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Article

Selection of an Animal Model for the Production of Leukocyte-Fibrin Rich Platelet Membranes (L-PRF); Standardized Protocol Proposed for Clinical Use and the Use of L-PRF Wound Box®

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Abstract

Fibrin rich of platelets (PRF®) of Choukroun represents a new step in the therapeutic concept of platelet gel with a simplified processing and biochemical changes little artificial. 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

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production of L-PRF in horse directing it to human production. Our hypothesis is that the L-PRF is easy to produce in the horse, without modifications 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®. The optical microscopy, most cell bodies were highlighted concentrated in the proximal portion of each membrane, the last 1/4 was observed at the center; 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 art L-PRF depends entirely on the speed of blood collection and transfer in centrifuge within a minute and by a temperature of centrifugation and compression is higher than 21°C (between 21 and 30°C). Our experiments on the horse 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 or anticoagulants, thrombin or bovine, or any other gelling agent, which makes it no longer the natural blood centrifuged without additives [2].

Although platelets and leukocyte cytokines play an important role in the biology of this biomaterial, the fibrin matrix of support certainly constitutes the decisive factor head of real therapeutic potential of PRF.

Within a few minutes, the absence of anticoagulant allows the activation of most of the platelets contained in the sample to trigger the coagulation cascade. The fibrinogen is initially concentrated

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in the upper part of the tube, until the effect of the autologous thrombin circulating transforms it into a network of fibrin.

The result is a fibrin clot containing the platelets located in the center 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 to recover 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).

Are abundant on fibrin structure the adhesive proteins: Fibrinogen (Fg), Fibronectin (Fn), vitronectin (Vn), thrombospondin-1 (TSP-1).

Among the growth factors stored in platelets and essential for the repair of wounds there are PDGF, with the -AB and -C; They are also present as VEGF-A, TGF-β1, the FGF-2; the EGF, HGF and the insulin-like growth factor-1 (IGF-1).

Fibrinogen can improve the healing of a wound, increasing both the proliferation that cell migration, it is assembled with Fn into fibrils regardless of the formation of 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 (thickness of the fibers, number of branch points, porosity and permeability of the clot at the site of injury [3]. The platelet-rich fibrin clots also constitute a bioactive tank. After the first massive release of growth factors, platelets synthesize and secrete new ones for the rest of their lives (≅7-10 dd).

The fibrin gel are desirable as scaffolds in tissue engineering for several reasons. The most important reason is inherent compatibility with the fibrin of the cellular life, which is different according to the many components and processes involved in the manufacture of scaffolds.

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Although platelet growth factors play an important role in the biology of PRF, the architecture of the fibrin and content of leukocytes are two key parameters.

The distribution of platelets and leukocytes within the fibrin clot was evidenced through the haematologic counts, the photonic microscopy and SEM. A valid method of preparation 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 platelet activating membrane. Consequently if platelets are damaged during the production of PRF, not secrete more of the bioactive growth factors. In fact, they are particularly labile and sensitive to any kind of stressful event during the step of processing In fact, they are particularly labile and sensitive to any kind of stressful event during the step of processing and application; for this reason also the concentration of growth factors can be influenced by manipulation during processing of the blood. So it is crucial to standardize the preparation procedure and thus the type of that centrifugation is carried out, it must have certain characteristics, which are: the initial Start Low, the central phase in high rpm and a Stop final Low [4] and should take place at a given temperature and for a determined time and application; for this reason also the concentration of growth factors can be influenced by manipulation during processing of the blood. So it is crucial to standardize the preparation procedure and thus the type of that centrifugation is carried out, it must have certain characteristics, which are: the initial Start Low, the central phase in high rpm and a Stop final Low [4] and should take place at a given temperature and for a determined time.

The PRF protocol Choukroun is a simple technique developed in France by Choukroun et al.[1], can be regarded as a concentrate of the second generation platelet concentrate is because the 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.

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In this study, the experimental design is to standardize the production of L-PRF in the horse directing it towards human production.

And' 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 surround ding cells guests;
2) platelets must be stored in fibrin structure with minimal damage or activation;
3) the three-dimensional fibrin lattice should be used as a scaffold for surrounding host cells.

Materials and methods

How to Prepare

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 polymerizes so widespread in the tube and will be obtained only a small part of the blood clot 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 keeps well enough surgical sutures (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).

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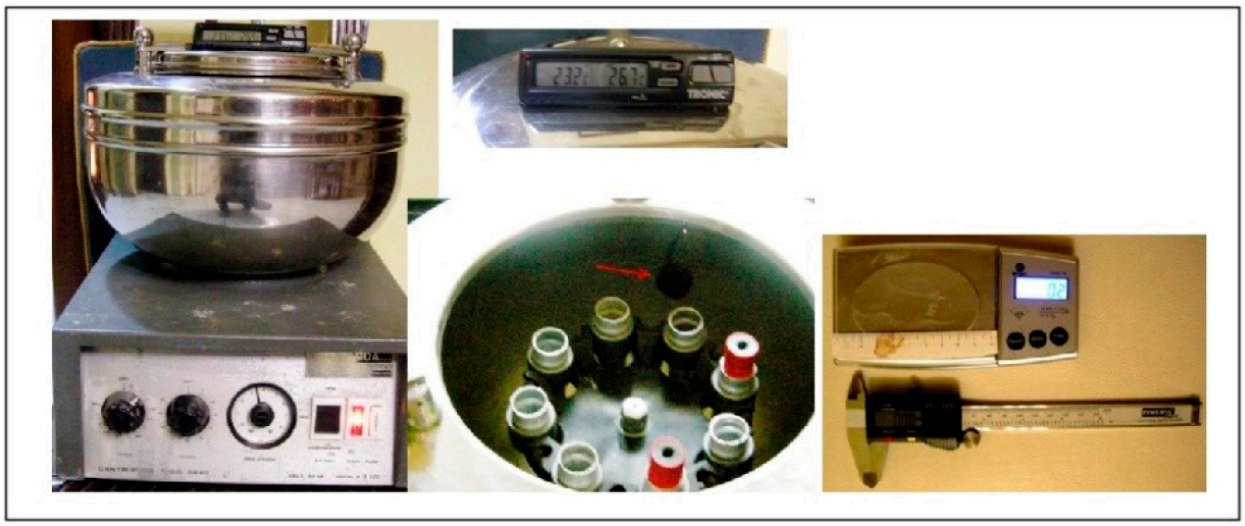

Fig.1 Centrifuge with thermometers, scales and digital gauge

The protocol is as follows: the blood samples are collected in tubes 9 mL, without anticoagulant, nor gel separator, and are immediately centrifuged according to the following program: 30" acceleration, 2' at 2700 rpm, 4' at 2400 rpm , 3' at 3000 rpm and 36" 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 by the PRF. The success of this technique depends entirely on the blood collection and the transfer speed in the centrifuge [7,8]. The 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 the horse, without modification of the human protocol, even allowing better standardization of the human protocol. A written consent of the owners has been provided for all the horses and the blood collection procedure was performed in accordance with the current AVMA guidelines.

E' was we performed a prospective study of equine blood. They have been used to test tubes without anticoagulant plastic coated glass, nor gel separator (BD Vacutainer tubes for serum 9.0 ml), for the production of L-PRF clots and membranes by n°6 healthy horses of various ages (average±SD, 10±4.1 years, range from 4 to 17 aa), gender and race.

The blood was collected quickly both with sterile syringes 10 cc with that needle class and shirt to Vacutainer tubes (22" average value, of less than 25" per tube) and immediately (within 1 minute)

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centrifuged according to the preceding description to a temperature more environmental than 21°C. The temperatures of the inner and outer surface of the centrifuge were recorded before and during centrifugation with a digital thermometer with internal probe ( ) (TRONIC)(Fig. 1).

A new device has been tested by us for the preparation and standardization of L-PRF in clots and membranes is the L-PRF Wound Box® (Fig.2). Using the PRF Box, the compression process of the membrane in the clots is performed through a slight compression, slow and homogeneous and the final membrane always remains homogeneously wet and soaked in serum.

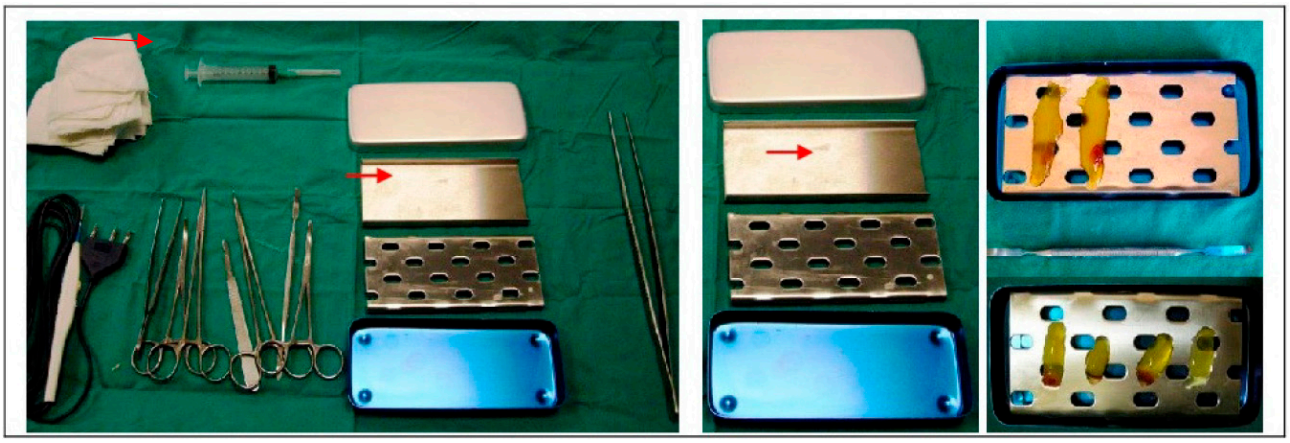

Fig.2 L-PRF Wound Box®

This mild method avoids the extraction and the loss of a significant amount of growth factors. The PRF Box existing on the market are of varying shape and exert through the compression plate a different pressure 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 x 7.6 x 2 cm containing a perforated steel plate of 150 x 68 x 1.5 mm, it is a second steel plate which acts as a compressor of 150 x 68 x 1.5 mm and weight of 148 grams (Fig.2). This second shaped plate |____| (→) it exerts a pressure of 142.437 Pa/cm^2. In this study the compression has been exerted on the clot for 2, 5, 10, 15 prime 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 buffy coat. Each fibrin clot L-PRF obtained was placed in a tray for measuring weight and size with a digital scale from goldsmith (Fig.1).

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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 to obtain from each clot, L-PRF membranes, which were individually weighed and measured with a digital gauge (Fig.1). The surface area in cm^2 of clots and the membranes was measured with the measurement software of "Calcderm" areas designed by us [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 on the midpoint of the membranes and were stained with hematoxylin-eosin. Each section has been divided into three equal-sized areas: Proximal (head), Center (body) 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 center of each observed area with a magnification of 25x, 40x, 60x, 100x (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 concentrated in the proximal area (head), closest to the red clot.

They were also examined smears of blood prepared by residual blood in the tubes for a morphological assessment, after removal of the PRF clot, with a spatula (two for each tube) differentiating the clot at 0' and 60' by centrifugation and fixing them with alcohol 90° for a coloring Gru nwald-May-Giemsa stain in order to identify the various elements corpuscular, in particular platelets and neutrophils to put them in relation to the examination blood count. blood chemistry analysis. From each horse he has also taken a blood sample to perform a blood count base using tubes K3E 5.4 mg EDTA (VacuMed).

Following the study of Peck et al.[11] n°3 were taken blood samples from the jugular vein left of each horse through a needle 14-gauge, 2 for the production of PRF and 1 for the blood count. After centrifugation the clot was removed in a group (A) immediately and in a group (B) after 60'. The supernatant derived from compression with Wound Box® membrane was differentiated between the

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0' and 60' and has been preserved in a test tube with K3E 5.4 mg EDTA for blood count. It was compared with the basal one and with the corpuscular elements of the counts performed on smears derived from red clot as indirect measurement of the platelet and the leukocyte concentration of the L-PRF. The two samples with 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 0' and 60' has been analyzed with a blood count in standard room. 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 counter Cell Dyn 3500 R (ABBOTT).

Determination of the parameters of the membranes

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

The size of the membranes were measured in height, length and width in mm with digital gauge and the areas in cm^2 with a software to calculate irregular areas (Calcderm) [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 on the supernatant and has performed on blood smears of red clot.

Statistical analysis

The statistical significance for the differences between two groups was calculated using the Student T-test and ANOVA for repeated measures for parametric variables and with the χ^2 for those not parametric.

Each value of $p < 0.005$ was considered statistically significant.

Data were analyzed using version 6.0 of the package for Discipline Biomedical Statistics of Santon-Glantz 2007.

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Results

The procedure was well tolerated in all animals. No significant differences were found in the basic haematological confrontation that had an average concentration of WBC 5.1x10^3/mL (±0.37 C.I. 95%) (p=0.24) and a platelet count average of 106.8x10^3/mL (±15.3 I.C.95%) (p=0.5). It was not possible to quantify directly the platelet concentration and WBC trapped inside the L-PRF clot and was derived indirectly by comparing the mean values of whole blood, mean values on the supernatant obtained after compression clot at 2' and average values obtained with the counts of smears of red clot after the removal of the clot of L-PRF.

The 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. In this comparison it was verified that there are significant differences in the characteristics of the clot, but these differences are canceled when you examine the membranes derived from the compression. This observation in our opinion would be assigned to a different content of exudate (weight exudate 1.47±0.13 gr in humans, 3.05±0.11 gr in the horse, p=0.000).

	Man (n=8)	Horse (n=6)		
Variable	Average (±SD)		T Student	Significance
Final T° test tube(°C)	27.5 (±0.66)			
Weigh Clot(g)*	2.09 (±0.19)	4.23 (±0.55)	P=0.000< 0.005	S
Weigh Membrane (g)	0.62 (±0.15)	0.78 (±0.08)	P=0.036> 0.005	NS
Exudate Weigh (g)	1.47 (±0.13)	3.05(±0.11)	P=0.000< 0.005	S
Lenght Clot (mm)	35.69 (±3.43)	44.38(±3.83)	P=0.000< 0.005	S
Width Clot (mm)	12.81 (±0.75)	14.74(±1.23)	P=0.003< 0.005	S
Height Clot (mm)		7.02 (±1.09)		
Surface Clot (mm^2)		4.10 (±0.86)		
Length Memb (mm)	34.81 (±2.95)	36.81(±3.18)✠	P=0.248> 0.005	NS
Width Memb (mm)	12.25 (±0.71)	13.02(±1.01)✠	P=0.119> 0.005	NS
Height Memb(mm)		3.02 (±0.51)✠		
Surface Memb (cm^2)		3.08 (±0.5)✠		
Weight ratio Clot/blod 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' compression at 30°C.

N.B.: the values are not in relation to the content of Hb and erythrocytes in whole blood.

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

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In this study the size of the membranes are not found to be in relation to the hemoglobin content or the content of Erythrocytes encountered in blood count baseline. In optical microscopy (Fig.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 center; the distal part had only residual traces of cell bodies. Optical microscopy has not, however, allowed to observe in more detail the exact state of these cell bodies.

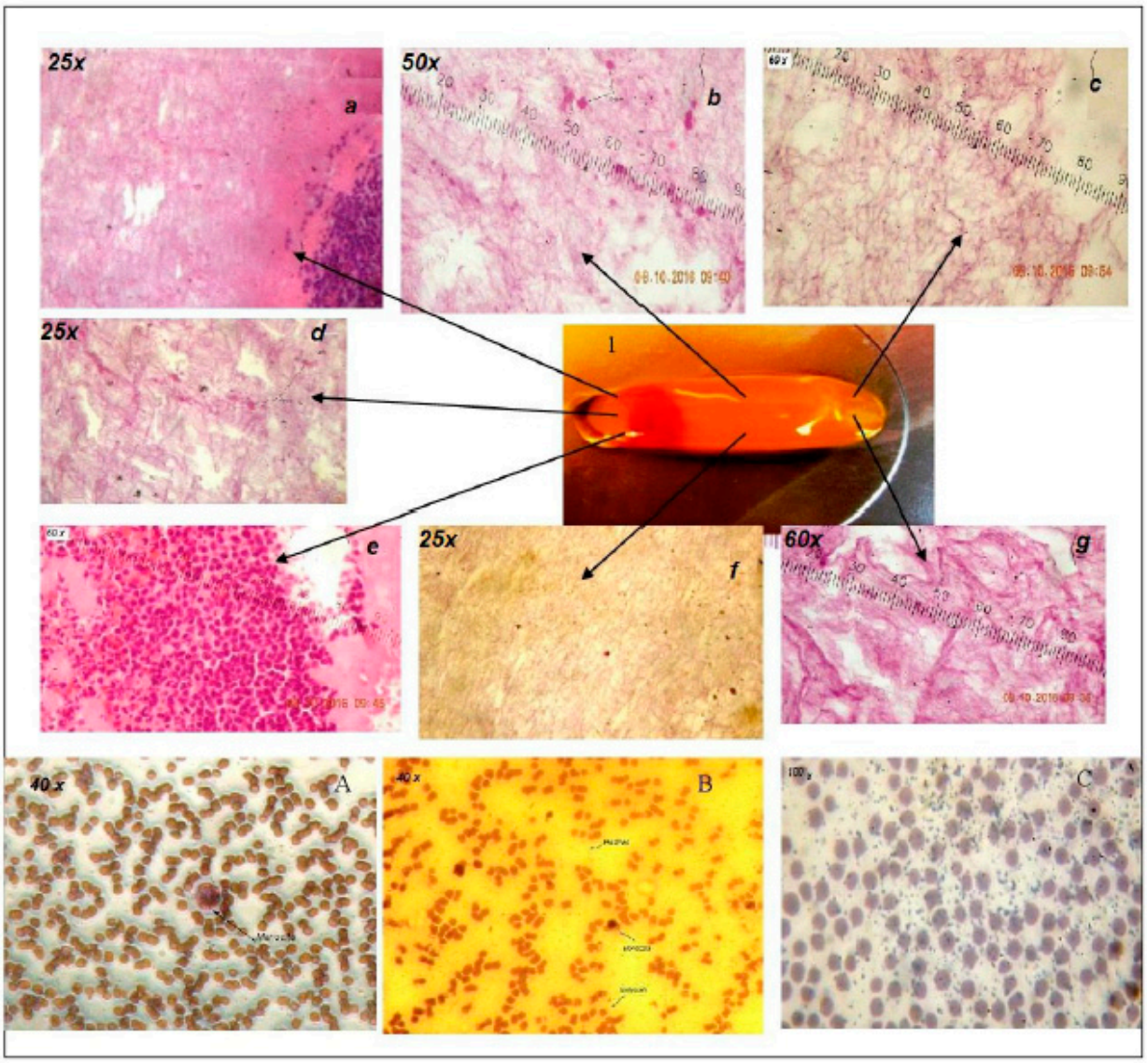

Fig. 3 Membrane L-PRF 0 minutes from the compression horsepower (hematoxylin-eosin staining). a) III proximal ingr.25x White Blood Cell-pattern Fibrin; b) medium-III ingr.60x Erythrocytes pattern Fibrin; c) III distal ingr.60x pattern Fibrin; d) III proximal ingr.25x Erythrocytes-Fibrin; e) III proximal ingr.60x Fibrin on the right, the center lymphocytes, erythrocytes and granulocytes neutrophils to left; f) average III ingr.25x pattern of fibrin; g) III distal ingr.60x pattern Fibrin; A) smear of red clot ingr.40x presence of Monocyte in a carpet of red cells; B) Red clot smear ingr.40x presence of red blood cells, monocytes and platelets; C) red clot smear ingr.100x presence of platelets in a carpet of red cells (Coloration May-Gru nwald-Giemsa);

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The Figure 4A shows the average characteristics of a membrane obtained with the L-PRF Wound Box to several minutes of compression (2, 5, 10, 15 minutes). The slight variations between M05', M10', M15', M02' compared to make us reach out towards the use of the membrane after two minutes into the compression (Tab.2).

Variable	between Basal and M02'		between Basal and M05'		between Basal and M10'		between Basal and M15'	
Parameter	*t-Student*	ANOVA	*t-Student*	ANOVA	*t-Student*	ANOVA	*t-Student*	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*
Lenght Memb (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 Memb (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 ✗
Heigth Memb(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 Memb (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' and M05'-M10'-M15' χ^2							
Weight (g)		p=1.000 ✗						
% Reducing Weight (g)		p=0.852 ✗						
Lenght Memb (mm)		p=1.000 ✗						
Width Memb (mm)		p=1.000 ✗						
Heigth Memb(mm)		p=1.000 ✗						
Surface Memb (cm^2)		p=1.000 ✗						

✗ p>0.005=No Significant Difference; * p<0.005=Significant Difference

Table 2. Test of Significance on the variations of the parameters of a membrane L-PRF to compressions with L-PRF Wound Box for 2, 5, 10, 15 minutes.

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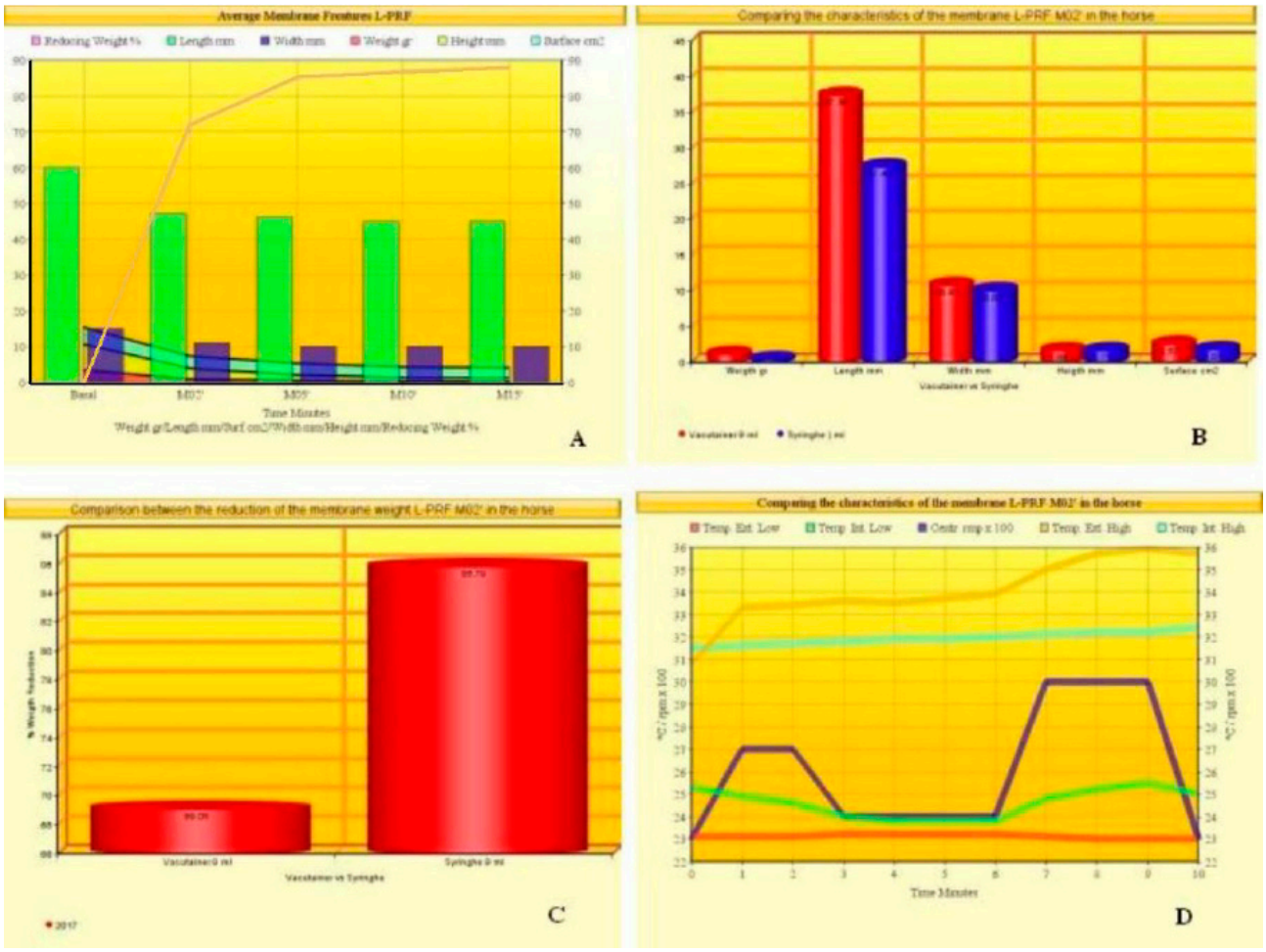

Figure 4 A) medium of a membrane features L-PRF obtained in the horse with L-PRF Wound Box to several minutes Compression: Weight (g) Height (mm) Length (cm) Width (cm), Size (cm^2),% weight reduction during compression. Picking with 9 ml Vacutainer system. B) Average Characteristics of an L-PRF membrane obtained in the horse with 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' compression.

The processing temperature should be >21°C since if lower than the clot is not produced. The Fig.4D shows the temperatures detected during centrifugation within and outside the low-temperature centrifuge (23°C) and high temperature (30°C) at various times. Note that increasing the speed of revolutions increases the internal temperature.

Using 9 ml syringes instead of the sampling system Vacutainer the membranes that are produced are smaller in size (Fig.4B) both in terms of weight, that of length, width and surface useful. After compression of 2', the reduction of the weight of the membrane with respect to the clot is 85% if it picks up the blood with a syringe, by 70% if it is taken with Vacutainer system (Fig.4C) probably because with syringe it is damaged the corpuscular part of the blood and fibrinogen.

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The results of the blood counts of whole blood, of the supernatant obtained from the clot compression 0' and 60' compared with the count of erythrocytes, platelets and WBC smear of red clot 0' and 60' are shown with the corresponding statistical tests in Table 3, while the microscopic images 60 'are shown in Figure 5.

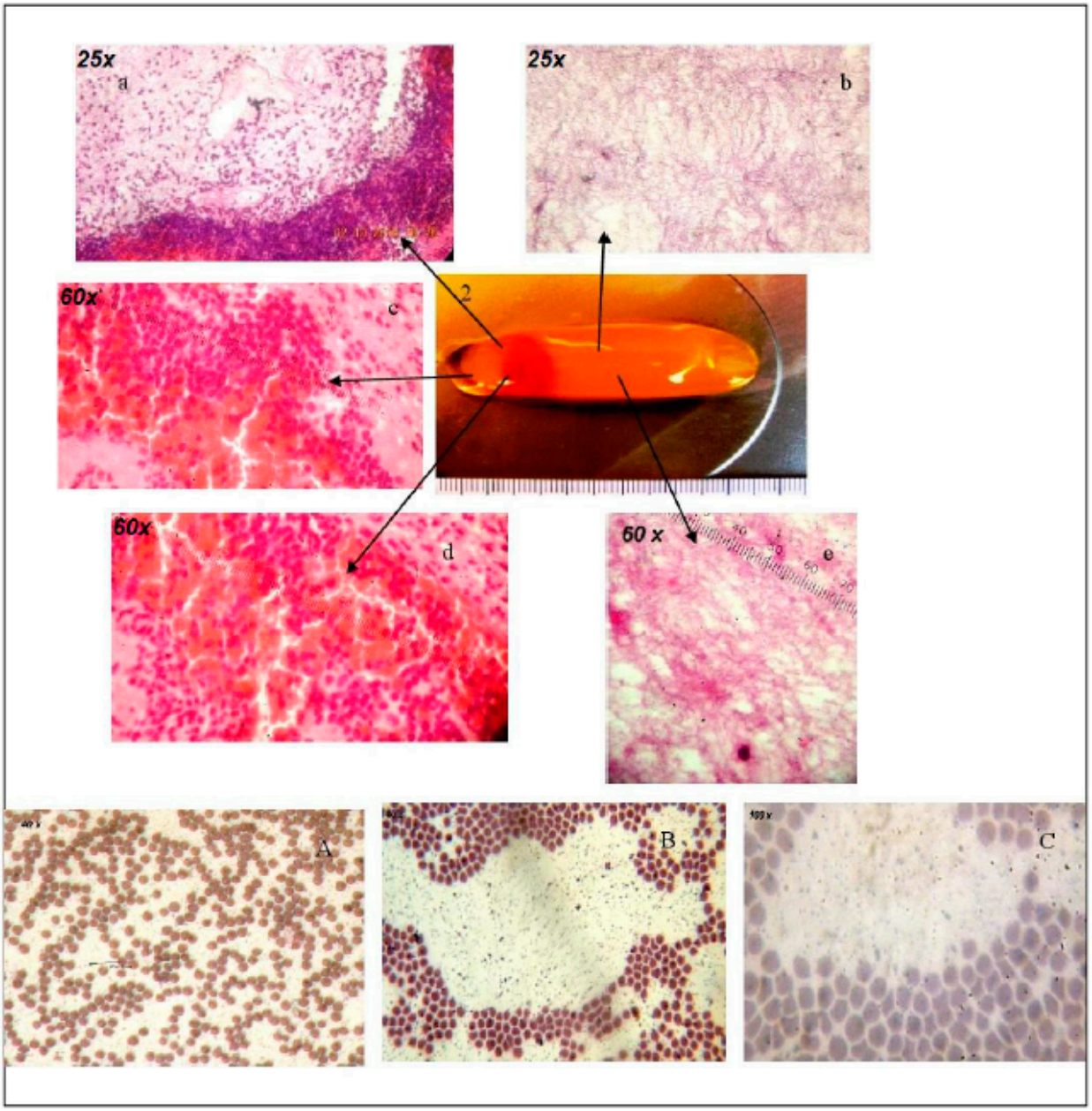

Fig.5 Membrane L-PRF of 60 minutes by the compression horsepower (hematoxylin-eosin staining). a) III proximal ingr.25x White Blood Cell-Erythrocytes-pattern Fibrin; b) medium-III ingr.25x pattern Fibrin; c) III proximal ingr.60x pattern of Fibrin on the right, the center lymphocytes, erythrocytes and granulocytes Neutrophils to left; d) III proximal ingr.60x Fibrin on the right, the center lymphocytes, erythrocytes and granulocytes neutrophils to left; e) average III ingr.60x pattern of fibrin with Lymphocyte; A) smear of red clot ingr.40x presence of platelets in a carpet of red cells; B) Red clot smear ingr.40x presence of erythrocytes and many platelets; C) red clot smear ingr.100x presence of many platelets in a carpet of red cells (May-Gru nwald-Giemsa staining);

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Table 3 demonstrates a 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.

Type	CBC blood	Smear C.R. 0'	Smear C.R. 60'	Between Red Clot smear 0-60'			
n°/µl	Average±D.S.	Average ±D.S.	Average ±D.S.	*t-test**		χ^2	
RBC	7648000±11309.81	7399440 ±27039.76	7322960±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	106780±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						
Lynphocites	1606±668						
Monocytes	490.2±138.06						
Eosinophils	5.4±5.37						

Type	CBC supern. at 0'	CBC supern.at 60'	Between CBC supernatant 0'-60'			
n°/µl	Average±D.S.	Average±D.S.	*t-test**		χ^2	
RBC	13428±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
Lynphocites	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=0.631>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	Membran 0'		Membran 60'		between Membran L-PRF 0-60'			
	n°/µl	%	n°/µl	%	*t-test**		χ^2	
RBC	216012	0.0028%	193966	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	NS	p=0.993>0.005	NS
PLT	105801	99.00%	97153	91.00%	p=0.002<0.005	S	p=1.000>0.005	NS

Table 3. Results of the blood counts of whole blood, of the supernatant obtained by compression of the clot at 0' and 60' compared with the counts of Erythrocytes, platelets and WBC of red clot smear at 0' and 60' with tests of significance.

* processing performed on two comparisons. hypothetical content of RBC, WBC, PLT in the L-PRF membranes at 0' and 60' with significance tests.

p>0.05 = +0.5% non-significant difference; p<0.01 = -1% significant difference.

It also shows a statistically significant difference between the content of RBC, WBC and PLT in smears obtained from red clot at 0' and 60' also shown in Figures 3 and 5 A, B, C at various magnifications. The Table 3 also shows the contents of RBC, WBC and PLT hypothetical in L-PRF membranes derived from the difference of these elements corpuscular between the whole blood, the supernatant at 0' and 60' and the smear of red clot to 0' and 60'. The t-test shows significant differences between RBCs at 0' and 60' and between PLT at 0' and 60' in L-PRF membranes.

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The content of RBC in the membranes is of 0.0028% with respect to the whole blood, that of WBC is of 99.24%, that of PLT is 99,0% compared to the content in whole blood. The membranes after 60 minutes of compression have a content of RBC, WBC and PLT lower than those at 0 minutes.

Discussion

The study performed by McLellan et al. [13] It has shown that the PRF equine similarly to that of humans provides an immediate and constant source of tissue growth factors. Our study has tried to standardize the preparation of L-PRF procedure, which while remaining a technique of easy execution, 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 for the abundance of growth factors.

The L-PRF represents a huge advance in the evolution of platelet concentrates since it is essentially a fibrin membrane with platelets and leukocytes trapped. 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 is constantly will form when the steps described above are strictly observed.

One of the important considerations in generating a good membrane L-PRF is the delay in time between blood collection and centrifugation, and the processing temperature. The success of the technique L-PRF depends entirely on the speed of collection of blood and the immediate transfer into centrifuge, usually within one minute and by a centrifugation temperature and higher compression at 21°C. You can not generate a clot of L-PRF well structured (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; is formed instead a little 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, can be subjected to mechanical forces and healing outcomes with success, they

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depend on the structural integrity of the L-PRF and therefore it is important to clarify its physical properties.

The L-PRF looks like the dense connective tissue with superior handling characteristics. Through an elastic modulus of 0.470 MPa (SD=0.107) of 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 architecture defined, impressive mechanical properties and 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.

Conclusions

The problems related to the conservation of these membranes will greatly limit the use. Cryopreservation at 4°C we have seen 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) than fresh (3.5-3.8 days) [14].

The data concerning the maximum storage time and the PRF of the ideal temperature of conservation 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' and 60' by centrifugation using dry Vacutainer tubes vs Vacutainer tubes with Separating Gel analyzing the prepared even to the 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 box Wound Box can be used effectively to fix the grafts autologous (L-PRF membrane or dermis bio-conductive or bio-inductive).

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