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A. Crisci, D. Lombardi, E. Serra, G. Lombardi, F. Cardillo, M. Crisci

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SCRIPTA MANENT EDIZIONI

L-PRF: Standardized protocol proposed for the use of fibrin rich in leukocyte platelet and the use of L-PRF Wound Box. Selection of an animal model.

Alessandro Crisci

A. Crisci [1], D. Lombardi [2], E. Serra [3], G. Lombardi [4], F. Cardillo [5], M. Crisci [6]

[1] Unit of Dermosurgery Cutaneous Transplantations and Hard-to-Heal Wound "Villa Fiorita" Private Hospital Aversa (CE) Italy;
[2] Professor of Surgery, School of Medicine, University of Salerno Italy;
[3] Laboratory Clinical Patology and Microbiology, Institute for the Study and Treatment of Diabetes Casagiove (CE) Italy;
[4] General Surgery, Hospital "S.Maria della Pietà" Nola (Na) Italy;
[5] Veterinarian freelancer, Sulmona (AQ) Italy;
[6] Faculty of Medicine and Surgery-Vasile Goldis Western University of Arad, Romania.

Summary

L-PRF: standardized protocol proposed for the use of fibrin rich in leukocyte platelet and the use of L-PRF Wound Box. Selection of an animal model.

Platelet-rich fibrin (PRF®), as described by Choukroun, represent a new step in the therapeutic concept of platelet gel with a simplified processing and few artificial biochemical changes. A valid method of preparation of the PRF must effectively separate the plates by erythrocytes and concentrate without damaging or lysing the plates themselves. In this study, the experimental design is to standardize the production of L-PRF in horses, directing it to human production. Our hypothesis is that the leukocyte platelet-rich fibrin L-PRF is easy to produce in horses, without modification of the human protocol, thus allowing a better standardization of the human protocol. A new device for the preparation and the standardization of L-PRF clots and membranes is the L-PRF Wound Box®. Via optical microscopy, most cell bodies were highlighted and concentrated in the proximal portion of each membrane, with the last 1/4 being observed at the centre; the distal part had only residual traces of cell bodies. The L-PRF will form constantly when the phases described above are strictly adhered to. The success of the L-PRF depends entirely on the speed of blood collection and transfer to a centrifuge within a minute, by the temperature of centrifugation, and the speeze is higher than 21 °C (between 21 and 30 °C). Our experiments on horses will no doubt be able to improve our understanding on wound healing, in particularly in chronic skin lesions therapy.

KEY WORDS: *Autologous; Buffy coat; Growth factor level; Platelet-rich fibrin; Thrombocyte concentrate.*

Introduction

The platelet-rich fibrin (PRF) of *Choukroun* [1] is a new step in the therapeutic concept of platelet gel with a simplified design and little artificial biochemical changes. Unlike other platelet concentrates, this technique does not require anticoagulants, thrombin, or any other gelling agent, which makes it no longer natural blood centrifuged without additives [2]. Although platelets and leukocyte cytokines play an important role in the biology of this biomaterial, the supporting fibrin matrix certainly constitutes the decisive factor of real therapeutic potential of PRFs. Within a few minutes, the absence of an anticoagulant allows the activation of most of the platelets contained in the sample to trigger the coagulation cascade. Fibrinogen is initially concentrated in the upper part of the tube, until the effect of the circulating autologous thrombin transforms it into a network of fibrin.

The result is a fibrin clot containing the platelets located in the centre of the tube, just between the lower layer of red blood cells and the plasma acellular top. The PRF clot is then placed on the grill in the *PRF Box®* and covered with the compressor cover. This produces an economical membrane of autologous fibrin in approximately one minute. The *PRF Box®* is designed for the production of constant thickness membranes that remain hydrated for several hours and allows the recovery of the serum exudate expressed by fibrin clots which is rich in proteins, such as vitronectin and fibronectin [2]. The PRF clot is produced by a natural polymerization process during centrifugation, and its natural fibrin architecture seems to be responsible for a slow release of growth factors and glycoproteins from the matrix (≥7 dd). The adhesive proteins fibrinogen (Fg), fibronectin (Fn), vitronectin (Vn), and thrombospondin-1 (TSP-1) are abundant on the fibrin structure. Among the growth factors stored in platelets, and which are essential for the repair of wounds, are PDGF, with -AB and -C; they are also present as VEGF-A, TGF-β1, FGF-2; EGF, HGF, and insulin-like growth factor-1 (IGF-1).

Fibrinogen can improve the healing of a wound, increasing both proliferation and cell migration, and it is assembled with Fn into fibrils regardless of the formation of the fibrin. The fibrin is an important factor in wound healing; in fact, the result of the healing of a wound is influenced by the structure of fibrin (the thickness of the fibres, the number of branch points, the porosity, and the permeability of the clot at the site of injury) [3]. The platelet-rich fibrin clots also constitute a bioactive reservoir. After the first massive release of growth factors, platelets synthesize and secrete new ones for the rest of their lives (≦7–10 dd). Fibrin gels are desirable as scaffolds in tissue engineering for several reasons. The most important reason is the inherent compatibility with the fibrin of the cellular organism which is different according to the many components and processes involved in the manufacture of scaffolds. Although platelet growth factors play an important role in the biology of PRF, the architecture of the fibrin and the content of leukocytes are two key parameters.

The distribution of platelets and leukocytes within the fibrin clot was evidenced through the haematological counts, photonic microscopy, and scanning electron microscope (SEM). A valid method of preparation of PRF® must effectively separate the platelets from red blood cells and concentrate them without damaging or lyse the platelets themselves. Growth factors contained within the α-granules are not active during the discharge; they blend with the platelet-activating membrane. Consequently, if platelets are damaged during the production of PRF, they will not secrete more of the bioactive growth factors. In fact, they are particularly labile and sensitive to any kind of stressful event during the processing step and application; for this reason, the concentration of growth factors can also be influenced by manipulation during processing of the blood.

Thus, it is crucial to standardize the preparation procedure and the type of centrifugation that is carried out must have certain characteristics, which include: starting slowly, initially, a central phase at high rpm, and a final slow phase before stopping [4], and should take place at a given temperature and for a determined time. The PRF protocol is a simple technique developed in France by *Choukroun et al.* [1] and can be regarded as a second-generation platelet concentrate because it is a natural product without any anticoagulant or gelling agents. Platelets and white blood cells are collected with high efficiency using this method and the leucocytes are preserved in their entirety. However, platelets are activated during this process, which leads to a substantial embedding of platelets, leukocytes, and growth factors in the fibrin matrix.

In this study, the experimental design is to standardize the production of L-PRF in horses, directing it towards human production. It is necessary to establish a standard protocol for preparing L-PRF so as to meet the following criteria:

1) The growth factors present in the platelets must be stored to stimulate the surrounding cell guests;
2) Platelets must be stored in the fibrin structure with minimal damage or activation; and
3) The three-dimensional fibrin lattice should be used as a scaffold for the surrounding host cells.

Materials and Methods

Preparation

The blood clotting begins instantly when it comes in contact with the glass surface due to the lack of anticoagulant. If the time required to collect blood and the start of the centrifugation is extremely prolonged, the fibrin polymerization is so widespread in the tube and only a small part of the blood clot will be obtained without consistency (PRF-like). As a result, blood collection must be followed by an immediate centrifugation and is a prerequisite in the specification of the PRF output. It is formulated to produce a homogeneously-moisturized thick membrane and an exudate rich in platelets, leukocytes, vitronectin, and fibronectin expressed by fibrin clots [5].

Overall, the L-PRF is mechanically resistant, able to support loads, has a capacity of two times to stretch under tension and retains surgical sutures well enough (it deforms significantly before the laceration) [6]. The production technique of PRF is very simple and requires only a blood sample and a table centrifuge (Figure 1). The protocol is as follows: the blood samples are collected in 9 mL tubes, without anticoagulant nor gel separator, and are immediately centrifuged according to the following program: 30 sec acceleration, 2 min at 2700 rpm, 4 min at 2400 rpm, 3 min at 3000 rpm, and 36 sec deceleration and stopping.

After centrifugation, three parts are localized in the tube: the red blood cells at the bottom, a fibrin clot that represents the PRF in the middle, and the acellular plasma at the top. Extracting the matrix from the tube with forceps and removing the red clot can be obtained from the PRF. The success of this technique depends entirely on the blood collection and the transfer speed in the centrifuge [7, 8]. Equine blood was used by *Textor et al.* [9] for the production of platelet concentrate (PRF). Our hypothesis is that the L-PRF is easy to produce in horses, without modification to the human protocol, even allowing better standardization of the human protocol. A written consent of the owners has been obtained for all of the horses and the blood collection procedure was performed in accordance with the current AVMA guidelines.

We performed a prospective study of equine blood, which has been collected in test tubes without anticoagulant plastic-coated glass nor a gel separator (BD *Vacutainer* tubes for serum 9.0 ml), for the production of L-PRF clots and membranes by six healthy horses of various ages (average ± SD, 10 ± 4.1 years, ranging from 4 to 17 yy), gender, and breed. The blood was collected quickly both with 10 cc sterile syringes with that needle to *Vacutainer* tubes (22" average value, of less than 25" per tube) and immediately (within 1 min) centrifuged according to the preceding description to a temperature greater than 21 °C. The temperatures of the inner and outer surface of the centrifuge were recorded before and during centrifugation with a

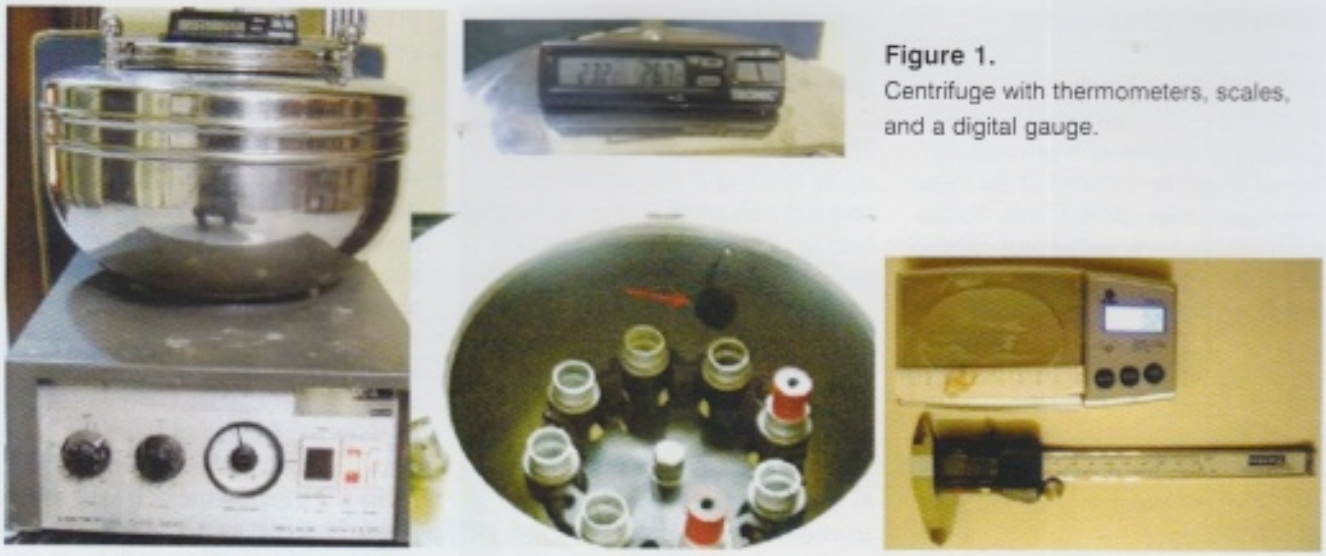

Figure 1.
Centrifuge with thermometers, scales, and a digital gauge.

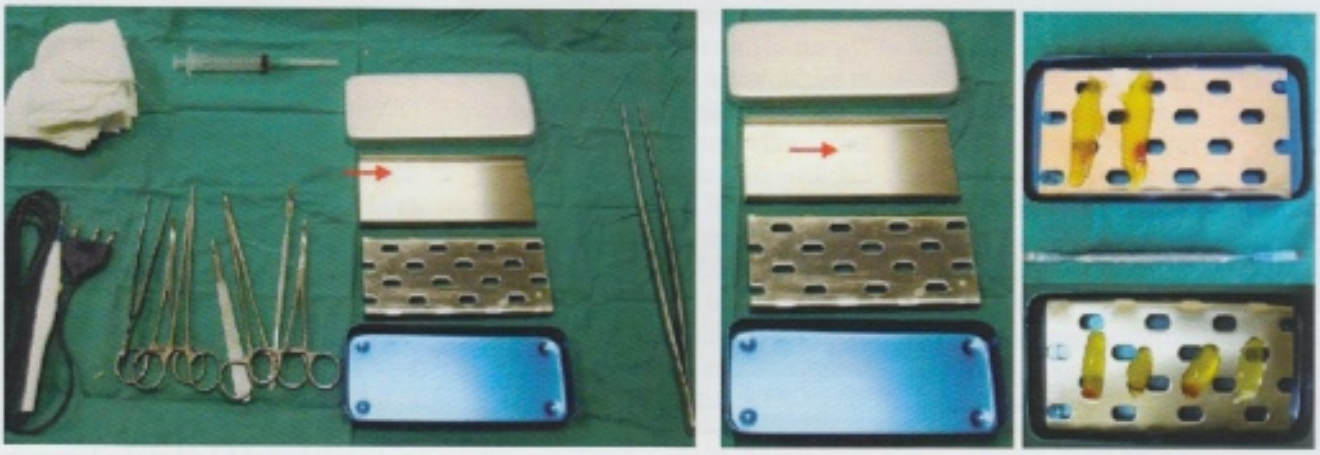

Figure 2. L-PRF Wound Box®.

digital thermometer with an internal probe (→) (*TRONIC*)(Figure 1).

A new device has been tested by us for the preparation and standardization of L-PRF in clots and membranes: the *L-PRF Wound Box*® (Figure 2).

Using the *PRF Wound Box*, the compression process of the membrane in the clots is performed through a slow and homogeneous slight compression, and the final membrane always remains homogeneously wet and soaked in serum.

This mild method avoids the extraction and the loss of a significant amount of the growth factors.

The *PRF Wound Box* that exists on the market come in a variety of shapes and exert, through the compression plate, different pressures according to the weight, giving rise to a membrane of varying thickness, width, and length.

The *L-PRF Wound Box*® designed by us is made from a metal container 17.5 × 7.6 × 2 cm containing a perforated steel plate of 150 × 68 × 1.5 m.

There is a second steel plate which acts as a compressor, 150 × 68 × 1.5 mm, with a weight of 148 grams (Figure 2). This second shaped plate exerts a pressure of 142.437 Pa/cm^2. In this study, the compression has been exerted on the clot for 2, 5, 10, and 15 minutes to produce membranes.

Macroscopic Analysis

After centrifugation, the L-PRF clot was removed from the test tube using sterile tweezers and a smooth spatula to gently release the red clot from the buffy coat. Each L-PRF clot obtained was placed in a tray for measuring the weight and size with a digital scale from a goldsmith (Figure 1).

The compression of the clot was carried out with the *L-PRF Wound Box*® we designed with a pressure of 142.437 Pa/cm^2 constant for two, five, ten, fifteen minutes. This method allowed us to obtain, from each clot, the L-PRF membranes, which were individually weighed and measured with a digital gauge (Figure 1).

The surface area in cm^2 of clots and the membranes were measured with the Calcderm measurement software [10].

Optical Microscopy Procedure

The membranes were fixed in 10% neutral buffered formalin for 24 h at ambient temperature for inclusion in paraffin. Subsequent sections of 4 μm were performed along the midpoint of the membranes and were stained with haematoxylin-eosin. Each section was divided into three equal-sized areas: proximal (head), centre (body), and distal (tail). Each area of these sections was observed through an optical microscope and analyzed by counting the visible cell bodies (marked in dark purple, mostly leukocytes) at the centre of each observed area with a magnification of 25×, 40×, 60×, and 100× (immersion). The total number of counted cell bodies were used to correlate their distribution between the three membrane areas (head, body, and tail). Most of the cells were concentrated in the proximal area (head), closest to the red clot. Smears of blood prepared by residual blood in the tubes were also examined for a morphological assessment after removal of the PRF clot with a spatula (two for each tube), differentiating the clot at 0 min and 60 min by centrifugation and fixing them with alcohol 90% for a *Grunwald-May-Giemsa* colouring stain in order to identify the various corpuscular elements, in particular platelets and neutrophils, to compare them in relation to the examination blood count. A blood samples was also taken from each horse to perform a blood count using K3E 5.4 mg EDTA tubes (*VacuMed*).

Following the study of *Pech et al.* [11] three blood samples were taken from the left jugular vein of each horse through a 14-gauge needle, two for the production of PRF and one for the blood count. After centrifugation, the clot was removed immediately (group (A)) and after 60 min (group (B)). The supernatant derived from compression with the *Wound Box®* membrane was differentiated between the 0 min and 60 min and was been preserved in a test tube with K3E 5.4 mg EDTA for blood count analysis. It was compared with the basal one and with the corpuscular elements of the counts performed on smears derived from the red clot as an indirect measurement of the platelet and the leukocyte concentrations of the L-PRF. The two samples taken with the *Vacutainer system* without gels were randomly assigned to two groups (A and B), using a coin toss.

The supernatant derived from the pressing of L-PRF at 0 min and 60 min were analysed with a blood count in standard lodging. Since a direct measurement of platelet concentration of the PRF is not yet possible, we calculated the residual platelet concentration. The examinations were performed with a Cell Dyn 3500 R cell counter (ABBOTT).

Determination of the Parameters of the Membranes

Immediately after formation, the L-PRF membranes were stored at 4 °C until delivery to the laboratory in order to avoid damage from storage.

The size of the membranes was measured in height, length, and width in mm with a digital gauge, and the irregularly-shaped areas were measured in cm2 with Calcederm software [10].

The content of erythrocytes, platelets, WBCs (neutrophils, lymphocytes, monocytes, basophils) present in the membrane is derived by comparing the basic blood count with that obtained from the supernatant, as performed on blood smears of the red clot.

Statistical Analysis

The statistical significance for the differences between two groups was calculated using Student mins t-test and ANOVA for repeated measures for parametric variables and with the x^2 for non-parametric variables. p values < 0.005 were considered statistically significant.

The data were analysed using the *Discipline Biomedical Statistics of Stanton-Glantz 2007 software package, version 6.0.*

Results

The procedure was well-tolerated in all animals. No significant differences were found in the basic haematological confrontation, which had an average concentration of RBC 9.8 × 10^6/mL (range:7-13), WBC 5.1 × 10^3/mL (range:5-13) (± 0.37 C.I. 95%) (p = 0.24) and a platelet count average of 106.8 × 10^3/mL (range: 100-350) (±15.3 I.C.95%) (p = 0.5). It was not possible to directly quantify the platelet concentration and WBC trapped inside the L-PRF clot, which was derived indirectly by comparing the mean values of whole blood, the mean values of the supernatant obtained after compression of the clot at 2 min, and the average values obtained with the counts of smears of the red clot after the removal of the L-PRF clot.

The PDGF and TGF-β1 are the most abundant growth factors contained with in the alpha-granules of platelets and are released into the extracellular space after platelet activation. These cellular factors direct the proliferation, cell differentiation, matrix production, angiogenesis and wound contraction, supplementation of growth factors improves the survival and differentiation of cells transplanted into a number of materials and treated tissues (Figure 2b). The average quantities of produced PDGF-AB are significantly higher in each experimental time and the TGF-β1, Vitronectin and VEGF are significantly higher during the first 4h [8].

Table 1 compares the characteristics of clots and membranes of L-PRF obtained in humans (centrifugal Intraspin) reported by *Pinto et al.* [12] and those we observed in the horse model. In this comparison, it was verified that there are significant differences in the characteristics of the clot, but these differences are eliminated when the membranes derived from the compression were examined. This observation, in our opinion, would be assigned to a different content of exudate (weight of the exudate is 1.47 ± 0.13 g in humans, 3.05 ± 0.11 g in horses, p = 0.000).

In this study, the size of the membranes is not found to be in relation to the haemoglobin content or the content of erythrocytes encountered in the blood count baseline. In optical microscopy (Figure 3), most of the cell bodies (stained in dark purple for the nuclei) were concentrated in the proximal part (head) of each membrane, the last 1/4 was observed at the centre, and the distal part had only residual traces of cell bodies. Optical microscopy has not, however,

allowed the observation of the exact state of these cell bodies in greater detail.

Figure 5A shows the average characteristics of a membrane obtained with the L-PRF Wound Box after several minutes of compression (2, 5, 10, and 15 minutes). The slight variations between M05 min, M10 min, M15 min, M02 min are compared to make us reach out towards the use of the membrane after two minutes into the compression (Table 2).

The processing temperature should be >21 °C since the clot is not produced if the temperature is lower than this. Figure 5D shows the temperatures detected during centrifugation within and outside the low-temperature (23 °C) and high-temperature (30 °C) centrifugation at various times. Note that increasing the speed of the revolutions increases the internal temperature.

Using 9 ml syringes instead of the Vacutainer sampling system the membranes that are produced are smaller in size (Figure 5B), both in terms of weight, that of length, width and surface useful. After compression for 2 min, the reduction of the weight of the membrane with respect to the clot is 85% if it picked up with a syringe, and by 70% if it is taken with the *Vacutainer system* (Figure 5C) probably because the corpuscular part of the blood and fibrinogen is damaged with the syringe. The results of the blood counts of whole blood and of the supernatant obtained from the clot after 0 min, and after 60 min of compression are compared with the counts of erythrocytes, platelets, and the WBC smear of the red clot after 0 and 60 min, and are shown with the corresponding statistical tests in Table 3, while the microscopic images after 60 min are shown in Figure 6.

Table 3 demonstrates the statistically significant difference between the content of RBC, WBC, and platelets between the supernatant derived from the compression of the clot at 0 and 60 minutes.

There is also shows a statistically significant difference between the content of RBC, WBC, and PLT in smears obtained from the red clot at 0 min and 60 min, as shown in Figures 3 and 5A–C at various magnifications. Table 3 also shows the hypothetical contents of RBC, WBC, and PLT in L-PRF membranes derived from the difference of these corpuscular elements between whole blood, the supernatant at 0 min and 60 min, and the smear of the red clot at 0 min and 60 min. The t-test shows significant differences between RBCs at 0 min and 60 min, and between PLT at 0 min and 60 min in L-PRF membranes. The content of RBC in the membranes is 0.0028%, that of WBC is 99.24%, and that of PLT is 99.0%, compared to the content in whole blood. The membranes after 60 min of compression have a content of RBC, WBC, and PLT lower than those at 0 min.

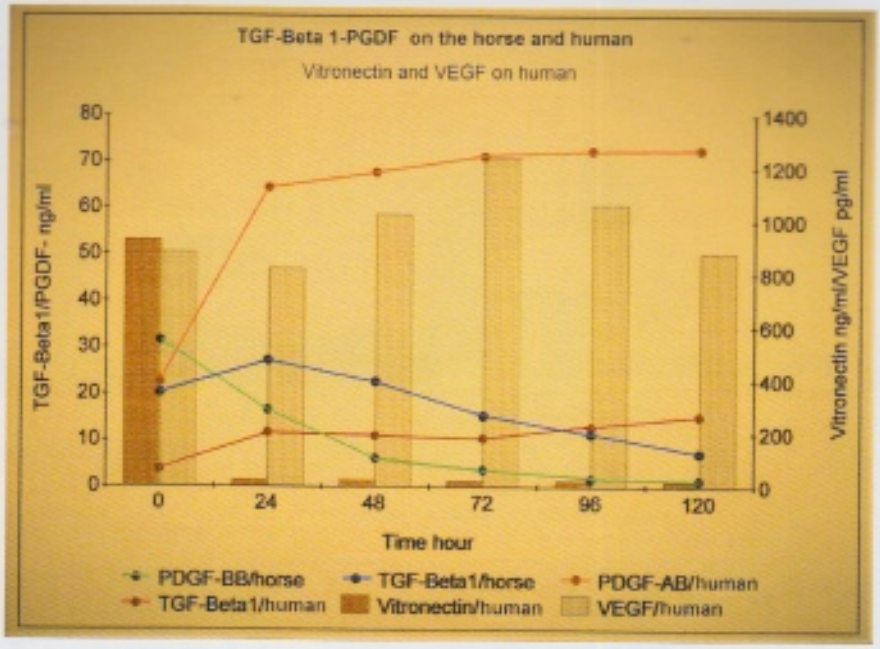

Figure 3. Comparison of TGF-B1 and PGDF growth factors on horse and man and Vitronectin and VEGF levels in humans [8].

Table 1. Comparison of membranes obtained from human blood (*Pinto et al., 2014*) and from equine blood.

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

* The difference of the weight clot is due to a difference of exudate content; ♦ Average values (±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.

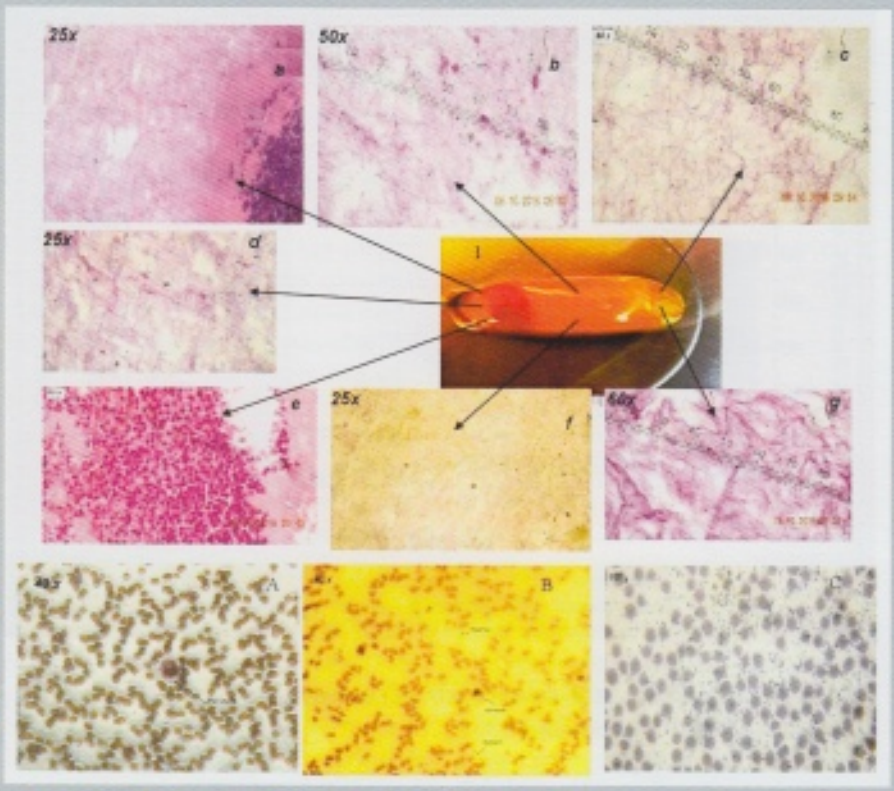

Figure 4. Membrane L-PRF 0 minutes after compression (hematoxylin-eosin staining). (a) III proximal 25× white blood cell-pattern fibrin; (b) medium-III 60× erythrocytes pattern fibrin; (c) III distal 60× pattern fibrin; (d) III proximal 25× erythrocytes-fibrin; (e) III proximal 60× fibrin on the right, the center lymphocytes, erythrocytes, and granulocytes neutrophils on the left; (f) average III 25× pattern of fibrin; (g) III distal 60× pattern fibrin; (A) smear of red clot 40× presence of monocyte in a carpet of red cells; (B) red clot smear 40× presence of red blood cells, monocytes and platelets; (C) red clot smear 100× presence of platelets in a carpet of red cells (coloration: *May-Grunwald-Giemsa*).

Table 2. Test of significance on the variations of the parameters of the membrane L-PRF to compression with the L-PRF Wound Box for 2, 5, 10, and 15 minutes.

Variable	Between Basal and M02 min		between Basal and M05 min		between Basal and M10 min		between Basal and M15 min	
Parameter	Student's *t*	ANOVA	Student's *t*	ANOVA	Student's *t*	ANOVA	Student's *t*	ANOVA
Weight (g)	$p = 0.006$ ♀	$p = 0.006$ ♀	$p = 0.002$*	$p = 0.005$*	$p = 0.002$*	$p = 0.005$*	$p = 0.001$*	$p = 0.006$ ♀
% Reducing Clot (g)	$p = 0.000$*	$p = 0.000$*	$p = 0.000$*	$p = 0.000$*	$p = 0.000$*	$p = 0.000$*	$p = 0.000$*	$p = 0.000$*
Length of the Membrane (mm)	$p = 0.031$ ♀	$p = 0.004$ ♀	$p = 0.039$ ♀	$p = 0.002$*	$p = 0.019$ ♀	$p = 0.002$*	$p = 0.021$ ♀	$p = 0.000$*
Width of the Membrane (mm)	$p = 0.015$ ♀	$p = 0.021$ ♀	$p = 0.001$*	$p = 0.005$*	$p = 0.001$*	$p = 0.006$ ♀	$p = 0.001$*	$p = 0.008$ ♀
Height of the Membrane (mm)	$p = 0.018$ ♀	$p = 0.054$ ♀	$P = 0.005$*	$p = 0.020$ ♀	$p = 0.002$*	$p = 0.014$ ♀	$P = 0.002$*	$p = 0.008$ ♀
Surface Area of the Membrane (cm^2)	$p = 0.506$ ♀	$p = 0.137$ ♀	$p = 0.083$ ♀	$p = 0.079$ ♀	$p = 0.058$ ♀	$p = 0.078$ ♀	$p = 0.038$ ♀	$p = 0.066$ ♀

between M02 min and M05 min-M10 min M15 min χ^2	
Weight (g)	$p = 1.000$ ♀
% Reduction in Weight (g)	$p = 0.852$ ♀
Length of the Membrane (mm)	$p = 1.000$ ♀
Width of the Membrane (mm)	$p = 1.000$ ♀
Height of the Membrane (mm)	$p = 1.000$ ♀
Surface Area of the Membrane (cm^2)	$p = 1.000$ ♀

♀ $p > 0.005$ = no significant difference; * $p < 0.005$ = significant difference

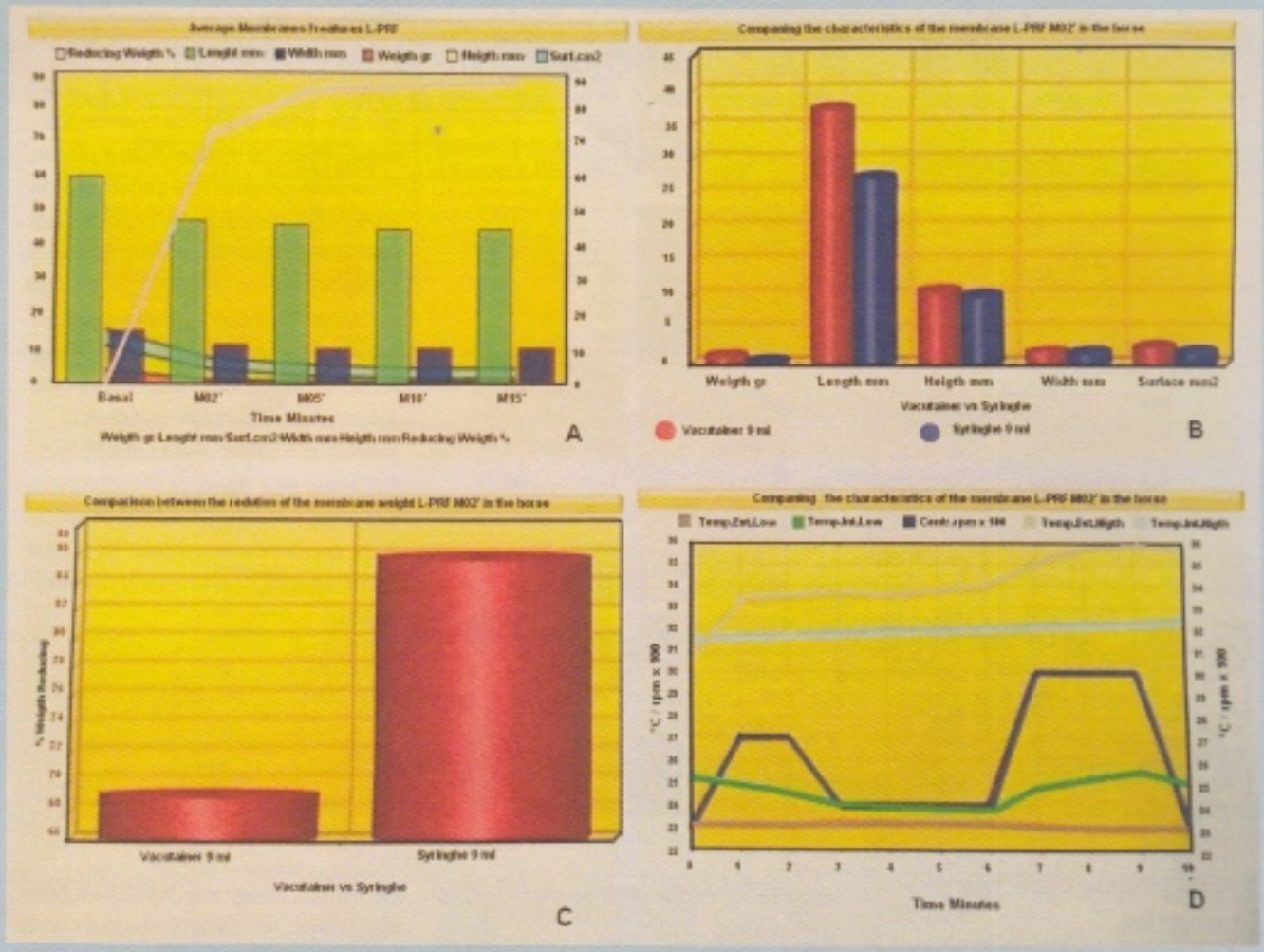

Figure 5. (A) Average characteristics of the L-PRF membrane features obtained in the horse model with the L-PRF Wound Box for several minutes of compression: weight (g); height (mm); length (cm); width (cm); size (cm^2); and % weight reduction during compression. Picking with 9 ml Vacutainer system. (B) Average characteristics of an L-PRF membrane obtained in the horse model with the L-PRF Wound Box: weight (g); height (mm); length (mm); width (mm); surface area (cm^2). (C) % weight reduction during compression. (D) Variations of external and internal temperature during centrifugation at 23 °C (B) and at 30 °C (A). Picking with *Vacutainer system* and with 9 mL syringe after 2 min of compression.

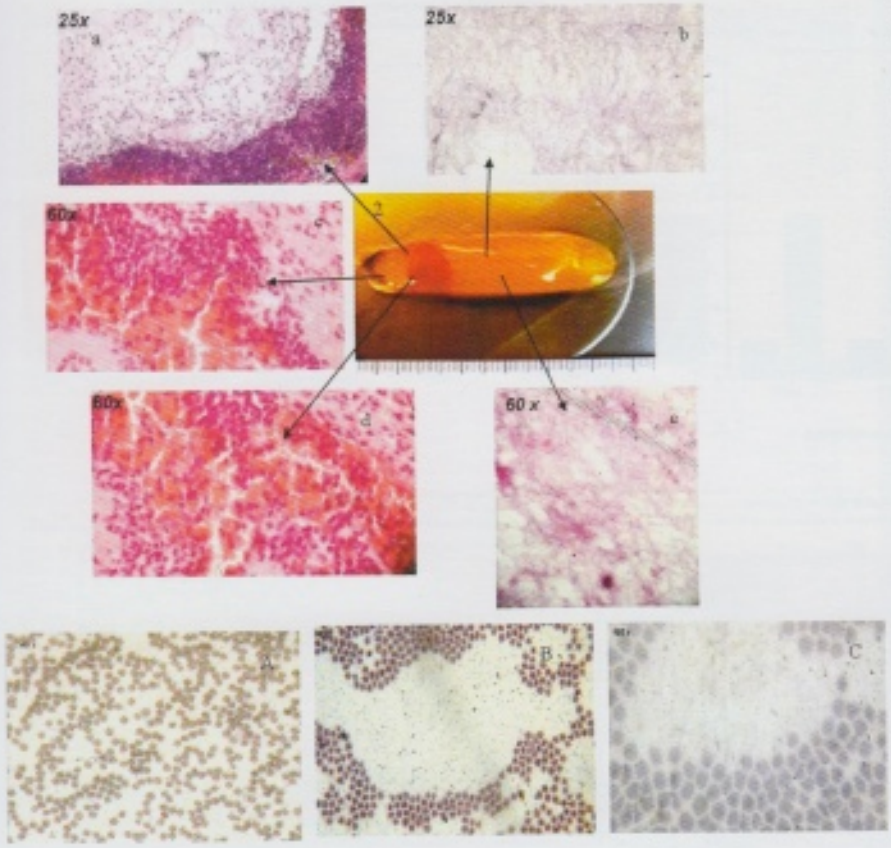

Figure 6. Membrane L-PRF of 60 minutes by the compression horsepower (hematoxylin-eosin staining). a) III proximal 25× White Blood Cell-Erythrocytes-pattern Fibrin; b) medium-III 25× pattern Fibrin; c) III proximal 60× pattern of Fibrin on the right, the center lymphocytes, erythrocytes and granulocytes Neutrophils to left; d) III proximal 60× Fibrin on the right, the center lymphocytes, erythrocytes and granulocytes neutrophils to left; e) average III 60× pattern of fibrin with Lymphocyte; A) smear of red clot 40× presence of platelets in a carpet of red cells; B) Red clot smear 40× presence of erythrocytes and many platelets; C) red clot smear 100× presence of many platelets in a carpet of red cells (*May-Grunwald-Giemsa staining*).

Discussion

The study performed by *McLellan et al.* [13] has shown that the equine PRF is similar to that of humans, providing an immediate and constant source of tissue growth factors. Our study has attempted to standardize the preparation of the L-PRF procedure which, while remaining a technique of easy execution and low-cost, does not require specialized equipment, but has a certain constancy in the production of a membrane in terms of L-PRF macroscopic and microscopic features. The autologous platelet concentrates are promising in the field of regenerative medicine due to the abundance of growth factors.

The L-PRF represents a very significant advance in the evolution of platelet concentrates since it is essentially a fibrin membrane with platelets and leukocytes trapped within. These solid membranes possess excellent handling characteristics, and can be firmly sutured in an anatomically-desired location during open surgeries. However, the physical and biological properties are relatively unknown. The L-PRF will form when the steps described above are strictly observed.

One of the important considerations in generating a good L-PRF membrane is the delay in the time between blood collection and centrifugation, as well as the processing temperature. The success of the L-PRF technique depends entirely on the speed of collection of blood and the immediate transfer into a centrifuge, usually within one minute, and by a centrifugation temperature and higher squeeze at 21°C. You cannot generate a clot of well-structured L-PRF (with its specific cellular content, architecture of the matrix, and profile of the release of growth factors) if the collection of blood is prolonged and not homogeneous, or if the centrifugation temperature is below 21°C; instead, it will form an inconsistent, crumbly mass of fibrin with unknown contents. The L-PRF functions as a provisional extracellular matrix, which is transformed into functional tissue during healing, and can be subjected to mechanical forces and healing outcomes with success, which depends on the structural integrity of the L-PRF and, therefore, it is important to clarify its physical properties.

The L-PRF looks like dense connective tissue with superior handling characteristics. With an elastic modulus of 0.470 MPa (SD = 0.107) the L-PRF membrane stretches to twice its initial length before breakage (of 215% strain). These data confirm the published literature [6] who reported a low rigidity (1–10 MPa) and a high voltage (up to 150%) before breaking down.

On the basis of these results, it is clear that L-PRF is a new biomaterial with unique features: the anticipated preparation of autologous blood, the simplicity of the protocol, the defined architecture, the impressive mechanical properties, and the abundance of derived growth factors from activated platelets. Our experiments on equine blood will no doubt be able to improve our understanding of healing, as well as contribute to advancing the field of personalized medicine.

Table 3. Results of the blood counts of whole blood, of the supernatant obtained by compression of the clot at 0 min and 60 min compared with the counts of erythrocytes, platelets, and WBC of the red clot smear at 0 min and 60 min, with tests of significance. * processing performed on two comparisons. Hypothetical content of RBC, WBC, PLT in the L-PRF membranes at 0 min and 60 min with significance tests. $p > 0.05$ = +0.5% non-significant difference; $p < 0.01$ = ÷1% significant difference.

Type	CBC blood	Smear C.R. 0 min	Smear C.R. 60 min	Between Red Clot smear 0-60 min			
	Average ± D.S.	Average ± D.S.	Average ± D.S.	*t-test**		χ^2	
RBC	7,648,000 ± 11,309.81	7,399,440 ± 27,039.76	7,322,960 ± 81119.29	P = 0.333>0.05	NS	p = 0.000<0.005	S
WBC	5150 ± 369	8.5 ± 2.12	0.5 ± 0.71	P = 0.037<0.05	S	p = 0.908>0.005	NS
PLT	106,780 ± 153.51	500 ± 707.11	6000 ± 1414.21	P = 0.039<0.05	S	p = 0.000<0.005	S
Neutrophil	3046 ± 857						
Basophils	4.2 ± 1.3						
Lymphocites	1606 ± 668						
Monocytes	490.2 ± 138.06						
Eosinophils	5.4 ± 5.37						

Type	CBC supernatant at 0 min	CBC supern.at 60 min	Between CBC supernatant 0-60 min			
	Average ± D.S.	Average ± D.S.	*t-test**		χ^2	
RBC	13,428 ± 21345	73714 ± 186233	p = 0.411 > 0.05	NS	p = 0.000 < 0.005	S
WBC	30 ± 27.99	10914 ± 172.95	p = 0.255 > 0.05	NS	p = 0.000 < 0.005	S
PLT	479 ± 77.614	3627 ± 3401	p = 0.031 < 0.05	S	p = 0.000 < 0.005	S
Neutrophil	0.29 ± 0.76	1.29 ± 2.21	p = 0.280 > 0.05	NS	p = 0.991 > 0.005	NS
Basophils	2.29 ± 2.14	2 ± 1.73	p = 0.785 > 0.05	NS	p = 0.611 > 0.005	NS
Lymphocites	19 ± 23.15	98.43 ± 167.69	p = 0.238 > 0.05	NS	p = 0.000 < 0.005	S
Monocytes	4.57 ± 7.68	2.86 ± 5.01	p = 0.651 > 0.05	NS	p = 0.928 > 0.005	NS
Eosinophils	4 ± 9.71	4.57 ± 7.96	p = 0.906 > 0.05	NS	p = 0.316 > 0.005	NS

Type	Membrane 0 min		Membrane 60 min		Between Membrane L-PRF 0-60 min			
	No./µl	%	No./µl	%	*t-test**		χ^2	
RBC	21,6012	0.0028%	193,966	0.0025%	p = 0.000 < 0.005	S	p = 0.266 > 0.005	NS
WBC	5111.15	99.24%	5036.86	97.80%	p = 0.007 > 0.005	N S	p = 0.993 > 0.005	NS
PLT	105,801	99.00%	97153	91.00%	p = 0.002 < 0.005	S	p = 1.000 > 0.005	NS

Conclusions

The problems related to the conservation of these membranes will greatly limit their use.

Cryopreservation at 4°C may be a solution to this problem, but at the moment the data reported in the literature are not encouraging because of the reduced half-life of cryopreserved platelets (< 2 days) compared to fresh (3.5 – 3.8 days) [14]. The data concerning the maximum storage time and the ideal temperature for the conservation of PRF are largely lacking. In future studies, we would like, therefore, to test the hypothesis of *Peck et al.* [11] comparing the parameters observed at 0 min and 60 min by centrifugation using dry *Vacutainer* tubes *vs Vacutainer* tubes with separating gel, analysing the prepared product with a scanning electron microscope.

We would also consider how long the PRF (clot or membrane) is stable after its establishment and if the exudate collected in the *L-PRF Wound Box* can be used effectively to fix the autologous grafts (L-PRF membrane, or bio-conductive or bio-inductive dermis).

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