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## Contents



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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.

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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

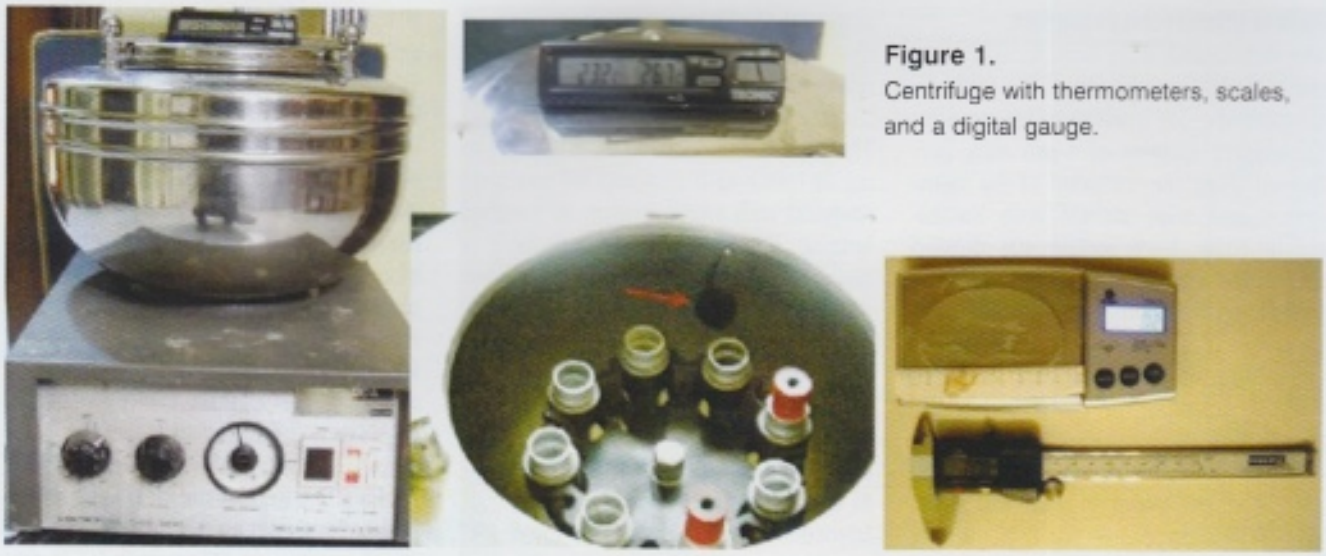

**Figure 1.**
Centrifuge with thermometers, scales, and a digital gauge.

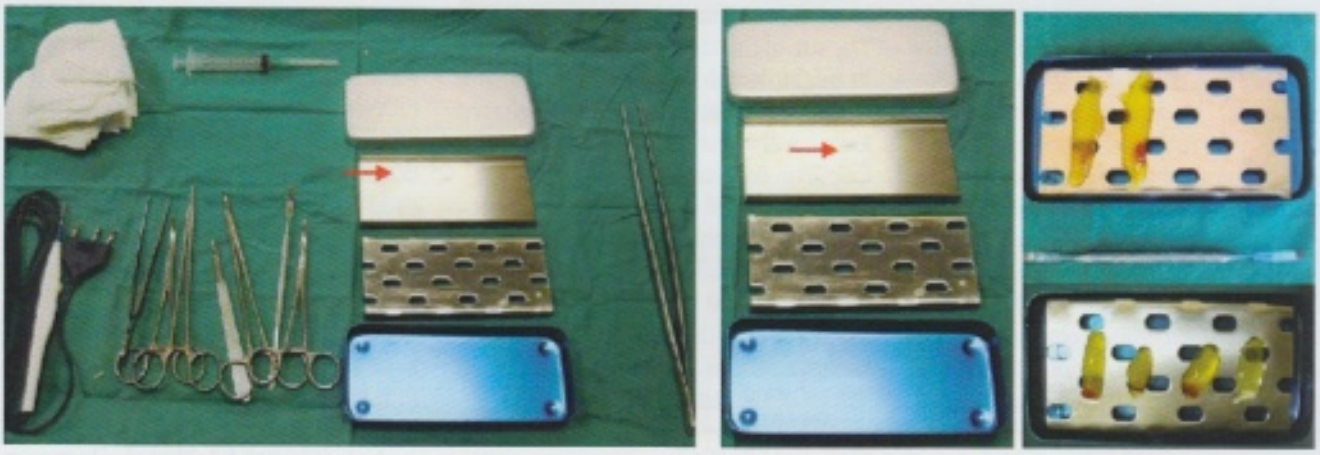

**Figure 2.** L-PRF Wound Box®.

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].