ISSN 2035-5092

UDPS UpDate in Plastic Surgery

Contents

Endorsed by

ASSECE EUROPEAN ASSOCIATION OF AESTHETIC SURGERY

SCRIPTA MANENT EDIZIONI

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

Alessandro Crisci

Alessandro Crisci, Francesco Placido, Michela Crisci, Annamaria Bosco

Service of Dermosurgery and Skin Transplantation, Nursing Home "Villa Fiorita" Aversa (Ce), Italy.

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-