



## **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**

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### **Authors' contributions**

*This work was carried out in collaboration among all authors. All authors read and approved the final manuscript.*

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### **ABSTRACT**

Developing a multidisciplinary field of tissue engineering aims to recover, improve or supplant typically damaged or missing tissues for a collection of conditions brought about by trauma, malady and old age. To guarantee that tissue engineering techniques are generally relevant in the clinical setting, it is important to adjust them so that they are promptly accessible and moderately simple to use in the everyday clinical schedule. Consequently, the steps between preparation and application

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must be limited and improved to make them practical in application. The general objective of creating platelet concentrates of natural origin can be delivered near the patient to quicken the implantation procedure, being monetarily practical for the patient and the health framework. Fibrin rich in platelets and leukocytes (PRF) and its derivatives (L-PRF, A-PRF, i-PRF) has been utilized in a wide collection of medical fields for delicate tissue restoration. Practically all platelets (> 97%) are missing within test-tubes in groups tested after PRF membrane extraction. Growth Factors liberated by platelets contained in derivatives of L-PRF induce and control the proliferation and migration of other cell types, associated with tissue repair, similar to smooth cell muscles (SMCs) and mesenchymal stem cells (MSCs). Conclusively, the outcomes of this work feature the positive effects of PRF on wound healing after regenerative treatment for the administration of different delicate tissue defects found in wound care.

**Keywords:** *Blood derivatives; growth factors; injectable platelet-rich fibrin; leucocyte platelet-rich fibrin; L-PRF wound box; stem cells.*

## 1. INTRODUCTION

The latest scientific proof proposes that platelets could assume a new role in tissue repair and in vascular remodeling, other than being dynamic actors in inflammatory and immune reactions. Platelets discharge naturally dynamic proteins and different contents, ready to impact a progression of procedures, favoring cell intake, growth and morphogenesis. These contents are discharged or uncoated on the surface of induced platelets. The platelet ability to discharge contents within a clot makes the clot itself a natural autologous basis of growth variables and cytokines, which can be remedially used to quicken and accelerate physio-scientific physio-clinical healing forms. A considerable lot of these contents are assembled and stored in platelets  $\alpha$ -granules, effectively related to Scanning Electron Microscope (SEM) and with immunofluorescence staining.

The fine fibrin fibers contained in HPC (Human Platelet Concentrate) could be identified with the high initial concentration of platelets in HPC ( $3-5 \times 10^{11}$  platelets/l), where local procoagulant action could even be improved through the beginning of pro-thrombotic stimuli enhancement, and it prompts a practically dangerous thrombin generation, therefore causing an expansion in fibrinogenesis on the surface of platelets, which thus prompts fibrin growth and polymerization [1].

The presentation of blood concentrates, for example, Platelet-Rich Plasma and Fibrin (PRP/PRF) was the initial move toward meeting clinical necessities [2] (Fig. 1). This blood concentrate is gotten from the patient's fringe blood after one-step centrifugation without anticoagulants to produce a platelet- and

leucocyte-rich framework. The presence of platelets, leucocytes, and fibrin was recently demonstrated to be basic for wound healing [3,4]. Furthermore the capability of leucocytes to impact angiogenesis and lymphomagenesis, this fibrin network, containing leucocytes and platelets, is the basis of cytokines and growth factors, which are primary membranes during the process of injury healing [5]. The utilization of explicit plastic tubes favors non-clotting PRF and brought about the improvement of a fluid PRF-based network (fluid PRF) created without the requirement for anticoagulants. Beforehand, a precise study explored the impact of the applied relative centrifugal force (RCF) on the synthesis and bioactivity of PRF-matrices. This fibrin platform, which has no cytotoxic potential, is acquired from 9 ml of the patient's blood after 1 phase of centrifugation and contains a collection of blood cells – including platelets, B and T lymphocytes, monocytes, stem cells, and neutrophil granulocytes – Furthermore growth factors.

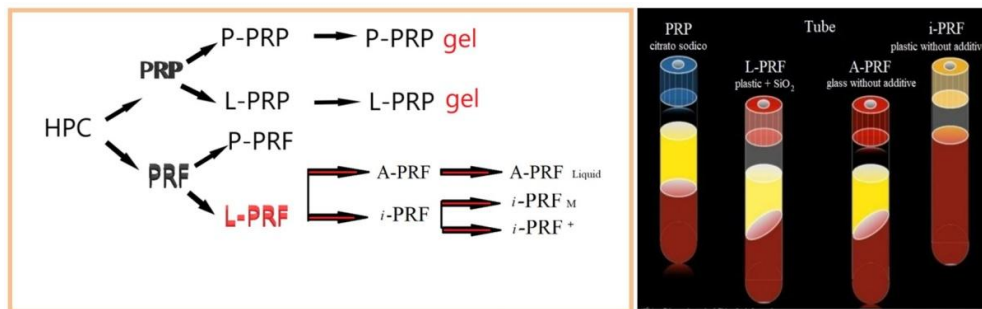
Moreover, L-PRF (likewise called leucocyte-PRF) contains white blood cells, necessary cells that are significant during the injury healing process [6]. In addition, since white blood cells, including neutrophils and macrophages, are among the first kinds of cells present in wound sites, their role additionally incorporates phagocytic fragments, microbes, and necrotic tissue, accordingly avoiding infection. Macrophages are likewise key cells derived from the myeloid genealogy and are viewed as one of the key cells associated with growth factor secretion during wound healing, including the transforming growth factor-beta (TGF- $\beta$ ), platelet derived growth factor (PDGF) and growth factor vascular endothelium (VEGF) (Fig. 2). These cells, together with neutrophils and platelets, are key

membranes in wound healing and in mix with their growth factors/discharged cytokines can encourage tissue restoration, the solution of fresh blood vessels (angiogenesis) and the infection prevention. The prevention of infections can be explained by the action of phagocytosis and apoptosis, which have the function of killing infectious cells and necrotic cell residues [3].

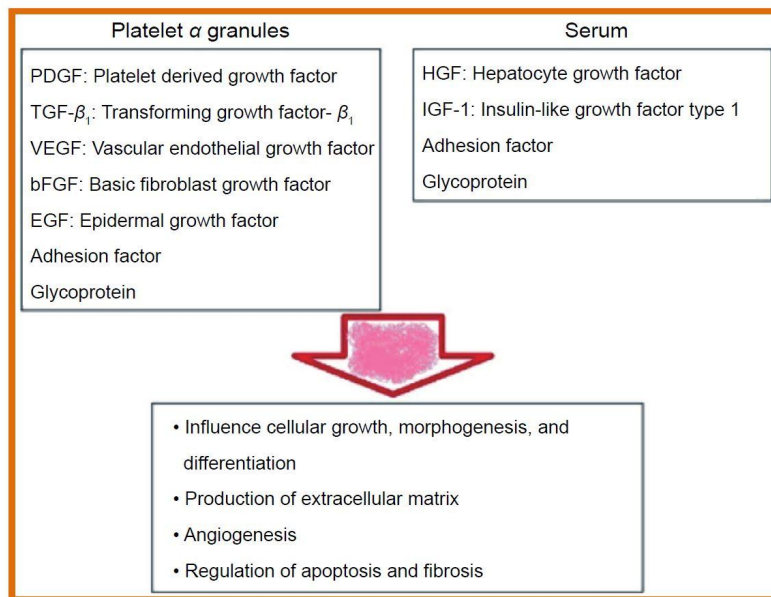
**L-PRF:** In the longitudinal area of the L-PRF coagulum, created in accordance with the standard centrifugation protocol (30" of speeding up, 12' at 2700 rpm [816 g-force] and 36" of deceleration and stopping) [6], a thick fibrin clot is available with negligible inter-fiber space. Cells

are observed all through the blood clot, albeit diminishing towards the most distal fragments of the PRF clot (Figs. 1 and 3).

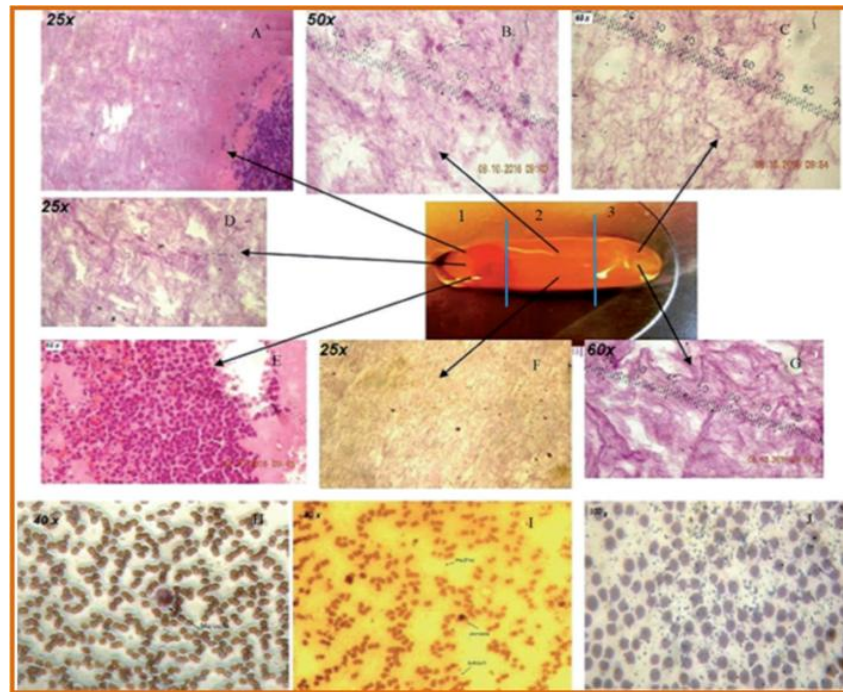
**Advanced-PRF:** The PRF clots framed with the A-PRF centrifugation protocol (Advanced-PRF) (1300 rpm, 8 minutes)(189 g-force) [7] indicated a more liberated structure with more inter-fiber space and more cells can be included in the fibrin-rich clot. Moreover, the cells are more uniformly dispersed in the clot than L-PRF, and a few cells can likewise be found in the most distal fragments of the clot. A representative picture for cellular circulation within A-PRF is presented in Fig. 4.



**Fig. 1. Different types of human platelet concentrates (HPCs): PRP (Platelet-rich plasma); PRF (Platelet-rich fibrin); P-PRP (Pure Platelet-rich plasma); L-PRP (Leukocyte and Platelet-rich plasma); P-PRF (Pure Platelet-rich fibrin); L-PRF (Leukocyte and Platelet-rich fibrin); i-PRF (Injectable Platelet-rich fibrin); A-PRF (Advanced Platelet-rich fibrin)**



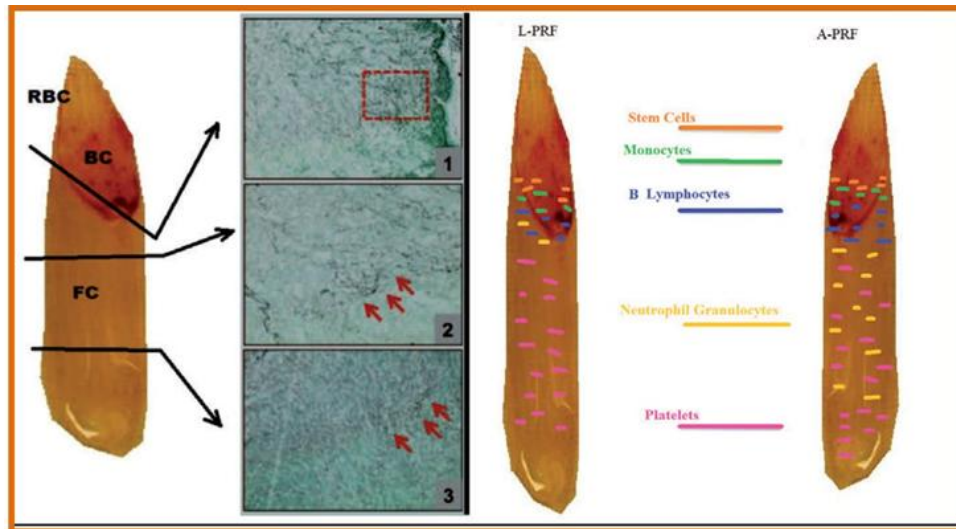
**Fig. 2. Function of the platelets in wound healing**



**Fig. 3. Horse L-PRF membrane at 0 minutes from compression (eosin-hematoxylin coloring)**  
 The L-PRF membranes were fixed in 10% formalin buffered neutral solution at pH 7.2 for 48 hours and combined in paraffin as indicated by the standard method. Twenty sequential areas (7  $\mu$ m thickness) of each example were cut utilizing a microtome. An) III proximal ingr. 25 $\times$  white blood cells - fibrin reticulum; B) III average ingr. 60 $\times$  erythrocytes-fibrin design; C) III distal ingr. 60 $\times$  fibrin reticulum; D) III proximal ingr. 25 $\times$  erythrocytes-fibrin; E) III proximal ingr. 60 $\times$  fibrin on the right, lymphocytes in the center, erythrocytes and neutrophil granulocytes on the left; F) III medium ingr. 25 $\times$  fibrin lattice; G) III distal ingr. 60 $\times$  fibrin reticulum; H) Red clot smear ingr. 40 $\times$  presence of monocita in a carpet of erythrocytes; I) smear red clot ingr. 40 $\times$ presence of erythrocytes, monocytes and platelets; J) smear red clot ingr. 100 $\times$ platelets in a carpet of erythrocytes (May-Grünwald-Giemsa stain). Duplicated from Crisci et al. [4] authorized under the formulations of Creative Commons Attribution 4.0 International License

*PRF injectable formulation:* The growth of an injectable formulation of PRF (referred to as i-PRF) [8,9] (centrifuged at 700 rpm [60 g-force] for 3 minutes) was sought after with the objective of conveying a platelet concentrate that is simple to use by specialists in fluid formulation that can be utilized alone or effectively combined with different biomaterials. Exploiting increasingly slow centrifugation speeds, a more prominent presence of regenerative cells with higher concentrations of growth variables can be observed contrasted with other PRF formulations utilizing higher centrifugation rates. Ghanaati et al. [7] reported that speed and time do not influence monocyte and stem cell concentrations, yet impact platelet and neutrophil concentrations. Subsequently, A-PRF contains more platelets; mostly found in the distal piece of the L-PRF and membrane which incorporate more neutrophils.

This kind of concentrate can possibly improve angiogenesis by communicating the enzymatic lattice metalloproteinase-9. In this manner, the incorporation of neutrophils in the PRF could be considered if angiogenesis is of interest. Investigation of the examination by Ghanaati et al. [7] additionally uncoated that the platelets were the only ones present in every coagulum zone up to 87 $\pm$ 13% in the L-PRF group and up to 84 $\pm$ 16% in the A-PRF group (Fig. 4). Moreover, the outcomes indicated that T lymphocytes (L-PRF: 12 $\pm$ 5%, A-PRF: 17 $\pm$ 9%), B lymphocytes (L-PRF: 14 $\pm$ 7%, A-PRF:12 $\pm$ 9%), CD34 positive stem cells (L-PRF: 17 $\pm$ 6%, A-PRF:21 $\pm$ 11%) (Fig. 5), and Monocytes (L-PRF: 19 $\pm$ 9%, A-PRF: 22 $\pm$ 8%) not over 30% of the total length of the clot have been found past a specific point since they are conveyed close to the BC produced by the centrifugation procedure.



**Fig. 4. Advanced-PRF (A-PRF) total scan of a fibrin clot along with its longitudinal centrifuge (Masson-Goldner staining). RBC represents the part of red blood cells. The buffy coat (BC) is the change zone between the fraction of RBC and the fibrin clot and FC represents the fibrin clot. The three bars within the scan and the arrows show the main floors of the separate territories. The red arrows mark cells that are trapped within the fibrin system**

## 2. MATERIALS AND METHODS

### 2.1 L-PRF Processing

L-PRF<sup>®</sup> generation protocol is extremely basic: blood is promptly centrifuged within 2 minutes from withdrawal, following the consequent steps: 30" of increasing speed, 12' at 2700 rpm (816 g-force) and 36" of deceleration and capture. The outcome product is made of three membranes: PPP (Platelet-poor plasma at the top), PRF (central clot), Red Blood Cells (RBCs) at the base, with Duo centrifuge (Process for PRF, France) (Figs. 1 and 4). Resulting PRF clots are assembled and red blood cells are expelled with the aid of scissors, without macroscopical damage at PRF structure expense. There is no macroscopic damage to the membrane as the cut is made about 2 mm beyond the white clot.

We performed a prospective investigation of equine blood, which has been collected in test tubes without anticoagulant plastic-coated glass, nor a gel separator (Vacutainer tubes for serum 9.0 ml), for the generation of L-PRF clots and membranes by six healthy horses of different ages (average  $\pm$  SD,  $10 \pm 4.1$  years, ranging from 4 to 17 yrs), gender, and breed.

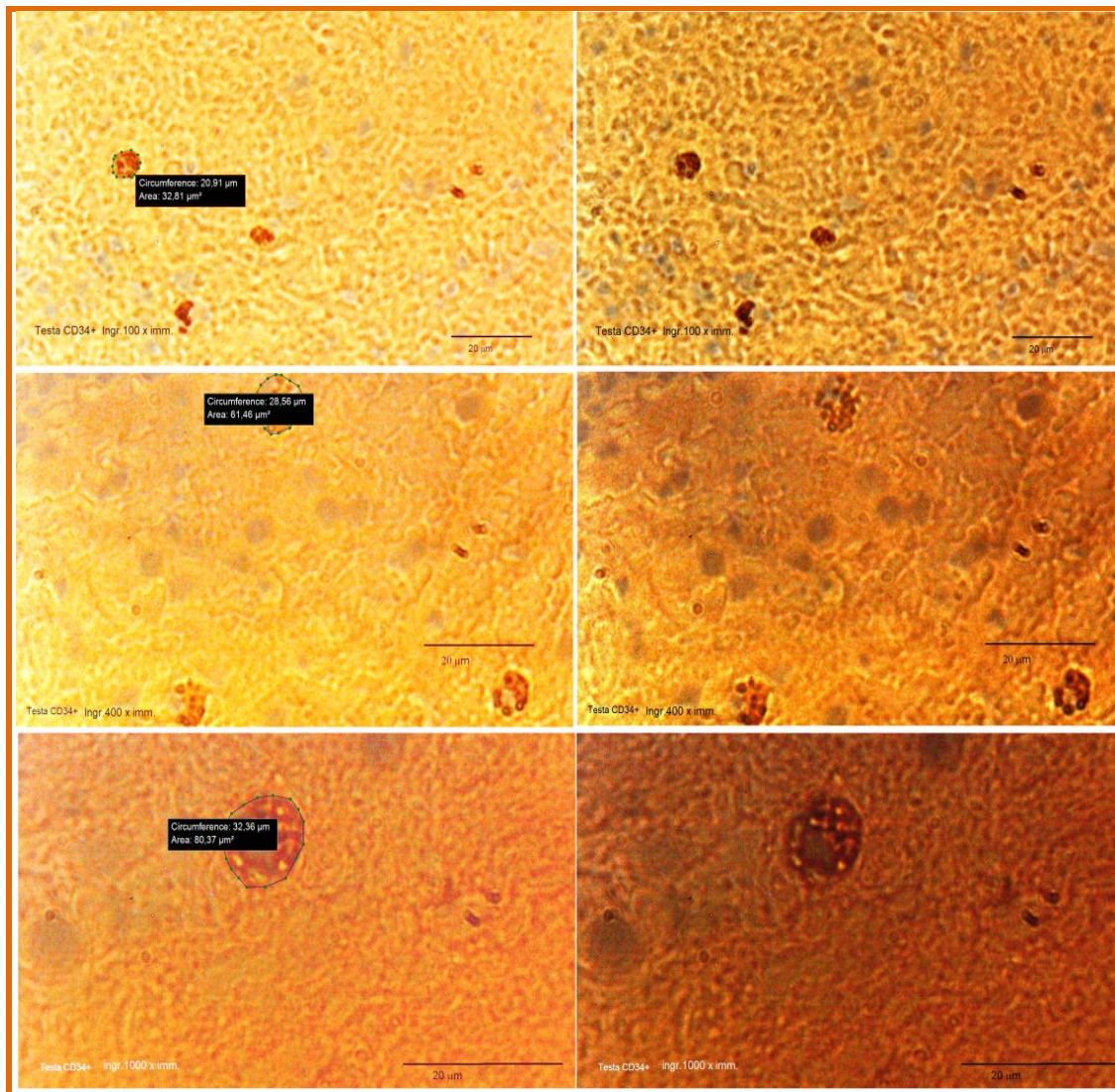
A composed assent of the proprietors has been accommodated for all horses and the blood collection method has been performed as per the current AVMA guidelines.

The blood was collected rapidly with 9 cc with needle 19G to Vacutainer tubes (22" average value, of under 25" per tube) and quickly (within 1 min) centrifuged by the previous depiction to a temperature greater than 21°C. Glass tubes coated with plastic without anticoagulant or gel separator (A-PRF+ Vacutainer tubes for Serum 9.0 ml) were utilized to deliver additionally A-PRF clots and membranes at an encompassing temperature above 21°C (21-30°C).

Fibrinogen is at first concentrated in the center and a predominant portion of the test tube, which is in between red blood cells (RBCs) at the base and the Platelet-Poor Plasma (PPP) at the top. Clot compression by means of a compression framework (L-PRF box) fundamentally stimulates cell proliferation and neovascularization [10].

Furthermore, with the standard formulations, PRFs can likewise be acquired in injectable form (i-PRF). i-PRF is gotten by creating a PRF, which isn't in this manner compressed. Profitably, this injectable material can coagulate following injection (within 10-12 minutes) to form a biomaterial and can be combined with any biomaterial of choice for non-covalent consolidation [11].

The PRF was subdivided into 3 regions, of equivalent length (Fig. 3) and platelet presence in every area was observed through S.E.M., through Optic Microscope in horse-derived preparations [12].



**Fig. 5. CD34+ stem cells found in the proximal part (head) of the self-compressed horse A-PRF membrane. Ingr. 100 ×, 400 ×, 1000 × immersion with measurements. The scale corresponds to 20 microns**

Region 1 is the region nearest to the red clot and shows a prominent number of platelets totals, showing a few lymphocytes and other white blood cells. Platelet count is decreased as the distance from the red clot is expanded. Within region 2 (central region), we observed fibrin fibers (primary and secondary fibers) and a few platelets. Within region 3, the fibrin mesh is very clear, while the platelet count is low (Fig. 3).

Under a clinical perspective, L-PRF and derivatives (A-PRF, i-PRF) shows great dealing with properties: single L-PRF clots can be transformed into membranes of fitting thickness

and measurements, on account of the "L-PRF Wound Box"; combining at least two membranes is valuable (can likewise be sutured) to make a bioactive membrane of greater measurements, to cover and form greater grafts. L-PRF membrane can likewise be cut and custom-fitted. Being adaptable enough, it adjusts to various anatomical regions.

L-PRF/i-PRF family adjusts to the necessities of the different surgical mediation. Much the same as clots and membranes, L-PRF has shape and volume effectively combined with the incredible greater part of surgical procedures, as filling

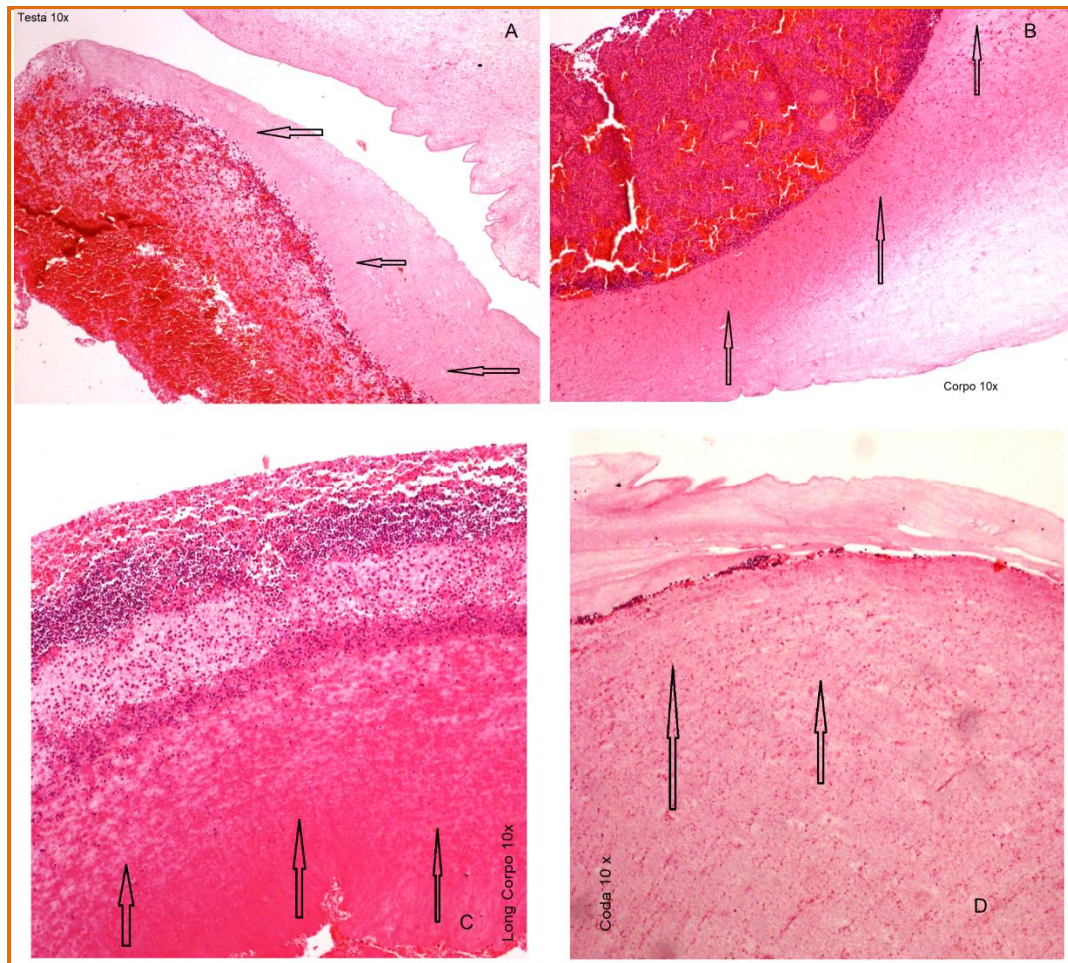
strategy and healing biomaterial apposition, or as protecting membranes for wound healing. Moreover, it is easy to prepare, additionally in extraordinary amounts, and cost-effective, making it especially suitable for ordinary clinical practice. It was effectively utilized by AA, particularly in the treatment of cutaneous diabetic ulcers, additionally with osteomyelitis presence [13].

## 2.2 Macroscopic Analysis

After centrifugation, the L-PRF and A-PRF clots (Fig. 4) product expelled from the test tube utilizing sterile tweezers and a smooth spatula to delicately discharge the red clot from the buffy

coat. Every L-PRF/A-PRF clot acquired was put in a tray for estimating the weight and size with a computerized scale from a goldsmith and a digital device.

The compression of the clot was done with the L-PRF Wound Box we designed with a compression of  $142.437 \text{ Pa/cm}^2$  constant for two, five, ten, fifteen minutes (no longer lasting compressions were tested on the grounds that they did not demonstrate to be useful in the past examination contrasted with the two-minute investigation). This strategy enabled us to obtain, from each clot, the L-PRF membranes, which were independently gauged and estimated with a computerized device.



**Fig. 6. Horse Advanced-PRF (A-PRF). Platelet distribution in PRF matrix prepared using glass tubes for A-PRF+ by low centrifugation (1300 rpm, 8 minutes). Sections of the 10 × regions of the head (A), body (B, C) and tail (D), stained with HE, are shown. The arrows indicate the direction of the centrifugal force. The upper margins of the PRF membrane, to which blood cells and serum proteins are attached, represent the region facing the inner wall of the tube**

At long last, they were captured with a computerized camera (Nikon) to have the option to ascertain the surface in  $\text{cm}^2$  as per the strategy we reported in the literature [14]. The surface region in  $\text{cm}^2$  of clots and the membranes were estimated with the Calcderm estimation software [14].

### 2.3 Optical Microscopy Procedure

The membranes were fixed in 10% neutral buffered formalin for 24 h. Briefly, the samples were dehydrated in a graded series of alcohol, treated with xylene, and embedded in paraffin. After deparaffinization and rehydration with xylene and graded ethanol (decreasing concentration), the samples were stained with histochemical stains. Segments of 4  $\mu\text{m}$  were performed along the midpoint of the membranes and were stained with Haematoxylin-Eosin (Fig. 6). Each segment was isolated into three equivalent estimated areas: proximal (head), center (body), and distal (tail). Every zone of these segments was seen through an optical microscope (Kern OBN-148) and examined by checking the noticeable cell bodies (marked in dark purple, for the most part leukocytes) at the central point of each observed zone with a magnification of 10 $\times$ , 25 $\times$ , 40 $\times$ , 60 $\times$ , and 100 $\times$  (immersion) (Figs. 3, 5 and 6). The complete number of counted cell bodies was utilized to correlate their appropriation between the three membrane regions (head, body, and tail). A large portion of the cells was concentrated in the proximal region (head), nearest to the red clot.

Smears of blood prepared by residual blood in the tubes were likewise analyzed for a morpho-scientific appraisal after expulsion of the PRF clot with a spatula (two for each tube), separating the clot at 0 min by centrifugation and fixing them with alcohol 90% for a Grönwald-May-Giemsa coloring stain so as to recognize the different corpuscular components, specifically platelets and neutrophils, to contrast them in connection with the assessment blood count.

A blood test was additionally taken from each horse to achieve a blood count utilizing K3E 5.4 mg EDTA tubes (VacuMed).

The supernatant got from compression with the Wound Box membranes L-PRF and A-PRF was separated between the 0 min and has been safeguarded in a test tube with K3E 5.4 mg EDTA for blood count examination. It was contrasted and the basal one and with the

corpuscular components of the counts performed on smears got from the red clot as an indirect estimation of the platelet and the leukocyte concentrations of the L-PRF.

The supernatant got from the squeezing of L-PRF/A-PRF at 0 min was analyzed with a blood count in standard lodging. Since the direct estimation of platelet concentration of the PRF is not yet conceivable, we determined the residual platelet concentration with the subtraction strategy as per the accompanying formula from Watanabe et al. [15].

$$PLT/WBC \text{ in A-PRF and L-PRF} = PLT/WBC \text{ in the whole blood} - [(PLT/WBC \text{ in red clot}) - (PLT/WBC \text{ in the serum over the clot of PRF}) - (PLT/WBC \text{ in the supernatant after compression of the clot PRF})]$$

The examinations were performed with a Cell Dyn 3500 R cell counter (ABBOTT).

### 2.4 Immunohistochemical Examination for Stem Cells

The freshly prepared PRF clots were gently, but not completely, compressed with a stainless steel PRF compression device (Wound L-PRF Box) for two minutes [6], washed three times with Buered Saline Phosphate (PBS), and fixed in 10% neutral buffered formalin. After splitting the fixed PRF membranes into 3 pieces (Fig. 3), they were processed in a Leica Biosystems processor. The location of the stem cells in the PRF matrices was determined using the immunohistochemical method.

A Ventana anti-CD34 antibody [CONFIRM anti-CD34 (QBEnd/10) Primary Antibody from Ventana<sup>®</sup> Medical Systems] was used: monoclonal mouse antibody (IgG1).

The antibody is designed for use in order to qualitatively identify CD34 antigen under a light microscope, on sections of tissue fixed in formalin and included in paraffin, in a Ventana auto stainer.

This antibody is optimized for use in combination with iVIEW<sup>TM</sup> DAB determination kits. No reconstitution, mixing, dilution or titration is required. Normally treated tissues, i.e. formalin-fixed and included in paraffin, can be used with this primary antibody when used with the Ventana determination kits and a Ventana autostain slide stained.



Each section was cut to a thickness of 4-5 µm and placed on a positively loaded slide. The slides with the tissue section were dried for at least 2 hours in an oven at 70°C ± 5°C.

The chromogen used is Diamminobenzidine (DAB) which gives an insoluble oxidation product coloured brown.

### 2.5 Statistical Analysis

The statistical significance for the contrasts between the two groups was determined utilizing Student's t-test for rehashed measures for parametric factors and with the  $\chi^2$  for non-parametric factors. P values < 0.005 were considered statistically noteworthy. The information was analyzed utilizing the Discipline Biomedical Statistics of Stanton-Glantz 2007 software package, version 6.0.

### 3. RESULTS

The methodology was well-tolerated in all animals. No significant contrasts were found in the primary hemato-scientific confrontation, which had an average concentration of RBC  $9.8 \times 10^6/\text{mL}$  (range:7-13), WBC  $5.1 \times 10^3/\text{mL}$  (range:5-13) ( $\pm 0.37$  C.I. 95%) ( $p=0.24$ ) and a platelet count average of  $106.8 \times 10^3/\text{mL}$  (run: 100-350) ( $\pm 15.3$  I.C.95%) ( $p=0.5$ ). It was impractical to directly evaluate the platelet concentration and WBC trapped within the L-PRF clot, which was derived in an indirectly by contrasting the mean estimations of entire blood, the mean estimations of the supernatant acquired after compression of the clot at 2 min, and the average values got with the counts of smears of the red clot after the evacuation of the L-PRF clot. Table 1 shows the number of leukocytes, red blood cells and platelets in the entire blood (control group) and red blood clot after collection of the PRF membrane (test group) liable for remedial potentiation.

Table 2 relates the features of clots and membranes of PRF acquired in humans (centrifugal Intraspin) reported by Pinto et al. [16] and those we observed in the horse model

(centrifuge DUO). In this examination, it was checked that there are significant contrasts in the attributes of the clot (weight), yet these distinctions are eliminated when the membranes got from the compression were inspected. This perception, as we would like to think, would be assigned to various content of exudate (weight of the exudate is  $1.47 \pm 0.13$  g in humans,  $3.05 \pm 0.11$  g in horses,  $p = 0.000$ ).

In this investigation, the size of the membranes is not seen as in connection to the hemoglobin content or the content of erythrocytes encountered in the blood count baseline. In optical microscopy (Fig. 3), the vast majority of the cell bodies (stained in dark purple for the nuclei) were concentrated in the proximal part (head) of every membrane, the last 1/4 was seen at the center, and the distal part had just residual traces of cell bodies. Optical microscopy has not, be that as it may, permitted the perception of the definite condition of these cell bodies in more prominent formulation.

The outcomes of the blood count's of entire blood and of the supernatant acquired from the clot after 0 min compression are contrasted and the counts of erythrocytes, platelets, and the WBC smear of the red clot after 0 min are shown with the comparing statistical tests in Table 3.

Table 3 exhibits the measurably noteworthy distinction between the content of RBC, WBC, and platelets between the supernatant got from the compression of the clot at 0' and the values acquired with the blood count. There is likewise statistically significant contrast shown between the content of RBC, WBC, and PLT in smears got from the red clot at 0 min, as shown in Fig. 3 A–C at different magnifications. Table 3 likewise shows the Theoretical content of RBC, WBC, and PLT in L-PRF/A-PRF membranes got from the distinction of these corpuscular components between entire blood, the supernatant at 0 min since centrifugation, and the smear of the red clot at 0 min. The Student t-test shows noteworthy contrasts between RBCs at 0 min and PLT at 0 min in L-PRF/A-PRF membranes and the blood count test.

**Table 1. Leukocytes, RBC and platelets number in whole blood (control group) and red clot after PRF membrane collecting (test group)**

Control	Leukocytes/ $\mu\text{L}$		RBC/ $\mu\text{L}$		Platelets/ $\mu\text{L}$	
	Mean	Range	Mean	Range	Mean	Range
	6.900	6.100-7.800	5.19 (106)	5.01-5.52 (106)	2.66 (105)	2.18-3.09 (105)
Group 1	3.500	3.000-3.800	5.89 (106)	5.75-6.08 (106)	6.000	4.000-8000
Group 2	3.600	3.300-4.000	5.84 (106)	5.78-5.91 (106)	7.000	6.000-9000

**Table 2. Comparison of membranes obtained from human blood (Pinto et al., 2014) and from equine blood**

Variable	Man (n = 8)	Horse (n = 6)	Student mins t	Significance
	Average ( $\pm$ SD)			
Final T° test tube (°C)	27.5 ( $\pm$ 0.66)	-	-	-
Weight of the Clot (g)*	2.09 ( $\pm$ 0.19)	4.23 ( $\pm$ 0.55)	$p = 0.000 < 0.005$	S
Weight of the Membrane (g)	0.62 ( $\pm$ 0.15)	0.78 ( $\pm$ 0.08)	$p = 0.036 > 0.005$	NS
Exudate Weight (g)	1.47 ( $\pm$ 0.13)	3.05( $\pm$ 0.11)	$p = 0.000 < 0.005$	S
Length of the Clot (mm)	35.69 ( $\pm$ 3.43)	44.38( $\pm$ 3.83)	$p = 0.000 < 0.005$	S
Width of the Clot (mm)	12.81 ( $\pm$ 0.75)	14.74( $\pm$ 1.23)	$p = 0.003 < 0.005$	S
Height of the Clot (mm)	-	7.02 ( $\pm$ 1.09)	-	-
Surface Area of the Clot (mm <sup>2</sup> )	-	4.10 ( $\pm$ 0.86)	-	-
Length of the Membrane (mm)	34.81 ( $\pm$ 2.95)	36.81( $\pm$ 3.18)†	$p = 0.248 > 0.005$	NS
Width of the Membrane (mm)	12.25 ( $\pm$ 0.71)	13.02( $\pm$ 1.01)†	$p = 0.119 > 0.005$	NS
Height of the Membrane (mm)	-	3.02 ( $\pm$ 0.51)†	-	-
Surface Area of the Membrane (cm <sup>2</sup> )	-	3.08 ( $\pm$ 0.5)†	-	-
Weight ratio Clot/Blood Sample (%) 10 ml	20.94 ( $\pm$ 2.4)	32.53( $\pm$ 0.54)	$p = 0.000 < 0.005$	S

\*The difference of the weight clot is due to a difference of exudate content; † Average values ( $\pm$ D.S.) after 2 min compression at 30°C; N.B.: the values are not in relation to the content of Hb and erythrocytes in whole blood

The content of RBC in the membranes is 0.0028%, that of WBC is 99.24%, and that of PLT is 99.0%, contrasted with the content in entire blood.

The examination performed by McLellan et al., 2014 [17] has demonstrated that the equine PRF is like that of humans, giving a quick and constant source of tissue growth factors. Our examination has endeavored to standardize the preparation of the L-PRF/A-PRF system which, while the residual procedure of simple execution and minimal effort does not require specialized hardware, yet has a specific steadiness in the generation of membrane as far as L-PRF/A-PRF macroscopic and microscopic characteristics. The autologous platelet concentrates are promising in the field of regenerative medicine because of the abundance of growth factors.

The L-PRF represents a critical advancement in the evolution of platelet concentrates since it is basically a fibrin membrane with platelets and leukocytes trapped within alongside stem cells. These solid membranes have great dealing with features and can be immovably sutured in an anatomically-desired area during open surgeries. Nonetheless, the physical and organic properties are moderately obscure and presently yet to be completely studied.

The L-PRF/A-PRF will frame when the steps portrayed above are stringently observed.

One of the significant contemplations in creating a decent membrane is the postponement in the time between blood collection and centrifugation, just like the processing temperature. The accomplishment of the procedure depends totally on the speed of collection of blood and the prompt exchange into a centrifuge, as a rule within one minute, and by a centrifugation temperature and higher squeeze at 21°C (between 21 and 30°C).

You cannot create a clot of well-structured L-PRF/A-PRF (with its particular cell content, design of the matrix, and profile of the release of growth factors) if the collection of blood is delayed and not homogeneous, or if the centrifugation temperature is underneath 21°C or more than 30°C; rather, it will form a conflicting, brittle mass of fibrin with obscure content.

The L-PRF/A-PRF capacities as a temporary extracellular network, which is changed into useful tissue during healing, and can be exposed to mechanical forces and healing results with progress, which relies upon the structural integrity and, therefore, it is important to explain its physical properties. The L-PRF/A-PRFs look

**Table 3. Outcomes of the blood counts of entire blood, of the supernatant acquired by compression of the clot at 0 min contrasted and the counts of erythrocytes, platelets, and WBC of the red clot smear at 0 min since centrifugation, with a trial of significance**

Type	CBC blood	CBC supernatant at 0 min	Smear C.R. 0 min	Between CBC blood and supernatant 0 min			
	Average ± D.S.	Average ± D.S.	Average ± D.S.	t-test*		χ <sup>2</sup>	
RBC	7,648,000 ± 11,309.81	13,428 ± 21345	7,399,440 ± 27,039.76	p = 0.411 > 0.05	NS	p = 0.000 < 0.005	S
WBC	5150 ± 369	30 ± 27.99	8.5 ± 2.12	p = 0.255 > 0.05	NS	p = 0.000 < 0.005	S
PLT	106,780 ± 153.51	479 ± 77.614	500 ± 707.11	p = 0.031 < 0.05	S	p = 0.000 < 0.005	S
Neutrophil	3046 ± 857	0.29 ± 0.76		p = 0.280 > 0.05	NS	p = 0.991 > 0.005	NS
Basophils	4.2 ± 1.3	2.29 ± 2.14		p = 0.785 > 0.05	NS	p = 0.611 > 0.005	NS
Lymphocytes	1606 ± 668	19 ± 23.15		p = 0.238 > 0.05	NS	p = 0.000 < 0.005	S
Monocytes	490.2 ± 138.06	4.57 ± 7.68		p = 0.631 > 0.05	NS	p = 0.928 > 0.005	NS
Eosinophils	5.4 ± 5.37	4 ± 9.71		p = 0.906 > 0.05	NS	p = 0.316 > 0.005	NS
Type	Membrane L-PRF/A-PRF 0 min						
	No./ μl	%					
RBC	21,6012	0.0028%					
WBC	5111.15	99.24%					
PLT	105,801	99.00%					

\*preparing performed on two correlations. The theoretical content of RBC, WBC, and PLT in the L-PRF membranes at 0 min with significance tests. p>0.05 = +0.5% non-significant distinction; p<0.01 = - 1% significant contrast

like dense connective tissue with predominant handling features. Thusly it is expected that, as in the L-PRF, additionally in the A-PRF there is a low rigidity.

With an elastic modulus of 0.470 MPa (SD±0.107) the L-PRF membrane stretches to twice its underlying length before breakage (of 215% strain). This information affirms the published literature [18,19] which reported a low rigidity (1–10 MPa) and a high voltage (up to 150%) before breaking down.

Based on these outcomes, plainly L-PRF/A-PRF is another biomaterial with one of a kind highlight: the foreseen preparation of autologous blood, the straightforwardness of the protocol, the characterized architecture, the noteworthy mechanical properties, and the abundance of derived growth factors from stimulated platelets. Our trials on equine blood will no doubt have the option to improve our comprehension of healing, as well as add to advancing the field of personalized medicine.

Limitations that were found in the clinical setting and utilization of these products include:

1. Since PRF is an autologous product, an expanded prerequisite for the biomaterial accessibility is troublesomely accomplished. Subsequently, its utilization in surgeries must be firmly controlled.
2. PRF contains circling immune cells, just as antigenic molecules that avoid its application as allogenic material; an expanded hazard for the transmission of infectious ailments is likewise to be considered.

Now, among the various parameters that were excluded from this sort of classification, we perceived: platelet concentration, leukocyte concentration and the corresponding measure of the distinctive leukocyte types. Platelet concentration-related issues are non-existent, as all platelets included in the blood test are activated and coordinated within the clot's fibrin framework.

Concerning the leukocytes' count and concentration, their impact ought to be studied with specific care, as their presence or absence could clarify the clashing outcomes we observed. An ongoing report by Kitamura Y. et al. [20] shows a technique for direct estimation of platelet count in PRF. These authors utilized a monetarily accessible recombinant t-PA,

Alteplase (GRTPA®; Mitsubishi Tanabe Pdamagea Corp., Osaka, Japan) through an absorption strategy. Here, they have demonstrated that t-PA is ground-breaking enough to have the option to count scattered platelets accumulated into platelet-improved insoluble fibrin frameworks.

Therefore, in study Crisci et al., [21], starting from the results obtained in the work of Kitamura, wanted to elaborate a simpler and inexpensive system to calculate the precise number of platelets and leukocytes present in the PRF, compared to that present in whole blood, starting from either the subtraction method that is from a simple blood count. In this study Crisci et al. it will be possible to deduce that, subtracting the value of the Platelet count by the subtraction method from the Emocrome value by 34.35%, and by 19.69% the value of the Leucocyte count by subtraction Method and by 34.12% value of the blood count, the value obtained with the method of digestion from t-PA will be obtained with a much simpler system, using a clinical method that can be applied quickly (max 15 minutes) and safely.

#### 4. CONCLUSIONS

In conclusion, we can assert that to accomplish a standard methodology for PRF preparation as graft material for tissue restoration purposes, we propose the work of PRF membrane's area with the most elevated conceivable platelet enhancement and, in addition, we recommend avoiding squeezing the entirety of the PRF clot plasma. Consequently, it is fitting to compress the clot with a compression gadget (L-PRF Wound Box). It's difficult, consequently, to control absolutely the human-derived materials' quality, as PRF preparations, however it is imperative to apply the most noteworthy conceivable quality-control check on PRF preparations before their clinical application.

Presently, their conveyance depends on ineffectively controlled bulk discharges. As a result, delayed medications require numerous treatments, for example, various injections i-PRF. These outcomes in firmly fluctuating growth factor concentrations, which hinders clinical consistency. Biomaterials can go about as controlled discharge gadgets, which will consider continued or even on-demand conveyance of these growth factor cocktails. Moreover, it very well may be imagined that biomaterials can covalently bind specific growth elements to locally hold significant levels of these molecules.

Further clinical, histo-scientific and statistical examinations are required to comprehend the advantages of this new platelet concentration method. Nevertheless, we cannot clear aside the fact that, when gotten from an autologous blood test, produced PRF is rare and just a limited volume can be utilized. This is an impediment for methodical PRF application in Regenerative Surgery interventions. Regardless of whether the potential uses of PRF are sufficient, exact information on the biomaterial is primary, including data for its biology, efficiency and limits, to upgrade its application in everyday clinical practice.

Cell migration assumes a critical role in the healing procedure. MSCs represent a cell pool, able to recreate the damaged tissue, and endothelial cells' contribution to angiogenesis. Migration models instigated by the supernatant of platelet concentrates' culture does not contrast between the two kinds of cells. The solidier MSCs and HUVECs migration were seen as an answer to L-PRF. The entirety of the above signifies that L-PRF could be valuable as a healing biomaterial, and as a natural anti-hemorrhagic agent to be utilized at surgical sites.

## CONSENT

It is not applicable.

## ETHICAL APPROVAL

The study was approved by the animal approved committee.

## COMPETING INTERESTS

Authors have declared that no competing interests exist.

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