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A new instrument aid of plastic surgeon: membranes L-PRF (Platelet-Rich-Fibrin)

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Summary

A new instrument aid of plastic surgeon: membranes L-PRF (Platelet-Rich-Fibrin)

The PRF is distinct in P-PRF (Platelet Rich Fibrin pure) and L-PRF (Fibrin Rich in Platelets with leukocytes). In vivo at the height of the formation of the fibrin clot, platelets bind to fibrin β-integrin and the clot shrinks. The PRF clot forms a strong fibrin matrix with a complex three-dimensional architecture. Quantifying PDGF-BB, TGF-1β and IGF-1 in the PPP and the PRF, the analyzes revealed that the slow fibrin polymerization during the processing of PRF leads to the intrinsic constitution from platelets of cytokines and glycan chains in the meshes of fibrin. The central part of the PRF has massively platelets trapped in the mesh of fibrin. The localization of platelets in the PRF was examined by immunostaining and Scanning Electron Microscope (SEM). Compared to the membrane PRF tablet dry gauze (G-PRF), the conservation of the level of the plasma, of the fibrin 3D, and platelets is more intact in preparations of membrane PRF with compression system (PRF-C) metal. The distribution of platelets in the membranes C and G-PRF-PRF was analyzed by SEM and immunocytochemistry. The fibrin rich platelet pure (P-PRF) and the leukocyte-platelet rich fibrin (PRF-L) are biomaterials solid fibrin or not containing leukocytes. The problem of the concentration of platelets does not exist in the PRF, in that all the platelets of the blood sample taken are activated and integrated in the matrix of fibrin in the clot. Approximately 97% of platelets, and more than 50% of the leukocytes are concentrated in the PRF clot that showed a specific three-dimensional distribution. Almost all platelets (>97%) were absent from tubes of the groups tested after extraction of the membrane PRF. In the red part of the PRF clot, clots have GR in fibrin network.

Key words: Growth Factors, Fibrin Rich of Platelets, L-PRF Wound Box.

Introduction

Many results suggest that platelets can have a new role in tissue repair and vascular plasticity, as well as active elements in immune and inflammatory responses. They secrete active proteins and other matters that are able to condition many processes supporting cells' intake, growth and morphogenesis. Activated platelets exude and show these matters. A clot is a natural source of growth factors and cytokines, thanks to substances releasing inside it by activated platelets, that could be used as therapy to speed up physiological healing. A lots of these stuffs are stocked in α-granules and may be easily recognize with SEM and immunofluorescence.

Exogenous adding of Platelets Rich Plasma (PRP) on wound's site not only speeds up physiological healing but also provides an additional substrate to tie up for others cells such as endothelial and smooth muscle ones, fibroblasts, leucocytes, keratinocytes and stem cells as well as platelets. Among benefits using PRP there's safety provided by platelets' antibacterial influence. They actually not only release substances counteracting bacteriums but also take part in bacterial disposal during sepsis.

Fairly amazing is the recent acknowledgement of platelets' aptitude to reduce pain. Molecular basis have to be study deeper, but an answer could be that platelets release PAR-4 which have antinociceptive properties.

In 1986 cicatrizant factors derived by human self platelets (PDWHF) have been proposed by *Knighton et al.*[1] to help healing of recalcitrating sores and induce formation of granulation tissue in early healing's step.

There are any morphological differences of thick fibers in PRP's kinds by SEM. There aren't many thin fibrin fibers in LPRP (lyophilized PRP) and FPRP (Freshly-made PRP), on the contrary they form a dense layer over thick fibers in HPC (Human Platelets Concentrate) (Figure 1).

Thin fibers present in HPC could be related with high initial platelets concentration in HPC (3-5 x 1011 platelets/l), when the local activity in favour of coagulation can be improved by amplification of prothrombotic stimulus, that leads to a such explosive production of thrombin with a consequential increase of fibergenesis on the platelets surface with fibrin's formation and its polymerization.

The HPC also has an higher concentration of fibrinogen (3,5 mg/ml) than FPRP and LPRP and it takes part in secondary net over tick fibers [2].

There are a lots of adhesive proteins on fibrin reticulum: fibrinogen (FG), fibrinonectin (Fn), vitronectin (Vn), thrombospondin-1 (TSP-1). Fn takes part to healing wounds and promote mitogen activity Platelets Derived Growth Factor (PDGF).

Among growth factors stored in platelets and useful wounds' healing there are PDGF (isoform -AB and -C); there are also Vascular Endothelial Growth Factor (VEGF), Transforming Growth Factor β1

(TGF-β1), basal Fibroblasts Growth Factor (bFGF) mainly FGF-2; Epidermal Growth Factor (EGF); Hepatocytes Growth Factor (HGF); Insulin-like Growth Factor (IGF). The members of TGFβ family are very important in wounds healing and formation of scar tissue. TGFβ function is up regulated by a secreting latent form, that can down regulate angiosperms even if it promotes matrix proteins' production. Platelets are a rich source of cytochines and chemokines, owning an important role in wounds healing. An example is RANTES, a chemokine released by a P-Selectine/Platelets dependent mechanism on inflamed endothelium.

Platelets are an important source also of matrix metalproteases (MMP) (MMP-2, MMP-9, ADAM- 10, ADAM- 17, ADAM TS-13) like also tissutale inibithors of metalproteases (TIMP 1-4). The MMPs are stored in α-granules and also in cytoplasmic membrane's vesicles.

The fibrinogen may improve wounds cicatrization, increasing both cells' proliferation and migration, it is linked with Fn in fibers regardless of the formation of fibrin. Fibrin is important in wounds healing, in fact the outcome of healing is influenced by the structure of fibrin in wound's site (thickness of the fibers, number of branch points, porosity and permeability of the clot) [3] (Figure 2).

If a wound doesn't heal in a quick and specific sequence, or if the healing process doesn't result in a structural integrity, the wound will be considered chronic. Chronic wound's healing is similar to the process of acute one, but granulation tissue will be made, often with excessive fibrosis that leads to scar contraction and loss of function.

Chronic wounds and their treatment are an adequate burden on the health system and this will continue to be a therapeutic challenge. Because of the heterogeneity of patients, multiple etiologies and lack of animal models, the research in this area is difficult and complex.

Despite this problems, various pathogenic local factor, cellular and molecular, have been identified. Medical therapy is still the standard choice in ulcers' treatment. If it fails, a surgery treatment is necessary. Skin grafting with partial depth is essential in chronic ulcers' treatment and is often used to cover or seal not healed extended ulcers.

Before the transplant, ulcer is debrided: skin is lipodermatosclerotic, tendons exposed and subcutaneous calcifications are removed to create a vascularized wound, suitable site for a skin cut mesh to make intimate contact with the site of the ulcer and facilitate the flow of blood. If the formation of a chronic ulcer creates problems, it will be possible to create an acute injury, because the morbidity background that first created the ulcer is still present.

It is clear that the fibrin clot rich in platelets constitute a bioactive reservoir. An high hematocrit or low level of platelets can be a limiting factor and more research are necessary to establish the exact number of platelets for their application. In addition to secrete proteins, platelets release diffusible stuffs of low molecular weight and large amounts of micro particles carrying proteins such as TF or IL-1 which are prothrombotic substances. So it could be not recommended the use in patients with thrombotic hereditary risk factors. The concomitant use of antiplatelet drugs could theoretically limit the effectiveness.

Aspirin reduces platelet secretion and should therefore be avoided in the days prior to the preparation of autologous PRP, because it inhibits COX enzyme. After first massive release of growth factors, platelets synthetize and release new ones for the rest of their lives (7-10 days). The PRF® (Fibrin Rich of Platelets) it's a new generation of platelets concentrate, obtained by centrifugation of autologous blood, without adding biological substances. It contains a polymer matrix of fibrin, leucocytes, cytokines and stem cells. It is distinguished in P-PRF (Pure Fibrin Rich in Platelets) and L-PRF (Fibrin Rich in Platelets with Leucocytes). The PRP have a transient effect on wounds healing and also bovine thrombin increases the risk of coagulopathy, that does not happen with the PRF. It happens a natural clotting process that allows an easy collection of leucocytes and PRF in clot. Fibrin gels are desirable as scaffolds in tissue engineering for many reasons. The main one is related with the compatibility with cell's life of fibrin, which is different in many components and manufacturing processes of scaffolds. Fibrin is a natural substance produced completely biodegradable, which facilitates the transition to a new extracellular matrix. In vivo at the peak of clotting platelets bind fibrin and β-integrin and the clot shrinks. It shrinks also against edge of wound's site creating tensions which direct new temporary matrix. PRF clot is produced by a natural polymerization process during centrifugation, and its architecture of natural fibrin may be responsible of slow release of growth factors and matrix's glycoproteins.

This slow release is unthinkable in most of PRP techniques because of platelets' sudden activation.

PRF clots are directly used to fill a cavity during a lipostructure in plastic surgery. Even if platelets growth factors have an important role in PRF biology, the architecture of fibrin and leucocytes' content are two data keys.

Platelets and leukocytes distribution inside fibrin's clot was evidenced by blood counts, photonic microscopy and SEM.

A good approach for PRF preparation must separate platelets and erythrocytes and concentrate them without any damage or lysing platelets, of course. Growth factors stored inside α-granules are not active during secretion, they fuse with membrane activating themselves.

So if platelets are damaged during production of PRF they won't produce bioactive growth factors. They are very unstable and sensible to all stressing issues during processing and application phases; according to this reason also growth factors' concentration could be influenced by manipulation during blood processing.

So it is important also the type of centrifugation, it must have specific features, such as Initial Start Low, high rpm in middle phase and Final Stop low [4].

It also have to be done at specific temperature and in a strict time

Material and methods

The protocol of PRF's preparation it's very easy: blood have to be centrifuged within 2 minutes following this program: 30" of acceleration; 2' at 2700 rpm; 4' at 2400 rpm, 3' at 3000 rpm; and 36" of deceleration and stop. PRF's clots have to be collected and RBC are removed with scissors without any PRF's damage at macro level (processing protocol, *Nice, France*). The resulting product it is made by three levels: PPP (Plasma Private of Platelets at the top) PRF (clot in the middle), RBC (in the bottom) (Figure 3).
Fibrinogen is concentrated in the middle and top of test tube at first, between RBC in the bottom and acellular plasma at the top. PRF's clot make a strong fibrin matrix with a complex tridimensional architecture (Figures 2, 3, 7), in which are concentrated the most of platelets and leukocytes. The compression of the clot with dry gauze (G-PRF) causes a reduction of isoforms of PDGF in comparison with C-PRF (PRF membrane made with a compression system) (PRF box) that stimulates in a more efficient way the cellular proliferation and neovascularization [5].
Quantifying PDGF-BB, TGF-β1 and IGF-1 in PPP and PRF, analysis revealed that slow polymerization of fibrin during manufacture of PRF leads to secretion of cytokines and glican chains by platelets inside fibrin's mesh. Analyzing three proinflammatory cytokines (IL-1β, IL-6, TNF-α), an inflammatory cytokine (IL-4) and angiogenesis promoter (VEGF), it have been show that PRF could be a *crux* in immune modulation with skills in inflammation control. PRF, in spite of other platelets concentrates, could be able to release progressively cytokines during remodeling of fibrin's matrix.
Many studies have proved that the L-PRP have antimicrobial effects, but without undesired inflammatory reactions. The PRF allows to surgeon to provide directly a natural healing response and can stimulate the formation of vital blood vessels, adipocytes, collagen deposition that seems to resist over time also without wound, of course [6].
Platelets' cytokines and leukocytes have an important role in this biomaterial, but fibrin's matrix and determinants are responsible of real therapeutic enhancement of PRF. Cytokines are immediately utilized in wounds healing.
A fibrin glue, enriched with cytokine (such as PRP) with a great uncontrollable effect have a short duration, it is better a physiological matrix of fibrin (like PRF), with better effects [7].
PRF's advantages over PRP are:
1- None biochemical blood manipulation;
2- Simplified and cheaper manufacture;
3- Use of bovine thrombin and anticoagulants are not requested;
4- Positive healing thanks to a slow polymerization:
5- More efficient cellular migration and proliferation;
6- PRF have a good effect on immune system;
7- PRF helps haemostasis.

The mechanism involved in PRF's formation it's fibrinogen concentrated at the top of tube test that combines itself with circulating thrombin produced by centrifugation to make fibrin. The centre of PRF shows many trapped platelets in fibrin's meshes. The success of this technique depends entirely by time elapsed between blood collection and its centrifugation that have to be done in less time as possible, also by manufacturing temperature and type of tube test. Blood samples have to be collected from patients with no assumption of aspirin or others anticoagulant drugs 2 weeks before. *Dohan et al.* (1988) revealed a slower release of growth factors and observed better healing skills in PRF than PRP. It have been proved also that cells are able to migrate in fibrin's mesh. The slow polymerization mode give to PRF's membrane a physiological architecture particularly favorable to support healing process. Platelets localization in PRF have been examined by Immune coloration and SEM. In previous studies clot's compression to make a PRF membrane have been done with a humid or dry gauze.
However they aren't worried about this compression, probably platelets damages and loss of Growth factors.
Su and Burnouf showed that high quantities of growth factors are removed by compression. So compression process could influence clinical efficacy and quality of PRF's compounds as graft material.
The levels of growth factors after different types of compression have been valued with biological dosages and cytokine-antibody techniques. Among PRF's membrane compressed with dry gauze (G-PRF), the conservation of plasma level, fibrin 3D mesh, and platelets it's more undamaged in PRF's membrane compounds with metal compression system (C-PRF).
The humid weight of PRF's membrane decreased from 2.18 g to 0.35 g with metal compression and 0.04 g with gauze compression (decrease of 98% vs 84%).
Among tested growth factors, the PDGF contained in C-PRF it's more and stimulates, significantly cell proliferation and neovascularization. The C-PRF could be useful for grafting, reducing loss of bioactive factors. An important skill of PRF is that resulting fibrin gel it's more stiff than PRF with adding of thrombin (PRP). Fibers density and branch points density of fibrin's mesh mainly regulate stiffness of fibrin's gel and these parameters are related with quantity of thrombin in dose-dependent mode (Figure 2).
It's necessary so establish a standard protocol to manufacturing PRF that satisfied these rules:
1- Platelets growth factors have to be preserved to stimulate surrounding patient cells;
2- Platelets have to be collected in fibrin's mesh with minimal damage or activation;
3- Fibrin's 3D mesh have to be used as scaffold for nearby patient cells.

The membrane samples PRF were examined by SEM and with the immunocytochemical method by *Kobayashi et al.* 2012 [5].
The C-PRF was divided into 3 regions of equal length and the presence of platelets in each region is been observed at S.E.M. The region 1 is the closest to the red clot and presents numerous platelets aggregated and there are some lymphocytes and other white blood cells. The number of platelets decreases with increasing dis-

tance from the clot red. In region 2 (center) present fibrin fibers and some platelets. In region 3 the reticle of fibrin is very obvious, while platelets are few (Figure 3).

The distribution of platelets in the membranes C-PRF and G-PRF of the region 1 was analyzed by SEM and with the immunocytochemical.

In the C-PRF the reticle of fibrin is completely covered by many aggregates platelets of and lymphocytes (Figure 4), whereas in the G-PRF the reticle of fibrin is fully pressed in a film form and can be observed few platelets. These results were verified by the detection. of positive cells to the antibody anti-CD41 through immunocytochemistry, in fact in the C-PRF on one side of membrane are accumulated numerous platelets CD41-positive and some platelets are located in the membrane.

On the opposite side of this there are few platelets. In the G-PRF instead to the platelets aggregates were not found on both membranes of PRF but only in it with a higher density than that of C-PRF.

In comparing the growth factors contained in C-PRF and G-PRF the first contains a higher concentration of TGF-β, PDGF-AB, PDGF-BB, EGF, FGF-4, IGF-II and VEGF-D.

The discovering of the study of *Kobayashi et al.* [5] is that the platelets are not equally distributed in and on the surface of the clot PRF, although it was considered to be a gel with a uniform concentration of platelets. Therefore in a clinical condition in which factors growth provided platelets are expected and desired, should be used an adjacent region to the red thrombus which is richer rich in platelets.

Based on the concept that the serum retained in the PRF clot may contain high levels of growth factors released from platelets that are more or less active during centrifugation, it wasn't attempted to squeeze all the plasma with full compression of the PRF clots.

The tendency to a higher level: growth factors in the C-PRF compared to G-PRF can be attributed to the growth factors (FG) Platelet-derived (PDGF-AA, PDGF-AB, PDGF-BB). This result may be due to fibrin because the reticle of fibrina can absorb directly the FG or could trap serum albumin or the heparin and thus indirectly to keep the FG.

It's almost impossible to count and adjust the number of platelets in the preparations of PRF before clinical use. Therefore, the clinically most effective way to control the quality of the results is to use the closest region to the PRF clot GR.

Results

The L-PRF in Surgery

The fibrin is a useful substrate for purposes of bioengineering and is one of the most popular hydrogel in the field of tissue engineering and regenerative medicine. The transplanted cells require specific signals from the extracellular matrix to survive. *Anoikis* is a term used to describe the premature death of cells that do not receive these signals mechanical and chemical from the matrix, and this phenomenon is considered a major cause of transplant cell failure.

The fibrin rich of platelets pure (P-PRF) and the Leukocytes-Fibrin are Rich of Platelets (PRF-L) are biomaterials solid fibrin or not containing leukocytes. In these techniques the platelet activation is part of the process of production and can be natural (L-PRF) or artificial (P-PRF), but it always occurs during centrifugation and leads to a strong final architecture of fibrin.

The L-PFR is a preparation with leukocytes and with a high density of the fibrin grid. These products exist in the form of activated gel and cannot be injected or used as traditional fibrin glue. However, because of their strong fibrin matrix can be treated as a solid material for applications that have been proposed, with interesting results in general surgery, but these applications are still in the experimental stage as they require to find a way to use clots in each specific surgical procedure.

Fibrin create a provisional matrix in the space of the transplant, but its fibers do not have directionality and tension, it has few growth factors associated content.

The fibrin gel instead have a regular arrangement of pores and reticular fibers with short and thin and not totally acellulate and at SEM have been observed platelets and leukocytes (Figure 5).

In studies performed on horse by *Textor* [8] (2014) and *Mc Callan* [13] (2014) the Fibrin Gel containing Platelets, (PRFG) have large dense fibers and randomly arranged (with average diameter of 117.7 ± 10.53 nm). The fibers of fibrin gel are smaller (56.8 ± 5.11 nm). The incorporation of platelets in fibrin gel thus resulting in structural alterations and an increase of the concentration of growth factors and can ultimately improve the performance of the insertion of cells in the scaffold after the transplant.

By virtue of the content of growth factors, platelets directly contribute to the growth, development and restoration of tissues. The PDGF and TGF-β1 are the most abundant growth factors contained within 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 (Figures 6, 7).

The addition of platelets to fibrin gels: (PRFG) implies an increase of the diameter of the fibers and the porous decreasing of the areas and increasing the rigidity of the fibrin gel. The measured diameter of the fibers has been associated with a lower concentration of platelets (100×10^3 platelets/μl) that approximates the systemic concentration of platelets in normal horses.

The explanation may be that less tension is applied to the fibers by the lower number of platelets. The porous areas and the percentage of porosity are important structural indices of any biological scaffold. The larger pores favor the inner growth and cell proliferation while the smallest pores promote cell adhesion due to a greater surface area. The concentration of leukocytes present in PRP and in PRFG is controversial. However, the specific effect of the concentration of leuko-

cytes on the formation of clot, on the formation of fibrin gel or on the rigidity of the clot has yet to be studied. The PRP used for the formation of PRFG has a concentration of WBC intermediate (average: 9.34 x 10^3 leukocytes/ml) (range: 3.2 x 10^3 to 16.0 x 10^3 leukocytes/ml) [8].

From a clinical point of view, L-PRF has excellent handling properties: the individual clots L-PRF are transformed into membranes of appropriate size and thickness thanks to the new "*L-PRF Wound Box*"; more membranes can be joined together and will serve to create a membrane bioactive larger to cover and form large insert. The membrane of L-PRF can be cut to measure. Being flexible enough adapts well to different anatomical areas.

From a legal point of view, a doctor being authorized to intravenously needle-stick is also authorized for blood sample. Moreover, by EU regulation, then with the law valid throughout Europe the L-PRF having no addition of substances is not an emo-derived but is included in the case of re-engagement of autologous cells.

As the healing that occurs with this technique is neither first nor second intention, it is called "*healing by second intention modified*" [9]. The family L-PRF fits the needs of the surgeries. As clots and the membranes L-PRF has a shape and a volume easy to combine with the majority of surgical techniques, such as filling and interposition of biomaterials of healing or as protection membranes for the healing of wounds. These membranes are also strong and provide a slow release of several growth factors for long periods. Finally, it is easy to prepare in large quantities and inexpensive, which makes it particularly suitable for daily clinical practice. It is used in particular in the treatment of skin ulcer.

The L-PRF in Vitro

In vitro behavior of a membrane and L-PRF and Gel P-PRP (Platelet Rich Plasma pure) (PRFG-endorest) were compared through the evaluation of the slow release of growth factors and matrix molecules.

These two families of gel were placed in a culture medium for 7 days, and the versions slowed of 3 factors of key growth. (TGF-β1, PDGF-AB, VEGF) and 3 coagulation proteins and matrix (TSP-1 [thrombospondin-1]) (Fn) (vitronectin) were quantified experimentally seven times (at 20', 1h, 4h, 24h, 72h, 120h, 168h) (Figures 9, 13) [10].

These studies revealed that the products have two very different profiles: the membrane L- PRF remained solid and intact after 7 days and continuously releases a large amount of growth factors, a significant part of it is produced by cells inside of the membrane.

In contrast the gel P-PRP clarified releases most of the growth factors in the early hours and is fully dissolved after 3 days (Figures 10, 13).

The leukocytes present in the L-PRF are not only inflammatory cells, as they also have anti-nociceptive effects through several chemokines, anti-inflammatory cytokines (IL-4, IL-10, IL-13) and opioid peptides (β-endorfine- dimorfina-A etc.) and therefore can promote a clinically relevant inhibition of pathological pain (the centrifugation process. can turn gently stimulate pathologically inflammatory state or destroy leukocytes). Many types of cells are present in these preparations.

The leukocyte formula is an important parameter: the lymphocyte populations are very different. does not have quite the same impact of monocytes and granulocytes. Also, many other cells, such as circulating stem, can be found in a platelet concentrate and not be neglected.

Was observed a certain amount of GF in the serum released by PRF (PRFR) and in the serum supernatant (SS) immediately after the formation of the PRF. It is a found an additional release from PRF GF up to 300' (5h). The content of GF in SS is ≅ 7 ng/mL (PDGF-AB), 9.5 ng/mL (TGF-β1), 0.1 ng/mL (VEGF) and constitutes a good indication of the basal level of GF in PRFR trapped in the clot. There concentration of GF issued by the PRF is significantly lower than that found in platelet lysates and is lower than the concentration present in the whole blood used to produce PRF. The PRF and the SS contain large amounts of GF and must not be discarded as they can bè useful in the treatment of the patient [11].

The inflammatory liquid in the PFR (rich in growth factors and serum protein) is collected in the container and, PRF membranes are kept in a humid environment of serum. This is an effective method from a biological point of view. A new device tested by us for the preparation and standardization of L-PRF clots and membranes is the L-PRF Wound Box.

The clot L-PRF contains almost all the platelets and more than 50% of the white blood cells initially collected, also has a strong architecture of fibrin and a special three-dimensional distribution of platelets and leukocytes. One solution is to keep the clot in a metal container and press them in sterile membranes with a sterile plate metal when necessary.

This device allows the preservation of the clot in a moist and sterile for 1h and allows an increased release of growth factors - total. The L-PRF Wound Box® is a versatile tool where PRF clots can be transformed into membranes. The average quantities of produced PDGF-AB are significantly higher in each experimental time and the TGF-β1 (Figure 10) and VEGF (Figure 11) are significantly higher during the first 4h. The explanation of this result is quite simple: by using the PRF Box, the compression process of the clots in the membrane is performed by a slight compression, slow and homogeneous, and the final membrane always remains homogeneously wet and soaked in serum.

This gentle method avoids the extraction and the loss of a significant amount of growth factors, and is particularly evident for the PDGF-AB, because this growth factor is released only by the platelets. On the contrary, will not influence other intrinsic factors of growth that are released slowly in high quantities for several days. The amount of released VEGF and TGF-β1 are produced by leukocytes massively.

A process for the collection of blood and preparation is not standardized, slow and inadequate, leads to a small mass of fibrin PRF-like, with the polymerization of fibrin unstable (resulting in weaker mechanical properties) and a growth factor

unknown and irreproducible. Also it is very difficult to separate these small masses of fibrin at the base of the GR, resulting in a heavy load of red blood cells in the product [12].

DISCUSSION

1- Being the PRF a product belonging to the same body; the availability of this biomaterial in greater quantities is difficult. Therefore, its use in surgical procedures must be well planned.
2- The PRF has circulating immune cells and antigenic molecules that prevent its use as allogenic material and there is a increased risk of transmission of infectious agents.

Platelets, fibrin and leukocytes naturally act in synergy to promote wound healing and regeneration tissue and the concept of platelet concentrates for surgical use is to multiply this effect coagulation/regeneration on a surgical site or wound. According to the classification POSEIDO all products in this category are grouped under the general term of platelet concentrates (HPC), whatever their form or content cellular, also it is important to highlight the key influence of leukocytes and the structure fibrin in the clinical potential or experimental effects of these products and that each product refers to a specific biological fingerprint.

At this point of our knowledge, among the parameters kept outside of this classification system we have: the concentration of platelets, the concentration of leukocytes and. proportions of various types of leukocytes. The problem of the concentration of platelets does not exist in PRF, in that all the platelets of the blood sample taken are activated and integrated in the matrix of fibrin in the clot.

Regarding the concentration of leukocytes and their formula, their influence has yet to be studied carefully, since their presence or absence may explain the contradictory results that are observed (Tables I, II).

The second generation of PRF, one with leukocytes (L-PRF) recently developed, is expressed as a three-dimensional biomaterial handle, which does not melt, but is destroyed by remodeling over time, similar to the natural blood clot.

- There were no statistically significant differences between the concentrations baseline WBC of 7.4 x 10^3/ml and platelets. 166 x 10^3/ml. There are correlations between blood levels and basal concentrations of FG. There is a significant correlation between the number of platelets and release of TGF-β1 ($p = 0.005$) and PDGF-BB ($P = 0.04$). The TGF-β1 is quantitatively greater in group PRF slow-release compared to immediate-release (Figure 6).

It was reported that WBC trapped in PRF matrix represents the main source of this additional release of TGF-β1, and WBC continue to produce this FG clot within the PRF for several days (about 7).

The PDGF-BB is almost completely contained in the alpha-granules of platelets and is released upon activation, which is why its production is highest in the PRP and PRF activated and reduces lens shapes. It is possible that the haematological values can not be used to predict the immediate or slow release of growth factors in the PRF [13].

In the study of *Dohan Ehrenfest et al.* [14], we wanted to determine the cellular composition and organization of this biomaterial three-dimensional autologous and usefulness of different tubes (Dry glass, coated glass, plastic) but all without gel. Approximately 97% of platelets, and more than 50% of the leukocytes are concentrated in the PRF clot that showed a specific three-dimensional distribution. The platelets and fibrin present in large quantities in the first millimeters of the membrane beyond the limit of the red blood cells. There is the presence of differences in the architecture of PRF using various types of tubes.

Analysis of platelets and leukocytes

Almost all platelets (> 97%) were absent from tubes of the groups tested after 'extraction membrane PRF. In the groups tested, the level of leukocytes decreased significantly compared to the control group ($p < 0.01$) more than half of leukocytes appeared to be disappeared (Table 1).

Platelets and leukocytes missing are trapped in the PRF matrix when using the method of collection with scissors.

The absence of a difference between the two groups tested ($p > 0.05$) seems to indicate that the brutal compression of the clot does not affect the possible release of cell bodies trapped within the fibrin matrix.

In the groups tested, the concentration of lymphocytes is significantly lower, while that of neutrophils was significantly higher ($p < 0.01$) in the control group (Table 2). This would indicate that the lymphocytes were more than other leukocytes trapped in the matrix of PRF. Finally, the mean platelet volume (MPV) is reduced significantly between the groups tested and control groups ($p < 0.01$), it decrease from 9 μm^3 (range: 8-11 μm^3) in whole blood to 4.7 μm^3 (range: 4.5 to 5.8 μm^3) in the groups tested. This phenomenon could be due to increased plasma osmolarity in the tubes after the activation of the coagulation cascade.

Study of Optical Microscopy

Histomorphometric analysis was performed using an optical microscope with a total magnification of 100 x. An eyepiece 10 mm with a pattern with 100 divisions was used to measure the percentage of coverage of the total length with at least one cell layer in each section. With hemalaun-eosin staining the fibrin matrix is homogeneous in pink, while the aggregated platelets are dark blue/purple (Figures 12a, b). The GR and cytoplasm of leukocytes are dark pink and not easily detectable. The nucleus of the leukocytes are colored in blue with hemalumen and are not easily distinguishable from platelet aggregates. With the tri-color Masson (*modified from Godman*) the aggregated platelets are still dark blue, but the GR are easily identifiable because colored red. Leukocytes are still difficult to highlight within platelet aggregates, however, the line of demarcation between GR and platelet aggregates/leukocytes is very clear (Figure 12c, d). The line of distinction between GR, leukocytes and platelet aggregates is not obvious.

Table 1. Leukocytes, RBC and Platelets number in whole blood (control group) and red clot after PRF membrane collecting (test group) (by Dohan Ehrenfest et al. 2010 modified).

	Leukocytes/µl		EtiologyRBC/µl		Platelets/µl	
	Mean	*Range*	*Mean*	*Range*	*Mean*	*Range*
Controll	6.900	6.100-7.800	5.19 (10^6)	5.01-5.52 (10^6)	2.66 (10^5)	2.18-3.09 (10^5)
Serie 1	3.500	3.000-3.800	5.89 (10^6)	5.75-6.08 (10^6)	6.000	4.000-8000
Serie 2	3.600	3.300-4.000	5.84 (10^6)	5.78-5.91 (10^6)	7.000	6.000-9000

Table 2. Leukocyte formula stabilized in whole blood (control group) and red clot after PRF membrane collecting (test group) (by Dohan Ehrenfest et al. 2010 modified).

	Whole blood (%)		Serie 1 (%)		Serie 2 (%)	
Tipo di cellula	*Mean*	*Range*	*Mean*	*Range*	*Mean*	*Range*
Neutrophils	51.8	49.7-53.2	72.1	66.1-77.1	66.4	60.9-71.4
Eosinophils	2.9	2.3-3.1	6.1	3.4-8.8	5.1	3.9-6.1
Basophils	0.5	0.3-0.8	0.1	0.0-0.3	0.4	0.1-0.9
Linfocytes	37.7	35.1-39.2	17.5	15.0-20.4	24.8	21.4-28.0
Monocytes	71.1	6.8-7.6	4.2	1.1-7.6	3.3	2.5-5.0
Total (Mean) /µl		6.900 (100%)		3.500 (100%)		3.600 (100%)

Rating at SEM

Observing the PRF clot at SEM at low magnification (x15) showed that the clot has a central concavity which is a result not real fixation. In the red part of the PRF clot, I have coagulates GR in fibrin network (Figure 13). The GR are normal but the fibrin grid appears immature.

The boundary between the red and the yellow of the clot (area buffy coat), the SEM examination showed leukocytes which appear as spherical structures with irregular surface (Figure 13 a).

The majority of these is small (6-8 µm in diameter) and thus may be mainly lymphocytes. Platelet aggregates appear along the fibrin filaments (Figure 4 b).

Beyond the area buffy coat are distinguished two different areas: the first consists of thick filament of fibrin and a few scattered GR probably from contamination; fibrin appears to be mature (Figure 14); the second consists of that vein of platelets thickened observed under the optical microscope, characterized by platelets and fibrin in dense clusters and large due to extensive aggregation and coagulation (Figure 15). This aggregate is constituted by a thick grid and solid and platelets appear to significantly activated during the preparation protocol of PRF.

A higher magnification fibrin is clearly organized in parallel fibers very thick and dense. In it is impossible to distinguish the cellular elements content.

Conclusions

The highest density of platelets and leukocytes was found in the first millimeter of the clot to limit yellow with the red. The delivery of platelets and leukocytes become lower as we move to the end of the clot and have not been found more platelets and leukocytes over the first half of the clot yellow.

In the first 2 mm over the edge between the red and the yellow clot distribution of platelets and leukocytes is fairly homogeneous on hall the length of the clot.

As you move from the edge of the yellow/red more platelets (and leukocytes) are grouped into the veins of platelet concentrations central or centrifuges. These veins have a high density of platelet/leukocyte to the interior of a matrix free of cells. This structure is similar in all coagulates, regardless of the patients, the type of test tubes and the compression method.

The platelet count showed clearly that there are almost no platelets let layer GR, the PPP and the inflammatory liquid after compression of the PRF clot. So most of the platelets from the whole blood sample is collected in the PRF membranes.

The white blood cell count has confirmed that more than half of leukocytes is trapped in the PRF membranes and The small lymphocytes seem attracted so selected as confirmed by SEM (Tables 1, 2). These leukocytes do not appear to be damaged during the preparation of PRF. This result has a strong clinical impact as the amount of leukocytes placed at the inside of the membranes is considerable and small lymphocytes are particularly efficient in the regulation of inflammatory

reactions. Furthermore, the cellular composition of the L-PRF implies that this biomaterial must be handled with care to ensure that the cell contents will be kept alive and stable.

The microscope has shown that the photonic platelet distribution and leukocytes in the clot is not uniform.

Platelets and leukocytes are concentrated in the layer which is located between the clot and the clot red fibrin and are arranged forming a coating on the surface of the macroscopic clot PFR. Therefore, the most useful part by a surgical point of view is the whitish layer intermediate. So it is necessary to preserve a small layer of GR at the end of the clot PFR which contains most of leukocytes and platelets.

The procedure can be done with scissors and is operator-dependent and this requires accurate knowledge of the structure of the PRF.

The slight compression of the fibrin matrix determines that the fibrin filaments are condensed and stick together. When the membranes are used in surgery PRF their reabsorption is slow and facilitates the remodeling of the fibrin matrix in a scar tissue.

For the standardization of the preparation PRF as graft material for tissue regeneration, we propose the use of the region of the membranes of PRF with the maximum enrichment platelet and, in addition, not to squeeze all the content in the plasma clot PRF. So it is advisable to compress the clot with a compression device (L-PRF Wound Box). therefore it is difficult to control accurately the quality of human-derived materials, such as preparations PRF, but it is very important to make the best quality control on PRF prepared before their clinical application.

Further clinical studies, histological and statistical are required to understand the benefits of this new platelet concentration. However, one can not ignore that the one obtained from a sample of autologous blood, the PRF product is scarce and only a limited volume can be used. This limits the systematic use of PRF in General Surgery. Although the potential applications of PRF are large, you need a thorough knowledge of the operation of the biomaterial, its biology, and efficiency advocate, limits, to optimize its use in daily clinical practice [15].

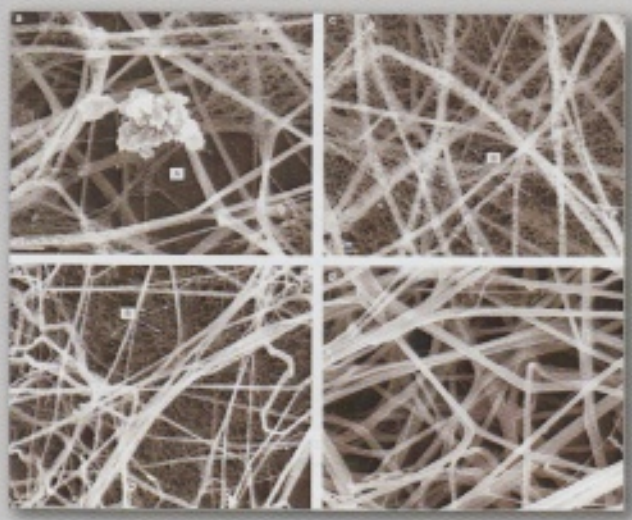

Figure 1.
SEM images showing a) Platelets aggregate in fibrin clot from Human Platelets Concentrate (HPC) (magnificent 10000x) and fibrin fibers. B label shows minor smooth fibers that constitute a secondary mesh on fibrin main fibers.

Figure 2.
Difference between PRP (a) and PRF (b) technique in fibrin molecular architecture. Look at the elastic difference between the structures.

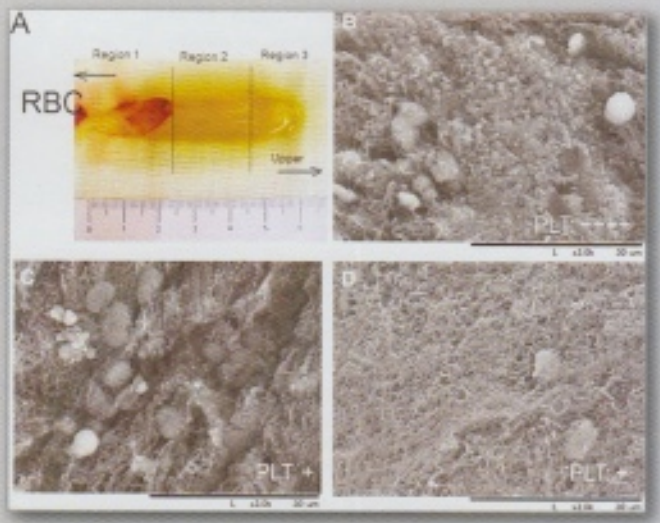

Figure 3.
The 3 areas of C-PRF and SEM's observations on membrane surface. (A) C-PRF has been divided into 3 regions: Region 1 closest to red clot (RBC), region 2 the middle one and region 3 that's the more far by red clot. Platelets localization has been observed in region 1 (A), region 2 (B) and region 3 (C). The concentration of platelets is higher in region 1 than 3. (*modified from Kobayashi et al. 2012*).

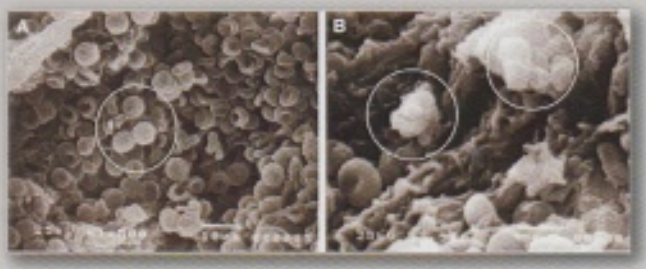

Figure 4.
A) on the edge between red zone and yellow clot by SEM. RBC and leukocytes group appear with spherical structure and irregular surface (white circles). The greatest part of them seems to be quite small (between 6 and 8 μm of diameter) and, so, could be lymphocytes. B) Platelets was often mixed in fibrin mesh, but sometimes, appear like aggregates (white circles) that have been easily identified. (Magnifier A) 1500x, B) 3500x.
(modified from Dohan Ehrenfest et al. 2010).

Figure 5 a, b.
PRF clot at SEM. The arrow shows thrombocytes
a) board=10 μm b) board=1 μm.

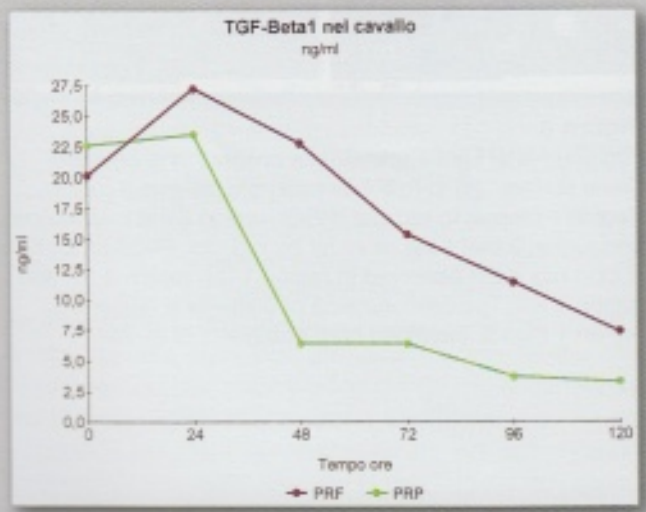

Figure 6.
TGF-β1 levels in horse
(modified from McLellan, Plevin, 2014).

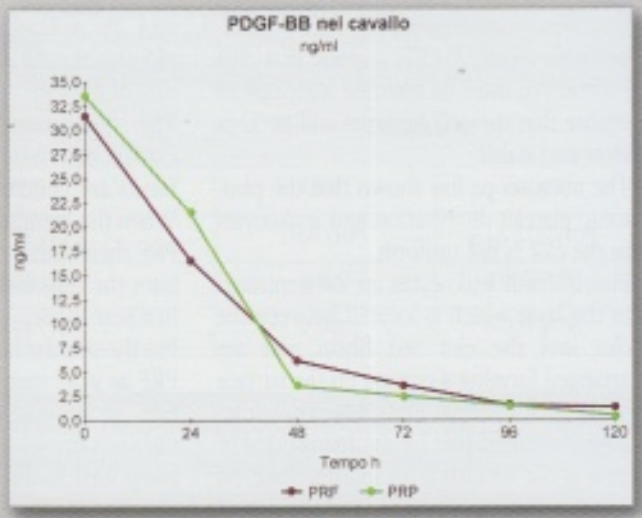

Figure 7.
PDGF-BB levels in horse
(modified from McLellan, Plevin, 2014).

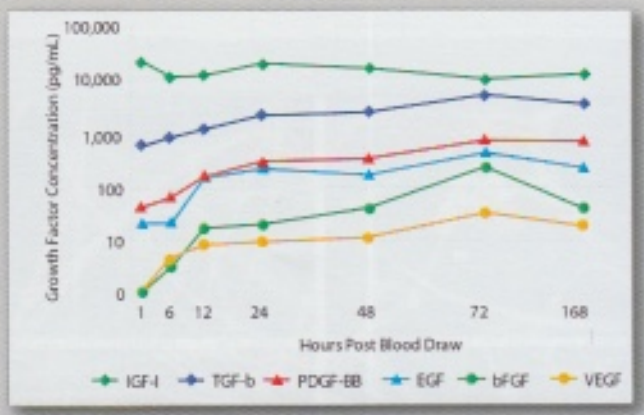

Figure 8.
Growth factors levels by hours post blood draw.

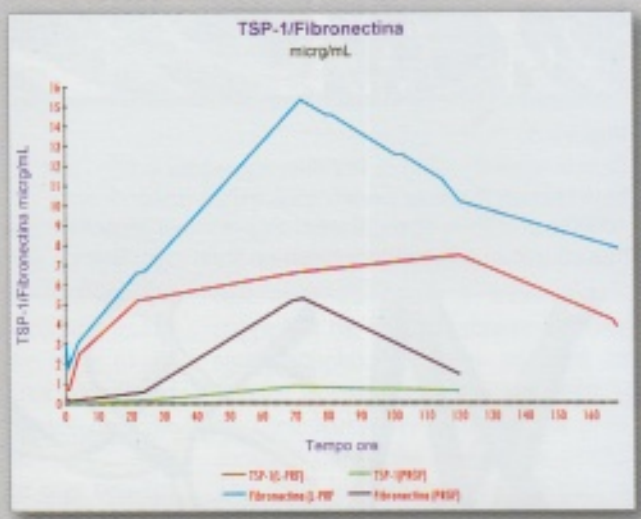

Figure 9.
TSP-1 and fibronectin variations by time. L-PRF versus PRGF (Plasma rich in Growth Factors=P-PRP)
(modified from Dohan Ehrenfest et al. 2012).

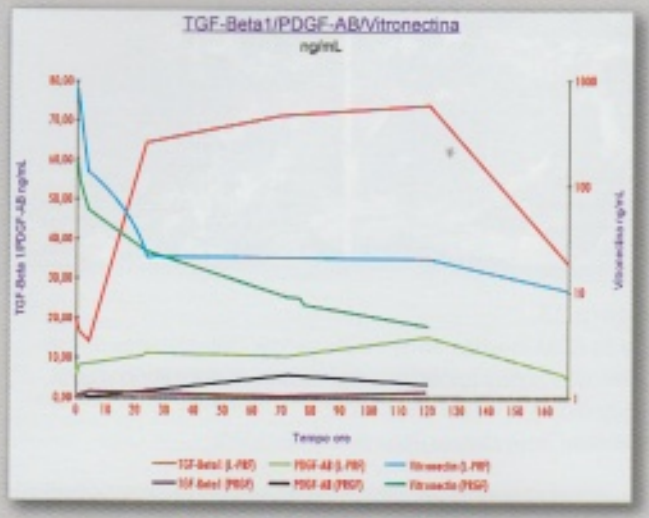

Figure 10.
TGF-β1/PDGF-AB and vibronectin by time. L-PRF versus PRGF (*modified from Dohan Ehrenfest et al. 2012*).

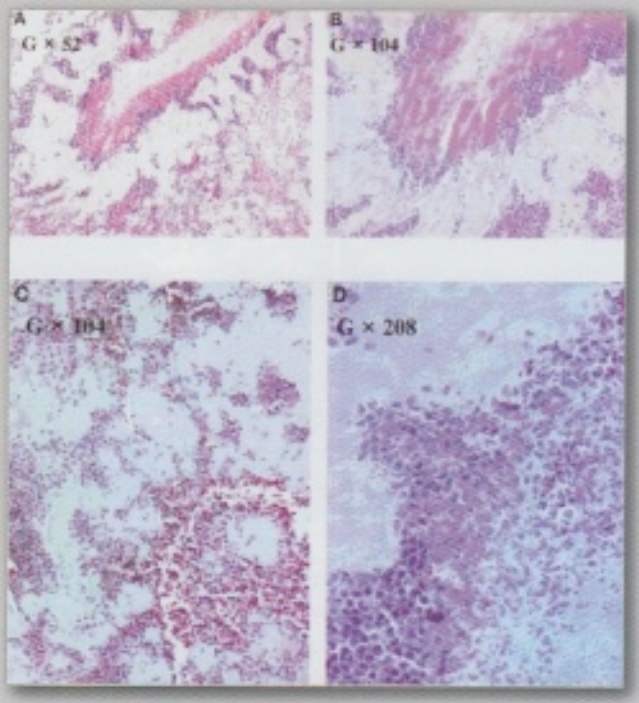

Figure 12.
Analysis by Optical Microscopy of PRF clots.
A e B) Hemalaun-eosin is not sufficient to distinguish various cell types trapped into fibrin matrix. C e D) Using tricolor Masson coloration it's easy to distinguish platelets aggregates by leukocytes (dark blue and RBC (red). Magnification (G)
(*modified from Dohan Ehrenfest et al. 2010*).

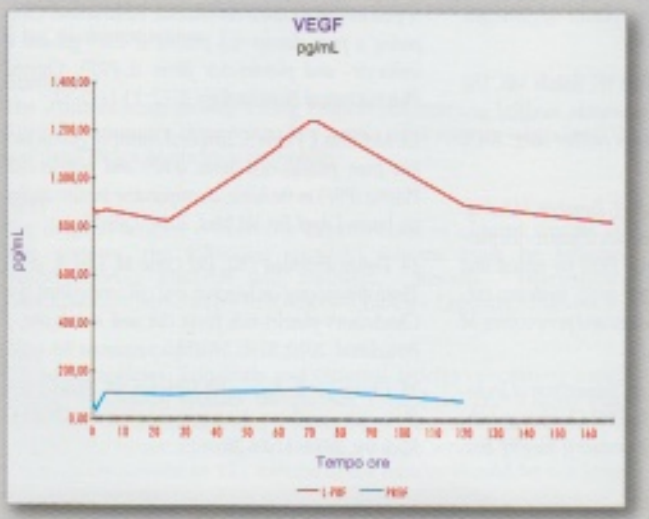

Figure 11.
VEGF variations by time. L-PRF versus PRGF
(*modified from Dohan Ehrenfest et al. 2012*).

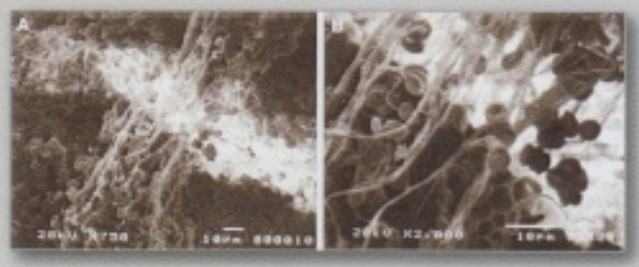

Figure 13.
A e B) red zone of PRF's clot. (SEM) contains many RBC trapped into an immature and very melted fibrin matrix (Magnification A x 750, B x 2000)
(*modified from Dohan Ehrenfest et al. 2010*).

Figure 14.
SEM analysis of fibrin yellow clot reveals a dense and mature fibrin matrix with a low quantity of identifiable trapped bodies (RBC, Leukocytes, Platelets aggregates) (Magnificent x 2000) (*modified from Dohan Ehrenfest et al. 2010*).

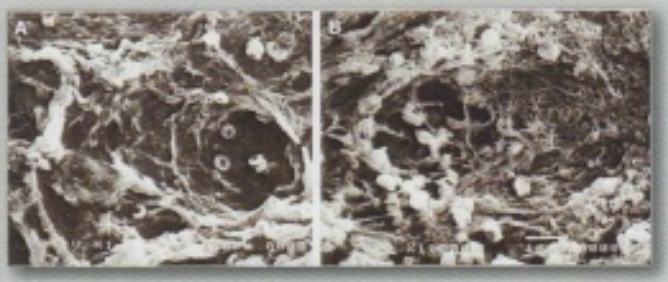

Figure 15.
A e B) SEM in white veins inside yellow clot, platelets aggregates was tightly joined in a dense and mature fibrin matrix. (Magnificent A x 1000, B x 1500)
(*modified from Dohan Ehrenfest et al. 2010*).

References

1. Knighton DR, Fiegel VD, Austin LL, et al. *Classification and treatment of chronic nonhealing wounds, successful treatment with autologous platelet-derived wound healind factors (PDWHF). Ann Surg, 1986; 204,322-29.*

2. Pretorius E, et al. *Ultrastructural comparison of the morphology of three different platelet and fibrin fiber preparations, The Anatomical Record, 2007; 290,188-98.*

3. *Del Corso M, Choukroun J, Simonpieri A, et al. Accelerazione dei processi di cicatrizzazione tissutale con un nuovo biomateriale: la fibrina ricca di piastrine (PRF), Odontoiatria, 2007; 4,361-66.*

4. *Nurden AT, Nurden P, Sanchez M, et al. Platelets and wound healing, Frontiers in Bioscience, 2008; 13,3523-48.*

5. *Kobayashi M, Kawase T, Horimizu M, et al. A proposed protocol for the standardized preparation of PRF membranes for clinical use, Biologicals, 2012; 40,323-29.*

6. *Zhao QM, Ding YJ, Si T. Platelet-rich fibrin in plastic surgery, OA Evidence-based Medicine, 2013; 1(1):3.*

7. *Naik B, Karunakar P, Jayadev M, et al. Role of platelet rich fibrin in wound healing: a critical review, J Conserv Dent, 2013; 16,284-93.*

8. *Textor JA, Murphy KC, Leach K, Tablin F. Ultrastructure and growth factors content of equine platelet-rich fibrin gels, A J V R, 2014; vol.75, n4, 392-401.*

9. *Desai CB, Mahindra UR, Kini YK, Bakshi MK. Use of platelet-rich fibrin over skin wounds: modified secondary intention healing, J Cutan Aesthet Surg, 2013; 6,35-37.*

10. *Dohan Ehrenfest DM, Andia I, Zumstein MA,et al. Classification of platelet concentrates (Platelet-rich plasma- PRP, Platewlet-rich fibrin - PRF) for topical and infiltrative use in orthopedic and sports medicine: current consensus, clinical implications and perspectives, M L T J, 2014; 1,3-9.*

11. *Su C-Y. How to optimize the preparation of leukocyte- and platelet-rich fibrin (L-PRP, Choukroun technique) clots and membranes: introducing the PRF box, Oral Surg, Oral Med, Oral Path, Oral Rad End, 2010; 110,278-80;*

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

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

14. *Dohan Ehrenfest DM, Del Corso M, Diss A, et al. Three-dimensional architecture and cell composition of a Choukroun's platelet-rich fibrin clot and membrane, J Periodontol, 2010; 81(4),546-55.*

15. *Chatterjee A, Agarwal P, Subhatah SK. Platelet rich fibrin: an autologus bioactive membrane, APOLLO Medicine, 2014; 11,24-26.*

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