American Journal of Engineering Research (AJER)
e-ISSN: 2320-0847 p-ISSN : 2320-0936
Volume-11, Issue-06, pp-107-117
www.ajer.org
Research Paper Open Access

Second generation solid platelet concentrates (L-PRF, A-PRF): morphometric characteristics. Experimental research on the horse.

Alessandro Crisci*[1,2,3] , Michela Crisci[4] , Flagiello Fabiana.[5]
[1] School of Medicine, University of Salerno Italy, 84084 Fisciano SA, Italy;
[2]Unit of Dermosurgery Cutaneous Transplantations and Hard-to-HealWound, "Villa Fiorita "Private Hospital, 81031 Aversa CE, Italy;
[3] Institute for the Studies and Care of Diabetics, Abetaia, 81020 Casagiove CE, Italy;
[4] Faculty of Medicine and Surgery, Vasile Goldis Western University of Arad, 310025 Arad, Romania;
[5] Analysis Laboratory "Villa Fiorita" Private Hospital Aversa (CE) Italy;
*Corresponding author: Prof. Alessandro Crisci, Department of Medicine, Surgery and Dentistry "Salernitan Medical School", University of Salerno, Fisciano (SA), Italy;

ABSTRACT
This prospective study with equine blood has been prepared to evaluate the characteristics of 2nd generation platelet concentrates in terms of Weight, Length, Width, Thickness, Clot Surface and diaphragms of L-PRF and A-PRF of L-PRF and A-PRF whith the aim that they can be are instantly usable and fairly simple to operate in to use in both human and veterinary daily clinical routine.
Equine blood was obtained in PET tubes without anticoagulants containing silica for coagulum production and L-PRF membranes and in glass tubes for coagulum generation and A-PRF diaphragms in six apparent well-being horses. The membranes and coagulum produced were examined.
Horse L-PRF/A-PRF membranes produced after centrifugation consisted of 66% Red Blood Cells, 1.56% White Blood Cells, and 32% Platelets, 0.0028%, 99.24%, and 99.0% of total amounts, respectively. Mean morphological characteristics (±D.S.) noticed between the diverse types of coagulum and membranes are: Clot weight gr. 3.52(±0.64); Exudate weight gr. 2.54(±0.48); Membrane weight gr. 0.84(±0.15); Clot length mm 40.8(±4.40); Clot width mm 13.89(±1.18); Clot thickness mm 6.48(±0.92); Clot surface mm^2 48.64(±5.65); Membrane length mm 34.37(±3.79); Membrane Width mm 11.55(±1.58); Membrane Thickness mm 2.78(±0.38); Membrane Surface mm^2 35.78(±5.67); Clot Weight/Blood Sample Ratio 10 ml % 22.15(±2.20). The most useful parameters in A-PRF in clinical conditions are those of clot surface 53.94(±14.39) mm^2 $p=0.001<0.05$ and membrane surface 43.52(±8.68) mm^2 $p=0.560>0.05$. Useful A-PRF membrane thickness is 3.19(±0.46) mm ($p=0.071>0.05$).
Our study sought to standardize the procedure for preparing L-PRF and A-PRF, which while remaining an easy-to-perform, low-cost procedure, does not require expert instrumentation and has some consistency in producing an L-PRF/A-PRF membrane in terms of macroscopic and microscopic characteristics.
KEYWORDS: *blood derivatives; growth factors; leukocyte and platelet-rich fibrin; advanced platelet-rich fibrin; L-PRF wound box; stem cells;*

Date of Submission: 28-05-2022 Date of acceptance: 09-06-2022

I. INTRODUCTION

Platelet-derived substances (PDPs) have increased in notoriety in recent years, mainly because of their high their high collections of biologically active molecules, such as growth factors and cytokines, play a vital role in tissue wound healing and repair. Recent indication suggests that platelets may have a novel role in tissue restore and vascular remodeling, as well as being active players in inflammatory and immune responses. They release proteins and other metabolically active materials that can influence several processes that promote cellular recruitment, growth, and morphogenesis: these are the growth factors, released or presented on the surface of activated platelets. Platelets' ability to discharge substances from within a clot (HPC-Human Platelet Concentrate) presents the latter as

a natural autologous source of growth factors and cytokines that can be used in the clinic to accelerate the physiological healing of a tissue injury (Fig.1)(1, 2).

Second-generation solid blood concentrate is derived from the subject's peripheral blood after one-step centrifugation without anticoagulants generating a matrix rich in platelets and leukocytes. The presence of platelets, leukocytes, and fibrin has been shown in the past to be essential for wound healing (3,4). In addition to the potential of leukocytes to influence angiogenesis and lymphogenesis, this flexible 3D fibrin network is a source of cytokines and growth factors

(5). The use of particular A-P glass tubes (A-PRF PRF-Process Nice France) promotes PRF coagulation and, with appropriate modifications of centrifugation speed, also led to the consequent formation of an injectable PRF-based liquid matrix (*i-PRF*).

In recent years, research has examined the impact of applied relative centrifugal force (RCF) on the composition and bio-activity of PRF matrices (6, 7). Because leukocytes, including neutrophils and macrophages, are among the first cell species present at wound sites, their role also includes the removal of phagocytic fragments, bacteria, and necrotic tissue, thereby preventing infection. Macrophages are the cells originated from the myeloid line and are also considered one of the key cells involved in the secretion of growth factors during wound healing, among them TGF-β, PDGF, and VEGF factors. Neutrophils along with platelets and in combination with their secreted growth factors/cytokines can facilitate tissue regeneration, formation of new blood vessels (angiogenesis), and prevention of infection (bacteriostasis).

Platelets can, therefore, be rapidly isolated and activated from peripheral whole blood, using specific centrifugation, and returned to the patient's peripheral circulation the same day. Chemokines released from triggered platelets also promote the recruitment, adhesion, and proliferation of adult stem cells to further aid in tissue repair (8).

Various types of second-generation concentrates have been produced by centrifuging blood drawn in different ways: ***-L-PRF:*** 30" of acceleration, 2' at 2700 rpm, 4' at 2400 rpm, 3' at 3000 rpm, and 36" of deceleration and stopping (9); ***-Advanced-PRF:*** 1300 rpm, 8 minutes (189 g-forces)(10);
-Injectable-PRF: 700 rpm (60 g-force) for 3 minutes) (11, 12);
-***Horizontal-A-PRF***: 1300 rpm (189 g-forces) for 8 minutes (13);

Ghanaati *et al.* (10) have reported that speed and time do not affect monocyte and stem cell populations, but they do affect platelet and neutrophil concentrations. Accordingly, A-PRF contains more platelets than *L-PRF*, a greater amount was found in the distal part of the PRF membrane, and more neutrophils are present. This type of concentrate has the power to increase angiogenesis by manifesting the matrix enzyme metalloproteinase-9.

Therefore, the insertion of neutrophils in PRF may be painstaking if angiogenesis is of interest.

Recently, the Authors, have studied an *L-PRF*, which appears to contain hematopoietic stem cells (HSCs)(14). The existence of these HSC cells was detected mainly by immunohistochemical analysis for the detection of specific CD34 cell markers. Hematopoietic stem cells were obtained without mobilization from equine peripheral blood (Figure. 2 A-B). Endothelial Progenitor Cells (EPCs) have been observed in the manufacturing of new vessels in the endometrium after ovulation, in vessels from colon tumors, and in skin wounds, this model induced significant vasculogenesis (15). MSCs (mesenchymal stem cells) found in wounds also represent a pool of stem cells, which can reconstruct damaged tissue, and in addition, endothelial cells contribute to angiogenesis. Models of migration caused by supernatant of platelet concentrates do not differ between two cell types MSCs and HUVECs (human umbilical vein endothelial cells).

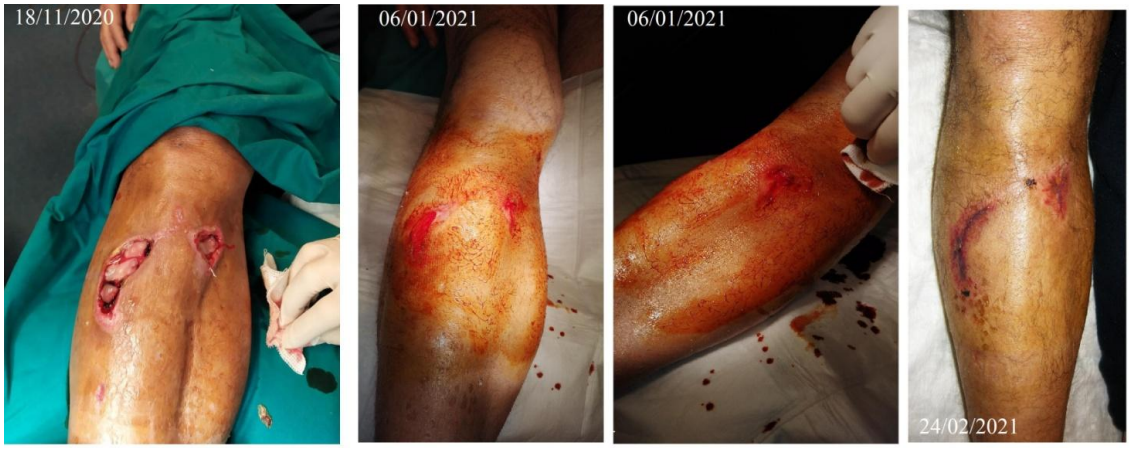

Figure 1. Regenerative therapy effect with A-PRF in a patient with ACOP ulcer and carrier Of leukemia thrombocytopenia (1 application).

The strongest migration of MSCs and HUVECs was observed in reply to *L-PRF* (Figure 3). All this implies that L-PRF might be as beneficial as useful as a therapeutic biomaterial and physiological antihemorrhagic agent for use at surgical sites (14,15).

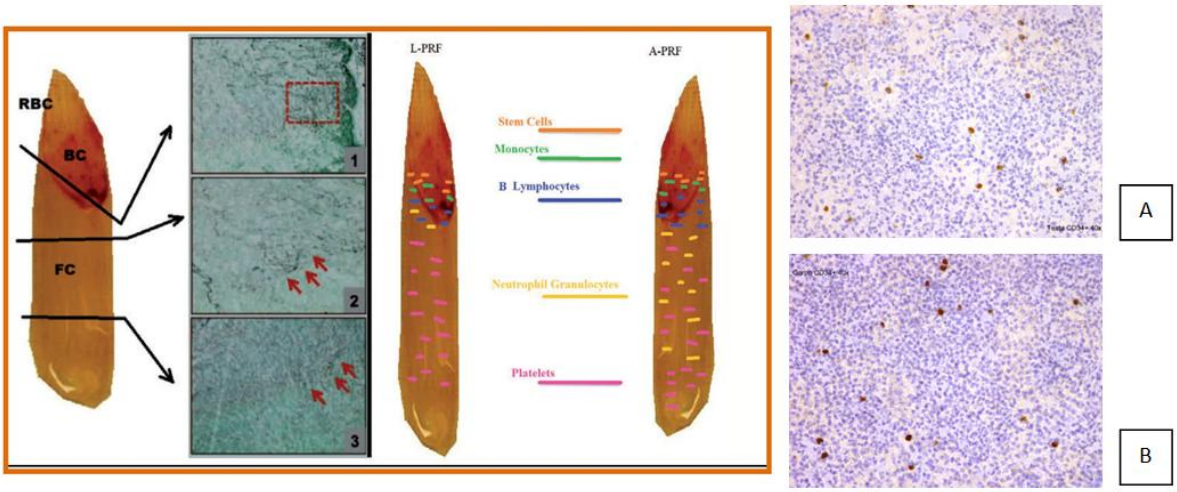

Figure 2. Advanced-PRF (A-PRF) compared with *L-PRF* full examination of a fibrin coagulum along its major axis (Masson-Goldner coloring). RBC denotes the RBC fraction. The *buffy coat* (BC) is the transformation zone between the RBC portion and the fibrin clot, while FC represents the fibrin coagulum. The three rows within the scan and arrows show the close-ups of the respective areas. The red arrows represent the cells ensnared in the fibrin network. The right content of different kinds of cells is in the two types of Platelet-rich fibrin. Right photomicrographs of the head (A) and body (B) of equine PRF membrane containing $CD34^+$ stem cells (ingr.40×)(18).

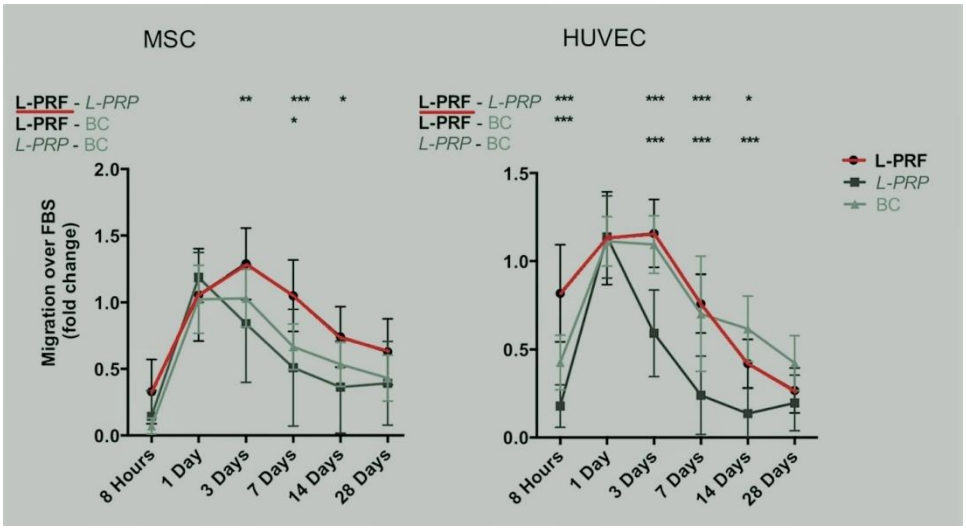

Figure 3. Translation of MSC and HUVEC is demonstrated in answer to factors expressed by *L-PRF*, *L-PRP* and blood coagulum (BC). MSC and HUVEC migration was analyzed in the Boyden chamber by means collected after 8 hours and 1, 3, 7, 14, and 28 days of *L-PRP*, *L-PRF*, and blood clots compared to carriers including 10% FBS and change expressed in turn. Data are presented as mean±SD from a triplicate of 11 samples. Statistical evaluation was performed using two-way repeated-measures ANOVA and Bonferroni's post hoc test. Significant differences for CSM and HUVEC migration between platelet concentrates at various time periods are shown: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. (from Schär et al. 2015 modified) (9).

In the current study, the AA. want to consider the the morphometric properties of various types of PRF (*L-PRF*, A-PRF, A-PRF+, A-PRF Vacu) (Exudate Weight, Weight, Length, Width, Thickness, and Surface of the coagulum as well as Length, Width, Thickness and Surface of the Membrane, % Ratio of Clot Weight/10 ml of Whole Blood).

II. MATERIAL AND METHODS

Blood was collected in PET tubes with silica without anticoagulant, nor a separating gel (Vacuaptaca clot activator tubes cod.30023 for Serum 9.0 ml [indicated as A-PRF-Vacu]) and glass tubes without anticoagulant, nor a separating gel (A-P tubes for A-PRF Vacutainer for Serum 9.0 ml, PRF-Process Nice France), for coagulum and diaphragm fabrication of L-PRF and A- PRF in six apparent well-being horses of various ages (mean ± SD, 10 ± 4.1 years, range: 3-20 years), sex, and breed.

Written consent from owners was provided for all horses, and the blood collection procedure was performed in accordance with current ARRIVE (Animal Research: Reporting of *In Vivo* Experiments) guidelines.

In Automated Counting, platelets artificially decrease if aggregates form. Pseudopodia may be observed on activated platelets (12). Equine platelet concentrations are among the lowest reported. Their concentration in a normal horse averages approximately 6-10 platelets per field in oil immersion (100× objective), normal values range from 100,000 to 350,000/µL. Repeated venipuncture, changes in blood flow, or delay in performing the analysis alters the count of these cells. Processing temperature and storage conditions also have an important correlation with significant effects on platelet aggregation.

The protocol for the training of *L-PRF*® in humans and animals is very simple: blood is immediately centrifuged within 2 minutes of the collection with a Duo centrifuge (Process for PRF, France) that has a rotor angle of 41.3° following the following schedule: 30" of acceleration, 8 min at 2700 rpm (816 g-forces) and 36" of deceleration and stop. The resulting product consists of three layers: PPP (Platelet-Free Plasma at the top), PRF (clot in the middle), and Red Blood Cells (RBC) at the bottom. The resulting PRF clots are harvested and the red clot portion is removed with scissors without any damage to the PRF macroscopically (7, 15).

When PRF preparation protocols are described in the literature, very often important parameters (e.g., whether anticoagulant and its volume is used, blood volume drawn and final PDP achieved, exercise temperature, time between sample collection/processing/analysis), leading to questionable and non-comparable results.

In our protocol, blood was collected rapidly with a 19 G needle into 9cc Vacutainer tubes using a vacuum system (average collection rate of 22", less than 25" per tube) and immediately (within 1 minute) centrifuged according to the above description at a temperature above 21°C.

L-PRF clot and membrane production tubes and A-PRF clot and membrane production tubes were used at an ambient temperature greater than 21°C (21-30°C), processing temperature adopted 21.3/22.5°C. Fibrinogen is originally concentrated in the middle and top of the pipe, right between the red blood cells at the bottom (RBC) and the acellular plasma at the higher (PPP). Clot compression with a metal compressed system (L-PRF box) more importantly stimulates cell multiplication and neovascularization (18, 19).

The *L-PRF* and A-PRF in this experiment were divided into 3 regions of equal length (Figs. 2, 4) and the presence of platelets in each region was observed by Optical Microscopy at various magnifications (Crisci A. et al.) (20-23). Region 1 is closest to the red clot (head) and has numerous aggregated platelets; there are some lymphocytes and other white blood cells. The number of platelets decreases with growing distance from the red clot. In region 2 (body) there are fibrin fibers (primary and secondary) and platelets in reduced numbers, in region 3 (tail) the fibrin lattice is very evident, whereas platelets are few (Fig.4).

Cold preservation of coagulum and membranes implies a deterioration of the biological and physical properties of the product, so the ideal minimum temperature for conservation for a few hours +4°C.

In this experimental design, the number of samples may seem a bit low, but the objectives of the study emphasized a natural mechanism, so it was not necessary to maximize statistical significance. It was also not designed as a clinical study, and thus is limited by the lack of a control group.

Macroscopic Analysis

After spinning, the *L-PRF* and A-PRF coagulums have been eliminated from test tube using sterile forceps and equally sterile scissors to to smoothly remove the red clot from the buffy coat (BC)(Figure 2). Each clot and each obtained *L-PRF/A-PRF* fibrin membrane were positioned in a serving tray to determine their weight and dimensions with digital device goldsmith scale and balance and digital scale three different times by three independent operators and averaged over the three measurements. Clot compression was performed with the "*L-PRF Wound Box"* with a pressure of 142.437 Pa/cm^2 constant for 120" (compressions of longer duration were not used because they did not prove useful in our previous study)(23).

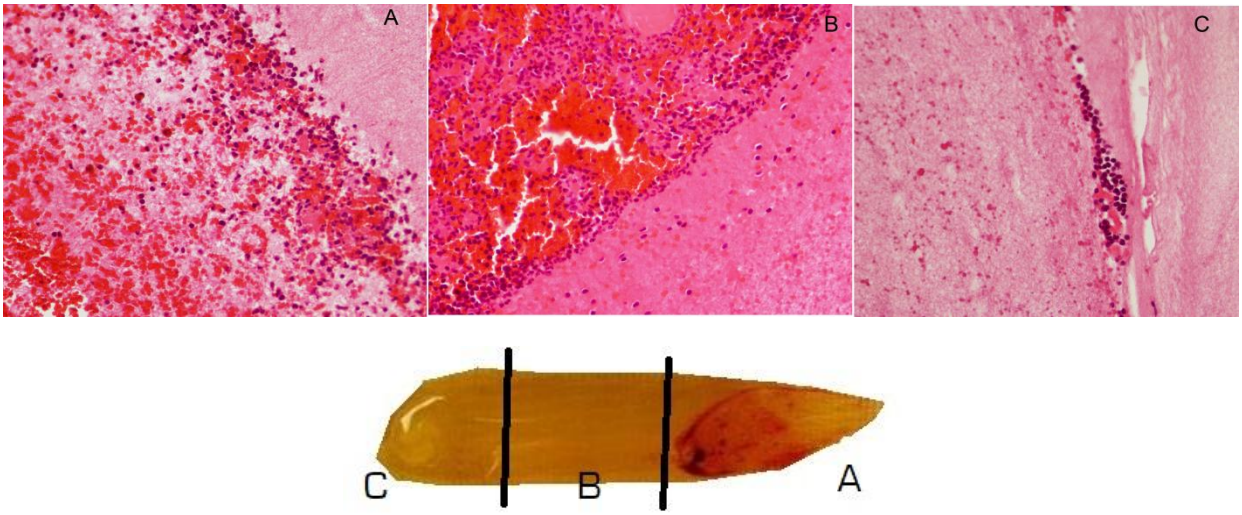

Figure. 4 Membrane microscopic study A-PRF after squeezing (hematoxylin-eosin coloration). The different layers of PRF were embedded in a 10% neutral formalin solution buffer pH 7.2 for 48 hours and embedded in kerosene according to standard procedure. Serial sections (7 µm thickness) of each sample were cut using a microtome.

***Above*: (A) Proximal III^ 40× fibrin on the right, central lymphocytes, erythrocytes, and neutrophil granulocytes on the left; (B) Middle III^ 25× fibrin pattern with abundant platelet and leukocyte content; (C) Distal III^ 60× fibrin pattern with scarce platelets and leukocytes (Crisci A. et al. 2017).**

***Bottom*: Head (A), Body (B), Tail (C) of self-compressed Horse A-PRF membrane centrifuged at a fixed angle**

Procedure for optical microscopy

PRF samples for histology were fixed with 10% neutral buffered formalin solution for 24 h at room temperature, embedded in kerosene, and sectioned in 3 µm using a microtome (Thermo® Microm HM355S, Thermo Scientific®). After this, Hematoxylin-Eosin staining was performed using automated staining equipment (Thermo Scientific® Microm HMS 740). Each clot was divided into three zones of equal size: Proximal (head), Central (body), and Distal (tail). The histopathological examination was conducted using a light microscope (Leica Microscope® DM750, Switzerland), and images were acquired with a NIKON 5000D digital camera and AxioVision V.4.8 software (Zeiss®, Oberkochen, Germany).

Each region of these sections was observed and analyzed by counting the visible cell bodies (marked in dark purple, mostly leukocytes) in the center of each region observed at a magnification of 25×, 40×, 60× and 100× (dip). The total concentration of cell bodies counted was utilized to correlate their allocation among the three membrane areas (head, body, and tail). We hypothesized that, unlike *L-PRF* in which most cells are concentrated in the proximal area (head), closer to the red clot, in A-PRF the cell distribution is more homogeneous, insofar as that the film can be applied in almost its entire length in the clinical location (Figure 4).

A blood sample was also taken from each horse to perform a blood count using K3E 5.4 mg tubes with EDTA (VacuMed).

Leukocyte and platelet concentrations of *L-PRF* and A-PRF were analyzed with a CBC in standard bays.

Blood counts were carried out with a Cell Dyn 3500 R cell counter (Abbott Laboratories; Abbott Park, IL, USA).

Statistical measurements and analysis
With regard to the measurement of various clot morphological parameters, statistical significance for differences between groups was calculated with *Student's* t-test for repeated measures for parametric variables and with the *Mann-*
Whitney signed-rank *U-test*. Any value of $p < 0.05$ was rated statistically significant for the Student's t-test, whereas $p<0.06$ was for Mann-Whitney's test.

The results were examined using version 6.0 of the Santon-Glantz 2007 Statistical Package for Biomedical Disciplines.

III. RESULTS

The procedure was well tolerated in all animals examined.
No significant differences were found in the baseline hematology comparison of the tested subjects, which had a mean
RBC concentration of 7.6×10^6/mL (±1.1 I.C.95%)($p=0.34$)(range:7-13×10^6 /mL), of WBC of 5.1×10^3 /mL (±0.37 I.C.95%)($p=0.24$)(range:5-13×10^3 /mL) and a Mean Platelet Count of 106.8×10^3 /mL (±15.3
I.C.95%)($p=0.15$)(range:100-350×10^3 /mL).
Tab.1 and Figs. 5 and 6 compare the characteristics of clots and PRF membranes obtained by us in the horse (DUO centrifuge) with the use of various types of tubes (glass A-P, coated PET Vacuaptaca). In this comparison, it was first established that there were significative differences in membrane characteristics (weight and surface area) after compression. The dimensions of the membranes were not found to be related to the hemoglobin content or the Erythrocyte content found in the Basal Hemochrome**.**

The average morphometric characteristics (±D.S.) measured among between the different typologies of coagulum and membranes are Clot Weight gr. 3.52(±0.64); Exudate Weight gr. 2.54(±0.48); Membrane Weight gr. 0.84(±0.15); Clot length mm 40.8(±4.40); Clot width mm 13.89(±1.18); Clot thickness mm 6.48(±0.92); Clot surface mm^2 48.64(±5.65); Membrane length mm 34.37(±3.79); Membrane Width mm 11.55(±1.58); Membrane Thickness mm 2.78(±0.38); Membrane Surface mm^2 35.78(±5.67); Weight Ratio Clot/Blood Sample 10 ml % 22.15(±2.20) (Table 1, Figs.5 e 6). The best clinically beneficial values are those derived in the A-PRF of clot surface 53.94(±14.39) mm^2 $p=0.001<0.05$ and membrane surface 43.52(±8.68) mm^2 $p=0.560>0.05$. The thickness of the A-PRF membrane is 3.19(±0.46) mm ($p=0.071>0.05$)(Table 3).

The student's *t-test* (Table 2) demonstrates significant differences ($p<0.05$) in several parameters (Clot Weight, Essudate and Membrane, Clot Weight to Whole Blood Ratio) between *L-PRF/A-PRF* Vacu membranes. These data are confirmed by the *Mann-Whitney U-Test* (Table 3).
The parameters derived from the comparison between A-PRF and *L-PRF were* then analyzed and significant differences were demonstrated in both Students' and Mann-Whitney tests for Clot Length and Thickness, Membrane Surface, and Clot Weight to Whole Blood Ratio. The comparison between *L-PRF* and A-PRF+ showed significant differences in Student's t-test and Mann-Whitney test for clot width and thickness, membrane length and width, and clot weight to whole blood ratio.

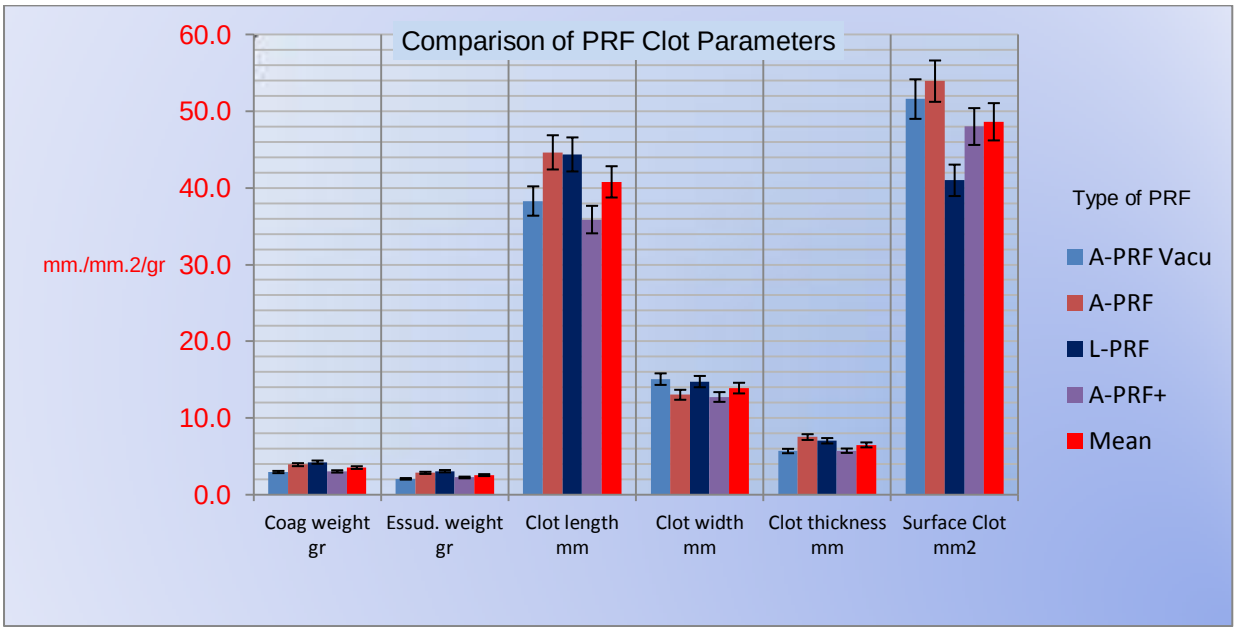

Figure 5. Comparison of parameters between the different types of PRF clots (*L-PRF*, A-PRF with A-P Tube, A-PRF+, A-PRF Vacu with Vacuaptaca Tube) .

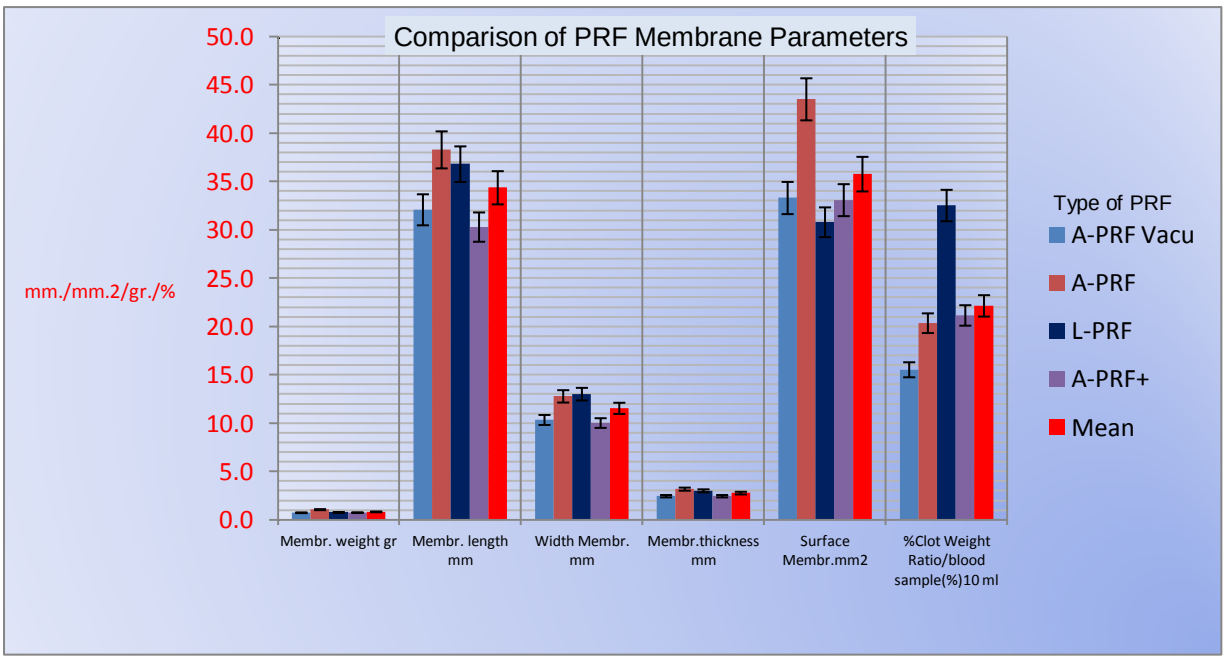

Figure 6. Comparison of parameters between the differnt types of PRF Membrane

(*L-PRF*, A-PRF with A-P Tube, A-PRF+, A-PRF Vacu with Vacuaptaca Tube) .

Table 1. Correlation of morphometric characteristics between various types of PRF membranes and clots obtained from equine blood by various methods (14)

Parameters	A-PRF Vacu	A-PRF	L-PRF	A-PRF+	Mean±DS
Coag weight gr	2,94(±0,72)	3,9(±0,61)	4,23(±0,55)	3,02(±0,42)	3,52(±0,64)
Essud. weight gr	2,05(±0,55)	2,83(±0,81)	3,05(±0,11)	2,23(±0,64)	2,54(±0,48)
Membr. weight mm	0,75(±0,32)	1,07(±0,29)	0,78(±0,08)	0,76(±0,21)	0,84(±0,15)
Clot width mm	38,3(±6,69)	44,65(±2,96)	44,38(±3,83)	35,88(±0,14)	40,8(±4,40)
Clot length mm	15,06(±1,87)	13,02(±0,48)	14,74(±1,23)	12,73(±0,30)	13,89(±1,18)

Clot thickness mm	5,68(±1,41)	7,5(±0,63)	7,02(±1,09)	5,72(±0,54)	6,48(±0,92)
Surface Clot mm2	51,6(±8,68)	53,94(±14,39)	41,0(±0,86)	48,02(±13,86)	48,64(±5,65)
Membr. length mm	32,09(±5,5)	38,29(±2,21)	36,81(±3,18)	30,30(±2,42)	34,37(±3,79)
Width Membr. mm	10,35(±2,26)	12,79(±0,06)	13,02(±1,01)	10,03(±0,37)	11,55(±1,58)
Membr.thickness mm	2,46(±0,72)	3,19(±0,46)	3,02(±0,51)	2,46(±0,35)	2,78(±0,38)
Surface Membr.mm2	33,31(±13,44)	43,52(±8,68)	30,8(±0,5)	33,09(±7,59)	35,78(±5,67)
%Clot Weight Ratio/ blood sample(%)10ml	15,54(±3,84)	20,36(±3,22)	32,53(±0,54)	21,16(±2,24)	22,15(±2,20)

The differences were found to be statistically significative by $p<0.05$.

Table 2. Student's Significance Test
Correlation of morphometric characteristics between various types of PRF membranes and clots obtained from equine blood (14).

			t-Student				
Parameters	**A-PRF vacu/A-PRF**	**A-PRF vacu/L-PRF**	**A-PRF vacu/A-PRF+**	**A-PRF/L-PRF**	**A-PRF/A-PRF+**	**L-PRF/A-PRF+**	ANOVA
Coag weight gr	p=0.072>0.05	p=0.000<0.05	p=0.887>0.05	p=0.391>0.05	p=0.180>0.05	p=0.016<0.05	p=0.002<0.05
Essud. weight gr	p=0.105>0.05	p=0.000<0.05	p=0.700>0.05	p=0.372>0.05	p=0.449>0.05	p=0.000<0.05	p=0.001<0.05
Clot length mm	p=1.66>0.05	p=0.077>0.05	p=0.968>0.05	p=0.011<0.05	p=0.292>0.05	p=0.803>0.05	p=0.229>0.05
Clot width mm	p=1.56>0.05	p=0.027<0.05	p=0.638>0.05	p=0.913>0.05	p=0.029<0.05	p=0.013<0.05	p=0.032<0.05
Clot thickness mm	p=1.03>0.05	p=0.668>0.05	p=0.131>0.05	p=0.041<0.05	p=0.461>0.05	p=0.051>0.05	p=0.081>0.05
Surface Clot mm2	p=0.065>0.05	p=0.037<0.05	p=0.971>0.05	p=0.49>0.05	p=0.048<0.05	p=0.140>0.05	p=0.055>0.05
Membr. weight gr	p=0.743>0.05	p=0.001<0.05	p=0.646>0.05	p=0.009<0.05	p=0.679>0.05	p=0.069>0.05	p=0.028>0.05
Membr. length mm	p=0.079>0.05	p=0.09>0.05	p=0.676>0.05	p=0.239>0.05	p=0.031<0.05	p=0.045<0.05	p=0.059<0.05
Width Membr. mm	p=0.117>0.05	p=0.005<0.05	p=0.859>0.05	p=0.710>0.05	p=0.000<0.05	p=0.003<0.05	p=0.008<0.05
Membr.thickness mm	p=0.149>0.05	p=0.071>0.05	p=1.00>0.05	p=0.619>0.05	p=0.167>0.05	p=0.176>0.05	p=0.144>0.05
Surface Membr.mm2	p=0.260>0.05	p=0.560>0.05	p=0.983>0.05	p=0.000<0.05	p=0.263>0.05	p=0.255>0.05	p=0.220>0.05
%Clot Weight Ratio/ blood sample(%)10ml	p=0.087>0.05	p=0.000<0.05	p=0.089>0.05	p=0.000<0.05	p=0.784>0.05	p=0.000<0.05	p=0.000<0.05
p>0.05=not sinificant difference;			p<0.05=significant difference;				

The differences were found to be statistically significative by $p<0.05$.

Table 3. Mann-Whitney U test of significance.
Correlation of morphometric characteristics between various types of PRF membranes and clots obtained from equine blood (14).

			U Test of Mann-Whitney			
Parameters	**A-PRF vacu/A-PRF**	**A-PRF vacu/L-PRF**	**A-PRF vacu/A-PRF+**	**A-PRF/L-PRF**	**A-PRF/A-PRF+**	**L-PRF/A-PRF+**
Coag weight gr	*p=>0.06*	*p=<0.06*	*p=>0.06*	*p=>0.06*	*p=>0.06*	*p=>0.06*
Essud. weight gr	*p=>0.06*	*p=<0.06*	*p=>0.06*	*p=>0.06*	*p=>0.06*	*p=>0.06*
Clot length mm	*p=>0.06*	*p=>0.06*	*p=>0.06*	*p=<0.06*	*p=<0.06*	*p=>0.06*
Clot width mm	*p=>0.06*	*p=>0.06*	*p=>0.06*	*p=>0.06*	*p=<0.06*	*p=<0.06*
Clot thickness mm	*p=>0.06*	*p=>0.06*	*p=<0.06*	*p=<0.06*	*p=>0.06*	*p=<0.06*
Surface Clot mm2	*p=>0.06*	*p=>0.06*	*p=>0.06*	*p=>0.06*	*p=>0.06*	*p=>0.06*

Membr. weight gr	*p=>0.06*	*p=<0.06*	*p=>0.06*	*p=>0.06*	*p=>0.06*	*p=>0.06*
Membr. length mm	*p=>0.06*	*p=>0.06*	*p=>0.06*	*p=>0.06*	*p=<0.06*	*p=<0.06*
Width Membr. mm	*p=>0.06*	*p=>0.06*	*p=>0.06*	*p=>0.06*	*p=<0.06*	*p=<0.06*
Membr.thickness mm	*p=>0.06*	*p=<0.06*	*p=>0.06*	*p=>0.06*	*p=>0.06*	*p=>0.06*
Surface Membr.mm2	*p=>0.06*	*p=>0.06*	*p=>0.06*	*p=<0.06*	*p=>0.06*	*p=>0.06*
%Clot Weight Ratio/ blood sample(%)10ml	*p=>0.06*	*p=<0.06*	*p=>0.06*	*p=<0.06*	*p=>0.06*	*p=<0.06*
p>0.06= not sinificant difference;			p<0.06= significant difference;			

The differences were found to be statistically significative by p<0.06**.**

IV. DISCUSSION

From a clinical perspective, *L-PRF* and derivatives (A-PRF, A-PRF+) have excellent handling properties: single *L-PRF* clots are transformed into membranes of appropriate size and thickness, thanks to the "*L-PRF Wound Box"*; more membranes can be joined together (even sutured) and will be used to create a larger bioactive membrane to cover and construct larger grafts. The PRF membrane can be cut to size and being flexible it adapts well to different anatomical areas.

The *L-PRF/A-PRF* group, therefore, fits the necessities of different surgical procedures. Like clots and membranes, PRF has a shape and volume which is easy to complete with most surgical techniques. Such as filling and interposition of healing biomaterials or as protective membranes for wound healing. Finally, it is simple to prepare in medium quantities and inexpensive, making it particularly suitable for everyday clinical practice. It has was used with satisfaction in humans by AA., particularly in the treatment of diabetic skin ulcers, including those with chronic osteomyelitis (24-26). The researches performed by McLellan et al. (28) demonstrated that equine PRF which is comparable to human PRF provides an instantaneous and continuing font of tissue growth factors. Our study sought to standardize the method of preparation of L-*PRF/A-PRF* procedure which remains the easy-to-perform, low-cost technique that does not necessitate sophisticated technology and has consistency in the generation of an *L-PRF/A-PRF* membrane in terms of macroscopic and microscopic specifications. Autologous platelet concentrates are promising in the sphere of of regenerative practice and surgery because of the abundance of growth factors. *L-PRF* and its derivatives represent a huge critical advancement in the progress of platelet concentrates, as it is basically a fibrin membrane with platelets and leukocytes entrapped with stem cells. These solid diaphragms possess a superior manipulation characteristics and can be sutured securely in an anatomically desired position during open surgeries.

However, the physical and biological properties are relatively unknown and have yet to be fully investigated. The *L-PRF/A-PRF* membrane is consistently formed when the phases discussed above are rigorously followed. One of the relevant considerations for producing a good membrane is, in addition to the type of specific tube used, the lag time between blood collection/centrifugation and processing temperature. The secret success of the technique depends completely on the rapidity of blood collection and its instantaneous transfer to the centrifuge, usually within one minute, and a centrifugation and compression temperature above 21°C (between 21 and 30°C).

You can't generate a well-structured *L-PRF/A-PRF* coagulum (with its specific cellular content, matrix architecture, and growth factor release profile) if blood collection is prolonged and inhomogeneous or if the centrifugation temperature is below 21°C or above 30°C; instead, a small, incoherent, friable mass of fibrin with unknown content substance is formed. The functions of *L-PRF/A-PRF* as a temporary extracellular matrix, transformed into functional tissue during healing, being able to be subjected to mechanical forces with successful healing outcomes, depend on its structural integrity and therefore it is important to clarify its physical properties.

L-PRF/A-PRFs resemble dense connective tissue with superior handling characteristics.

Therefore, it is hypothesized that as in *L-PRF* also in A-PRF there is low stiffness (1-10 MPa) and high tension (up to 150%) of the membrane before rupturing.

Based on these results, it is clear that both *L-PRF* and A-PRF are novel biomaterials with unique features: -a predictable preparation from autologous blood; -simple protocol; -defined architecture; -impressive mechanical properties; -and an abundance of growth factors produced by activated platelets.

Our equine blood trials are undoubtedly increase understanding of the mechanisms of healing, as well as advancing the field of personalized medicine.

Limitations found in the therapeutic setting and the use of these products includes:

1) Because PRF is an autologous product, it is difficult to achieve a greater need for biomaterial availability. Therefore, its use in surgical procedures must be strictly controlled and planned.

2) PRF contains circulating immune cells as well as antigenic molecules that prevent its use as an allogeneic material; an increased risk of transmission of infectious it is also important to consider pathologies if it is of heterologous origin.

At this point, between the different markers that were not incorporated into in this type of classification, we recognize platelet concentration, leukocyte concentration, and the proportional amount of the various kinds of leukocytes. Problems with platelet concentration are nonexistent since almost all platelets included in the blood sample (99%) are activated and integrated into the fibrin matrix of the clot. As for the leukocyte count and concentration, their influence should be studied with particular attention, because their presence or absence could explain the conflicting results that are observed in the various studies (29, 31, 32).

V. CONCLUSIONS

Concluding this research, we can state that to obtain a standard procedure for the production of PRFs as graft material for tissue regeneration, we suggest the use of the PRF matrix region with the highest possible platelet enrichment and, in addition, avoiding crushing all the plasma in the PRF clot. Therefore, it is appropriate to compress the clot with a compression device *(L-PRF Wound Box)*. It is difficult, therefore, to precisely control the quality of materials of human or equine origin, such as PRF preparations (*L-PRF/A-PRF*), but it is important to apply the highest possible quality control to PRF preparations before their clinical application.

Preparation of PDPs is extremely technically challenging because of the delicate nature of platelets that are rapidly activated during manufacturing. Therefore, special recommendations are required to maximize therapeutic effects of these blood products.

Currently, their delivery is based on the poorly controlled bulk release. As a result, prolonged treatments require multiple treatments, such as numerous *i-PRF* injections. This results in large fluctuations in growth factor concentrations, compromising clinical predictability. Bio-materials may act as controlled-release devices that allow for prolonged or even on-demand administration of these mixtures of growth factors. In more, biomaterials can be expected to they covalently bind specifically growth factors at the local level maintain elevated levels of these molecules.

Further clinical, histological, and statistical research is required to comprehend the benefits of this new platelet concentration technique. However, we cannot ignore the circumstance that, when obtained from an autologous blood sample, the PRF produced is scarce and can only be used in a limited volume. This is a limitation to the regular use of PRF in Veterinary and Human Regenerative Surgery. Although the potential spheres of application of PRF are broad, accurate knowledge of the biomaterial, including information about its biology, efficacy, and limitations, is needed to maximize its use in everyday clinical experience.

The effectiveness of authological PRF as a biological promoter of tissue healing has been validated in human treatments. The minimalist PRF production procedure (a safe, easy, and inexpensive strategy without the need for advanced manufacturing capabilities), combined with low inherent variables in its preparation methodology, high level of reproducibility, and consistent clinical performance, determine the success of this biological product.

REFERENCES

[1]. Pretorius, E. et al, Ultrastructural comparison of the morphology of three different platelet and fibrin fiber preparations, The Anatomical Record; 290:188-198. (2007)

[2]. Choukroun, J., Adda, F., Schoeffler, C., Une opportunite en paro-implantologie: le PRF., Implantodontie; 55-62. (2001)

[3]. Litvinov, R.I., Weisel, J.W., What Is the Biological and Clinical Relevance of Fibrin?, Semin Thromb Hemost; 42: 333-43. (2016) http://dx.doi.org/10.1055/s-0036-1571342.

[4]. Kitamura, Y., Watanabe, T., Nakamura, M., Isobe, K., Kawabata, H., Uematsu, K., Okuda, K., Nakata, K., Tanaka, T., Kawase, T., Platelet Counts in Insoluble Platelet-Rich Fibrin Clots: A Direct Method for Accurate Determination, Front Bioeng Biotechnol; 6:4, (2018) http://dx.doi.org/10.3389/fbioe.2018.00004.

[5]. Soloviev, D.A., Hazen, S.L., Szpak, D., Bledzka, K.M., Ballantyne, C.M., Plow, E.F., Pluskota, E., Dual Role of the Leukocyte Integrin M 2 in Angiogenesis, J Immunol; 193: 4712-4721. (2014) http://dx.doi.org/10.4049/jimmunol.1400202.

[6]. El Bagdadi, K., Kubesch, A., Yu, X., Al-Maawi, S., Orlowska, A., Dias, A. et al. Reduction of relative centrifugal forces increase growth factor release within solid platelet-rich-fibrin (PRF)-based matrices: a proof of concept of LSCC (low-speed centrifugation concept). Eur J Trauma Emerg Surg. Mar. (2017) https://doi.org/10.1007/s00068-017-0785-7

[7]. Choukroun, J., Advanced-PRF and i-PRF: platelet concentrates or blood concentrates? J Periodont Med Clin Pract;1:1-3. (2014)

[8]. Rubio, M.M., Longitudinal Assessment of FEV1 Change Following Autologous Cellular Therapy. J Reg Med Biol Res.;2(1):1-11(2021). http://dx.doi.org/10.46889/JRMBR.2021.2101.

[9]. Miron, R.J., Fujioka-Kobayashi, M., Hernandez, M., et al. Injectable platelet-rich fibrin (i-PRF): opportunities in regenerative dentistry? Clin Oral Invest;21:2619-27. (2017)

[10]. Ghanaati, S., Booms, P., Orlowska, A., et al. Advanced Platelet-Rich Fibrin: a new concept for cell-based tissue engineering by means of inflammatory cells. J Oral Implantol;40:679-89. (2014)

[11]. Schär, M.O., Diaz-Romero, J., Kohl, S., Zumstein, M.A., Nesic, D., Platelet-rich Concentrates Differentially Release Growth Factors and Induce Cell Migration In Vitro. Clin. Orthop. Relat. Res.; 473:1635-43. (2015) http://dx.doi.org/10.1007/s11999-015-4192-2.

[12]. Satué, K., Gardón, J.C., Muñoz, A., Interpretation of platelets in the horse. J. Hematol. Res,; 4: 19-25. (2017) http://dx.doi.org/10.12974/2312-5411.2017.04.3.

[13]. Miron, R.J., Chai, J., Zheng, S., Feng, M., Sculean, A., Zhang, Y., A Novel Method for Evaluating and Quantifying Cell Types in Platelet Rich Fibrin and an Introduction to Horizontal Centrifugation, J Biomed Mater Res; 107, 2257-2271,(2019). https://dx.doi.org/10.1002/jbm.a.36734.

[14]. Crisci, A., Barillaro, M. C., Lepore, G., Cardillo, F., L-PRF Membrane (Fibrin Rich in Platelets and Leukocytes) and Its Derivatives (A-PRF, i-PRF) are Helpful as a Basis of Stem Cells in Regenerative Injury Treatment: Trial Work on the Horse. International Blood Research & Reviews; 10(2): 1-14.(2020) https://doi.org/10.9734/ibrr/2019/v10i230117.

[15]. Crisci, A., Crisci, M., Boccalone, E., Final results of experimental research about a technique of measurement of skin lesions. Dermatological Experiences; 16: 147-52. (2014)

[16]. Watanabe, T., Isobe, K., Suzuki, T., et al, An Evaluation of the Accuracy of the Subtraction Method Used for Determining Platelet Counts in Advanced Platelet-Rich Fibrin and Concentrated Growth Factor Preparations, Dent J; 5: 7 (2017). http://dx.doi.org/10.3390/dj5010007.

[17]. Crisci, A., Kawase, T., D'Adamo, R., Crisci, M., Experimental research on a technique for quantification of platelets and leukocytes in second-generation platelet concentrates, International Journal of Current Medical and Pharmaceutical Research; 05(12): 4792-99. (2019) http://dx.doi.org/10.24327/23956429.ijcmpr201912804.

[18]. Kobayashi, M., Kawase, T., Horimizu, M., Okuda, K., Wolff, L.F., Yoshie, H., A proposed protocol for the standardized preparation of PRF membranes for clinical use, Biologicals; 40: 323-29. (2012)

[19]. Dohan Ehrenfest, D.M. et al, Do the fibrin architecture and leukocyte content influence the growth factor release of platelet concentrates? An evidence-based answer comparing a pure platelet-rich plasma (P-PRP) gel and leukocyte- and platelet-rich fibrin (L-PRF), Current Pharmaceutical Biotechnology; 13: 1145-52. (2012)

[20]. Crisci, A., Benincasa, G., Crisci, M., Crisci, F., Leukocyte Platelet-Rich Fibrin (L-PRF), a new biomembrane useful in tissue repair: basic science and literature review. Biointerface Res Appl Chem 2018;8:3635-43.

[21]. Crisci, A., De Crescenzo, U., Crisci, M., Platelet-Rich Concentrates (L-PRF, PRP) in tissue regeneration: control of apoptosis and interactions with regenerative cells. J Clin Mol Med;1:5-12. (2018)

[22]. Crisci, A., Lombardi, D., Serra, E., et al. Standardized protocol proposed for clinical use of L-PRF and the use of L-PRF Wound Box® . J Unexplored Med Data;2:77-87. (2017) http://dx.doi.org/10.20517/2572-8180.2017.17

[23]. Crisci, A., Manfredi, S., Crisci, M., Fibrin Rich in Leukocyte-Platelets (L-PRF) and Injectable Fibrin Rich Platelets (I-PRF), Two Opportunity in Regenerative Surgery: Review of The Sciences and Literature. IOSR Journal of Dental and Medical Sciences (IOSR-JDMS); 18 (4): 66-79. (2019) http://dx.doi.org/10.9790/0853-1804106679

[24]. Crisci, A., Marotta, G., Licito, A., Serra, E., Benincasa, G., Crisci, M., Use of leukocyte platelet (L-PRF) rich fibrin in diabetic foot ulcer with osteomyelitis (three clinical cases report), Diseases; 6:30. (2018) http://dx.doi.org/10.3390/diseases6020030.

[25]. Crisci, A., D'Adamo, R., Crisci, M., The Second-Generation Platelet Concentrates in The Treatment of Chronic Ostemyelitis: One Modern Regenerative Surgery. International Journal of Research -GRANTHAALAYAH, , 8(10), 112-122. (2020) https://doi.org/10.29121/granthaalayah.v8.i10.2020.1842

[26]. Giuliano, G., Crisci, M., D'Adamo, R., Crisci, A., Second-generation platelet concentrates in the therapy of chronic osteomyelitis: a modern regenerative surgery, Giornale Italiano di Ortopedia e Traumatologia;46:1-12; (2020) https://doi.org/10.32050/0390-0134-271

[27]. Hasan, F.K., Characterization of leukocyte-platelet rich Fibrin, a novel biomaterial, 2015. http://scholarscompass.vcu.edu/etd

[28]. McLellan, J., Plevin, S., Temporal release of growth factors from platelet-rich fibrin (PRF) and platelet-rich plasma (PRP) in the horse: a comparative in vitro analysis. Intern J Appl Res Vet Med; 1: 48-57. (2014)

[29]. Sato, A., Kawabata, H., Aizawa, H., Tsujino, T., Isobe, K., Watanabe, T., Kitamura, Y., Miron, J.R., Kawase, T., Distribution and quantification of activated platelets in platelet-rich fibrin matrices, Platelets 2020. https://doi.org/10.1080/09537104.2020.1856359.

[30]. Crisci, A., Kawase, T., D'Adamo, R., Crisci, M., Quantification of platelets and leukocytes in solid blood platelet concentrates for surgical use: study of an experimental technique. Chirurgia;34(4):141-53. (2021) https://doi.org/10.23736/S0394-9508.20.05129-3

[31]. Crisci, A., New Platelet Concentrates Useful in Tissue Repair. Platelet-rich Fibrin with Leukocytes (*L-PRF*), Advanced Platelet-Rich Fibrin (A-PRF) and Injectable Plateletrich Fibrin (*i-PRF*), BP International Edition, United Kingdom, 2021, https://doi.org/10.9734/bpi/mono/978-93-91473-15-0

[32]. Crisci, A., Crisci, M.**,** Second-Generation Platelet Concentrates (PRFs): Estimation of Cellular Content**,** Biointerface Research in Applied Chemistry, 12, 4, , 4747 - 4754, (2022) https://doi.org/10.33263/BRIAC124.47474754