDTA of all the enrolled animals undergone polymerase chain reaction test (PCR). DNA extraction was performed from each tube using the QIAamp Blood & Tissue Kit (Qiagen, Germany), according to the manufacturer's instructions. The DNA samples were stored at −20 °C until amplification. A multiplex PCR protocol amplifying the 18S ribosomal RNA gene was used to identify *Babesia caballi* and *Theileria equi* positive samples (Alhassan et al., 2005). A 50 μl of PCR reaction mix was prepared, containing 1× EmeraldAmp MAX PCR Master Mix (Takara, Japan), 2.5 pmol of primers Bec-UF2, Cab-R, Equi-R and 3 μl of template DNA. The PCR thermal profile has provided an initial denaturation at 96 °C for 10 min, followed by 40 cycles at 96 °C for 60 s, 60.5 °C for 60 s e 72 °C for 60 s and terminated with a final extension at 72 °C for 10 min. The amplified products were detected on a 1.5% ethidium bromide-stained low melting agarose gel (BIO-RAD, Spain).

2.5. Histopathologic evaluation

Membranes were preserved in 10% neutral buffered formalin (code no. 05-01007Q, Bio-Optica, Milan, Italy), dehydrated and embedded in paraffin (code no. 06–7920, Bio-Optica, Milan, Italy). Tissue sections were stained with haematoxylin and eosin (HE) for morphological analysis, and all histological evaluations were performed by the same experienced pathologist.

2.6. Statistical analysis

Data were recorded into an electronic spreadsheet (Microsoft Excel ver.16.53 2, Microsoft Corp., Redmond, WA, USA) before being imported into dedicated statistical analysis software (SPSS 27.01 for Windows, SPSS Inc., Headquarters, Chicago, IL, USA). Normality was assessed using Shapiro-Wilk's W test, and results were reported as mean ± standard deviation for normally distributed data or as median (range) for non-normally distributed data. A Student's *t* or a Mann-Whitney's *U* test was used to compare haematological parameters between mares and geldings and the measurements obtained with the two centrifuges. If clots and membranes obtained by the two methods of centrifugation did not differ, the size of FC products were related to selected variables,

including sex, RBCs, PCV, Hb, MCV, MCHC, RDW, WBCs, total neutrophiles and percentage, total lymphocytes and percentage, total monocytes and percentage, total eosinophils and percentage, total basophiles and percentage, PC, MPV and PCT, using the Spearman's rho r_s. The degree of correlation was classified as perfect (±0,59), poor (±0.29 - ±0.1) and none (<±0.1) (Chan, 2003). Significance was set at $p < 0.05$.

3. Results

Twenty Standardbred horses were enrolled in the study, comprising 13 geldings and 7 females. The median age was 4.5 (range 3–7 years). All the animals were clinically healthy, vaccinated against influenza, tetanus, and herpes virus,

All animals tolerated whole blood sampling well, and procedure did not induce any discomfort. The environmental temperature ranged from 15.8 to 17.2 °C; consequently, all the tubes were heated using a thermostat. Normal clots were formed from all blood samples (100% success).

The CBC was within physiological range in all animals. Medium clot and membrane surface obtained by HC were 482.3 ± 107.8 mm (range 246.88–688.28 mm) and 294.7 ± 81.6 mm (129.64–497.86 mm), respectively, whereas mean clot and membrane size obtained by FC were 492.8 ± 115.9 mm (225.12–696.17) and 285.1 ± 78.5 mm (134.25–491.81), respectively (Fig. 1). Statistical analysis showed no differences between clot and membrane surfaces obtained using the two centrifuges.

All the tested animals were negative for *Babesia caballi* and *Theileria equi*.

As shown in Figs. 2 and 3, there was no difference between clot and membrane weights, lengths, widths and heights among HC and FC centrifuges as well.

Haematological parameters showed no significant differences between sexes ($p > 0.05$ for all parameters) and there were no differences between *clot and membrane surfaces obtained by male and female horses ($p = 0.573$ and 0.903)*.

A fair correlation between age ($r_s = -0.401$), RBC (0.403), MCV (−0.388), monocyte concentration (−0.402) and percentage (−0.373) and clot's surface obtained with FC was obtained, as well as between membrane surface, PCV (−0.326) and monocyte concentration (−0.335).

Haemoglobin, neutrophils, lymphocytes and eosinophils percentage and concentration, PLT, MPV and ratio MPV/PC were poor correlated to clot's surface (0.252, −0.128, −0.235, −0.170, −0.114, −0.168, −0.163, 0.111, 0.154, −0.205, respectively). Red blood cells (−0.165), Hgb (−0.160), MCHC (−0.178), RDW (−0.239), neutrophiles concentration and percentage (−0.193 and − 0.241, respectively), lymphocyte concentration (−0.107), monocyte percentage (−0.151), basophiles percentage (0.154), PLT (−0.280), MPV (0.143), PCT (−0.190) and ratio MPV/PC (0.101) showed poor correlation to membrane size, as well.

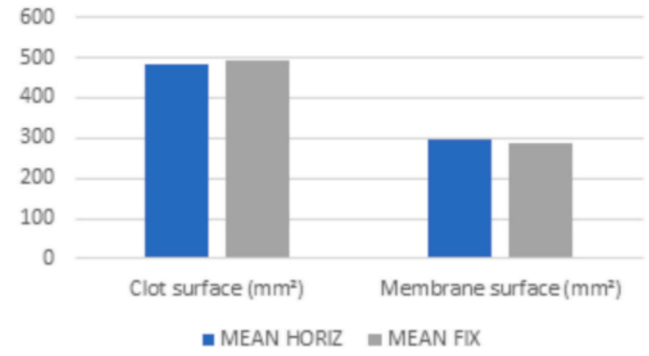

Fig. 1. Clot and membrane surface obtained with Horizontal and Fixed-angle centrifuges.

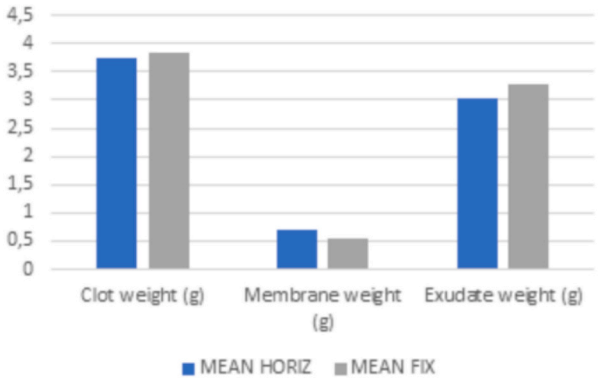

Fig. 2. Clots, membranes and exudate weight obtained with Horizontal and Fixed-Angle centrifuges.

The other haematological parameters showed no correlation ($r_s <$ ±0.1) to clot and membrane surfaces.

Histology allowed to observe, in all the examined A-PRF+ membranes, five layers according to the distribution and density of blood cells in this biomaterial. The first layer was primarily composed of erythrocytes, followed by a transitional zone of leukocytes and platelets, a layer of fibrin and fewer leukocytes and then a fibrin layer. Erythrocytes were stained red, whereas platelets and aggregates of platelets were stained in dark pink. The fibrin network was clearly distinguished with the near absence of colour and the scarce presence of cells (Fig. 4). No difference in cellular distribution was found between A-PRF+ produced with FC and HC.

4. Discussion

In this prospective study, haematological parameters demonstrated a low impact on the final size of the A-PRF+ clots and membranes. Additionally, there were no macroscopic and microscopic differences between clots and membranes obtained with HC and FC, as previously reported in the literature (Castro et al., 2021; Feng et al., 2020). Furthermore, the heating of the tubes allowed the retrieval of clots from all samples, independently of the environmental temperature.

All included animals were tested for Piroplasmosis. Equine piroplasmosis is a tick-born disease, with a high incidence in clinically healthy horses in Southern Italy (around 30%) (Del Pino et al., 2016; Manna et al., 2018; Coluccia et al., 2024). Infection caused by *Theileria equi* and *Babesia caballi* can produce several haematological disorders, including the decrease of red cells, platelets, and haemoglobin concentration (Camacho et al., 2005; De Waal, 1992) Therefore, horses were tested for the infection, including only the negative ones, because the infection could have negatively interfered with clot production and with the results of the research. The PCR technique was chosen for the diagnosis, because detection of pathogens using PCR has shown to have a higher sensibility and specificity than microscopic techniques and has been considered the best method for diagnosis of animals with chronic infection (Bashiruddin et al., 1999; Nicolaiewsky et al., 2001; Rampersad et al., 2003; Onyiche et al., 2019).

Studies in human medicine demonstrated that females and older patients usually produce larger L-PRF membranes compared to younger and male patients, probably associated with the lower haematocrit level (Miron et al., 2019b). Thus, assuming a similar influence of age and sex on the size of the A-PRF+, a sample of young animals, in a restricted age range, was included in the study. Moreover, haematological and clots/membranes differences between males and females were analysed. Unlike human medicine, a significant variation in blood parameters between sexes was not found and the sizes of equine A-PRF+ did not differ between mares and geldings. Further studies are needed to determine

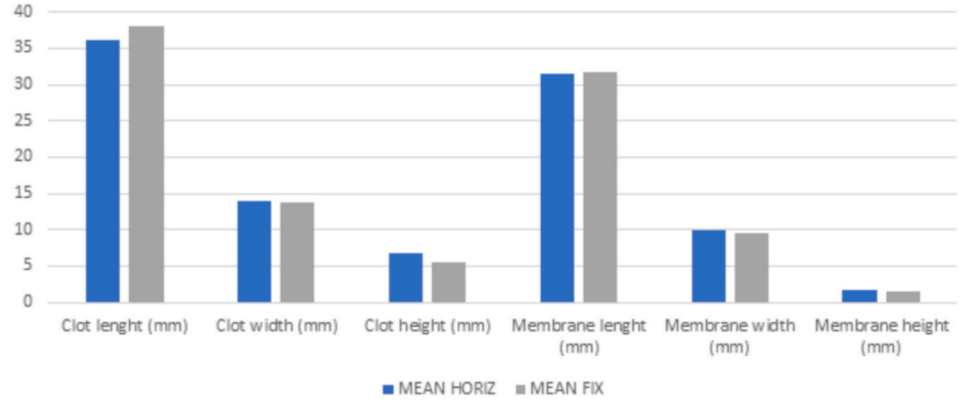

Fig. 3. Length, width and height of clots and membranes obtained with the Horizontal and the Fixed Angle centrifuges.

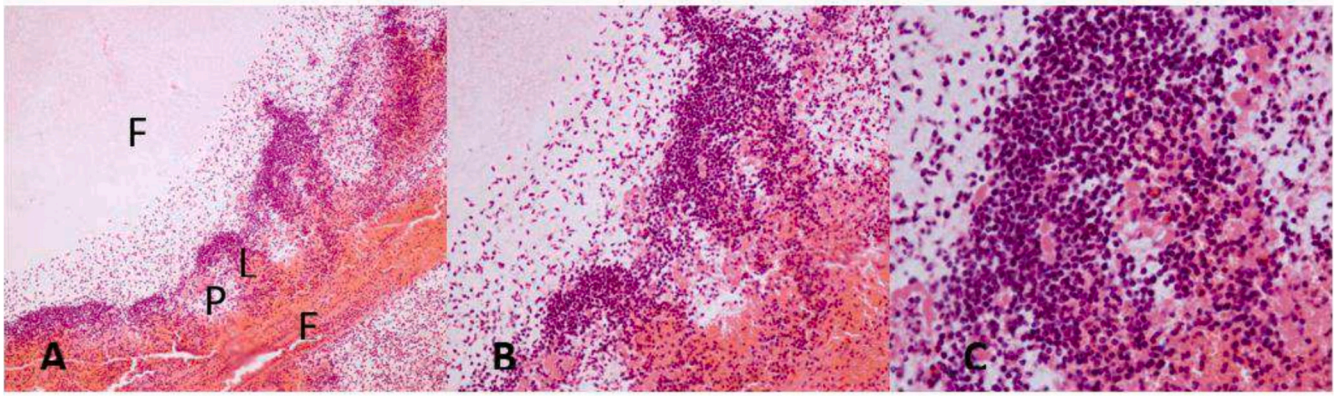

Fig. 4. Histology of A-PRF+ membranes. Cellular portion of A-PRF+ to x4 (A), to x20 (B) and x40 (C). P = platelets; E = erythrocytes; F = fibrin; L = leukocytes.

whether there are significant age-related changes in the size of clots and membranes in horses.

It was reported that PRF cannot be generated when whole blood is collected and processed at a temperature below 21 °C or above 30 °C (Crisci et al., 2017). Under such conditions, fibrin clots would either not be produced, or only small, incoherent, friable masses of fibrin would be obtained (Crisci et al., 2020). Nevertheless, the study was performed in late Autumn, with an environmental temperature of 15–17 °C. Heating the tubes for 15 min in a thermostat allowed us to get clots from all samples, despite the low external temperatures. Histological examination confirmed that membranes produced with blood collected in heated tubes has the same characteristics of normal membranes. This is an important issue, as it allows A-PRF+ production in field, even in the coldest seasons.

In the present research, a horizontal and a fixed-angle centrifuge were used for A-PRF+ production and it was found that, using the reported speed and the time of centrifugation, clots and membranes showed no significative differences within the two centrifuges. In human medicine, Dohan Ehrenfest et al. (2018) described the influence of the centrifuge characteristics on the macroscopic features, cellularity, concentration of GFs and fibrine architecture, highlighting significant differences in PRF clots obtained with different types of centrifuges (Intra-Spin centrifuge, Advanced PRF Process and LW -UPD8). Indeed, the Intra-spin centrifuge had the lowest level of vibration and produce the largest clot compared to the other centrifuges. On the contrary and in accordance with our study, Miron et al. (2019a) suggested that centrifugation device have no impact on the final size of L-PRF and A-PRF. Additionally, it has been showed a correlation between the platelet-rich fibrin size and the centrifugation tubes size, with significantly greater-sized clots produced using Process for PRF tubes when compared to other commercially available tubes (Miron et al., 2020). In the current study, A-PRF tubes Process were used, avoiding the impact of using different test tubes on the results of the research.

Time between sampling and centrifugation, between centrifugation and compression, compression pressure and time were standardized, as their influence on membrane size was demonstrated (Miron et al., 2019a; Castro et al., 2021; Caterino et al., 2022).

Only few haematological parameters showed a fair correlation to the surface size of clots and membranes, which the authors considered as no significant. Therefore, other blood components could influence clots and membranes size, as already proven in human medicine (Miron et al., 2019b). Fibrine clot formation, structure, and stability in the blood are strongly influenced by many features, such as pro- and anticoagulants concentration, fibrinogen variants, vascular and blood cells, heparin, and protamine levels (Kattula et al., 2017). Glycoprotein IIb-IIIa levels, pH, chloride and calcium ion concentration has demonstrated to have an influence on fibrine clot structure and mechanical properties, as well (Carr Jr et al., 1986; Carr Jr and Hardin, 1987; Carr Jr et al., 1985; Di Stasio et al., 1998; Weisel et al., 1992). Additional studies are required to investigate which of these parameters may possibly have an influence on the size of A-PRF+ in the horse.

Histological examination did not show any differences in cellular distribution among the two centrifuges, in contrast to what reported for L-PRF: in fact, horizontal centrifugation of L-PRF produced better cell layer separation and a higher percentage of immune cells when compared to those achieved with fixed-angle centrifuge (Miron et al., 2019a). This may be related to the more uniform cellular distribution that occur in A-PRF+ clots (Crisci et al., 2022), which is probably not affected by the angle of the centrifuge.

The average size of clots and membranes evaluated in this study were

sufficient to cover small lesions and are consistent with the measurements reported by Crisci et al. (2022) in horses. Albeit in human medicine A-PRF+ is widely used in several areas, such as oral and maxillofacial surgeries and for treatment of diabetic ulcers (Fan et al., 2020; Crisci et al., 2022), in veterinary medicine studies concerning the clinical application of A-PRF+ in horses are still lacking. In future, studies evaluating the use of this platelet product to enhance tissue healing in horses are advisable.

One limitation of the current study is that the time required for blood sampling was not recorded. It has been recently shown that all tubes should be collected within a 60–90 s, to avoid clots from decreasing in size (Miron et al., 2018). Although time has not been considered, the blood sampling performed by equine practitioners was carried out under the same conditions and was quick and without any complication. Thus, it was assumed that this parameter did not affect the size of A-PRF+ in the present research.

Another significant limitation of this study is the lack of evaluation of growth factor concentrations. Existing literature reports that low-speed centrifugation products, such as A-PRF+, exhibit higher concentrations and release of growth factors (particularly PDGF, TGF-B1, EGF, and IGF-1) compared to standard L-PRF. (Fujioka-Kobayashi et al., 2017). Furthermore, at day 10, A-PRF+ exhibited a significantly higher release rate of VEGF, EGF, and TGF-β1 compared to A-PRF and PRF (El Bagdadi et al., 2019). Based on the literature and our findings, we did not anticipate differences in the growth factors released by A-PRF+ produced by one centrifuge versus another. However, further studies are needed to confirm this hypothesis.

5. Conclusion

Haematological parameters have an unremarkable impact on A-PRF+ size. Macroscopic and microscopic features did not differ between clots and membranes obtained with HC and FC. Heating of the tubes made possible to obtain clots from all samples at environmental temperature, allowing the production of A-PRF+ even in field conditions. The mean size of A-PRF+ clots and membranes are adequate to cover a small lesion. Further studies are needed to understand the influence of other blood molecules on A-PRF+ size.

CRediT authorship contribution statement

Montano Chiara: Conceptualization, Data curation, Investigation, Methodology, Writing – original draft. **de Chiara Mariaelena:** Conceptualization, Data curation, Formal analysis, Investigation, Methodology. **Crisci Alessandro:** Investigation, Methodology, Validation, Writing – review & editing. **De Biase Davide:** Data curation, Validation, Writing – review & editing. **Ciuca Lavinia:** Investigation, Methodology. **Maurelli Maria Paola:** Investigation, Methodology. **Lamagna Barbara:** Investigation, Writing – review & editing. **Del Prete Chiara:** Investigation, Methodology, Writing – review & editing. **Fabiana Flagiello:** Methodology, Validation. **Pasolini Maria Pia:** Conceptualization, Data curation, Investigation, Methodology, Supervision, Writing – review & editing.

Declaration of competing interest

The authors declare no conflict of interest.

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