

World Journal of *Clinical Cases*

World J Clin Cases 2017 May 16; 5(5): 159-190





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INDEXING/ABSTRACTING

World Journal of Clinical Cases is now indexed in PubMed, PubMed Central.

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NAME OF JOURNAL

World Journal of Clinical Cases

ISSN

ISSN 2307-8960 (online)

LAUNCH DATE

April 16, 2013

FREQUENCY

Monthly

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E-mail: editorialoffice@wjgnet.com
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Suite 501, Pleasanton, CA 94588, USA
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PUBLICATION DATE

May 16, 2017

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<http://www.wjgnet.com/bpg/gerinfo/204>

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Evolution, current status and advances in application of platelet concentrate in periodontics and implantology

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Author contributions: Agrawal AA contributed all to this paper.

Conflict-of-interest statement: Agrawal AA declares no conflict of interest related to this publication.

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Manuscript source: Invited manuscript

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Received: September 12, 2016

Peer-review started: September 13, 2016

First decision: October 21, 2016

Revised: January 29, 2017

Accepted: February 28, 2017

Article in press: March 2, 2017

Published online: May 16, 2017

Abstract

Platelet concentrates (PC) [platelet-rich plasma (PRP) and platelet-rich fibrin (PRF)] are frequently used for surgical procedures in medical and dental fields, particularly in oral and maxillofacial surgery, plastic surgery and sports medicine. The objective of all these technologies is to extract all the elements from a blood sample that could be

used to improve healing and promote tissue regeneration. Although leukocyte rich and leukocyte poor PRP's have their own place in literature, the importance of non-platelet components in a platelet concentrate remains a mystery. PC have come a long way since its first appearance in 1954 to the T-PRF, A-PRF and i-PRF introduced recently. These PC find varied applications successfully in periodontics and implant dentistry as well. However, the technique of preparation, standing time, transfer process, temperature of centrifuge, vibration, *etc.*, are the various factors for the mixed results reported in the literature. Until the introduction of a proper classification of terminologies, the PC were known by different names in different countries and by different commercial companies which also created a lot of confusion. This review intends to clarify all these confusion by briefing the exact evolution of PC, their preparation techniques, recent advances and their various clinical and technical aspects and applications.

Key words: Platelet concentrates; Platelet rich plasma; Platelet-rich fibrin; Pure-platelet-rich fibrin; Leukocyte- and platelet-rich fibrin; Sticky bone; Platelet derived growth factors; Fibrin glue

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Core tip: Platelets concentrates are known to be a rich source of growth factors with added antimicrobial efficacy due to incorporations of leukocytes. But does that mean that platelets or platelet poor/depleted plasma do not have any antimicrobial role? Are the mixed results reported in the literature due to deviations from the manufacturing protocols and nomenclature of platelet concentrates (PC)? Does technical factors related to centrifuge speed, time, temperature, vibrations, resonance, *etc.*, affect the biological quality of the resultant platelet concentrate? A thorough knowledge evolution, preparation and applications of various PC will help clinicians to use this arsenal more efficiently.

Agrawal AA. Evolution, current status and advances in application of platelet concentrate in periodontics and implantology. *World J Clin Cases* 2017; 5(5): 159-171 Available from: URL: <http://www.wjgnet.com/2307-8960/full/v5/i5/159.htm> DOI: <http://dx.doi.org/10.12998/wjcc.v5.i5.159>

INTRODUCTION

An average baseline platelet count in humans is $200000 \pm 75000/\mu\text{L}$ with a half-life of 7-10 d. Platelets are irregularly shaped, small (2-4 μm) anuclear cells, derived from fragmentation of precursor megakaryocytes. They contain few mitochondria, many granules and 2 prominent membrane structures, the dense tubular system and the surface connected canalicular system. Activated platelets trigger their major effects by substances located in one of the three different types of platelet granules: A-granules, dense granules, and lysosomes. Alpha granules are the most abundant type and contain many different bioactive mediators. They are spherical or oval structures (200 to 500 nm), enclosed by a unit membrane. Upon contact with exposed endothelium (due to damage tissue or wound) the platelets get activated and are known to release key wound healing factors: Platelet derived growth factor (PDGF), vascular endothelial growth factor (VEGF), transforming growth factor (TGF) and epidermal growth factor (EGF). Platelets begin to actively secrete these proteins within 10 min after clotting, with more than 95% of the pre synthesized growth factor secreted within 1 h. For the balance of their life (5 to 10 d), the platelets synthesize and secrete additional proteins. As the direct platelet influence begins to subside, macrophages, which arrive by means of vascular ingrowth stimulated by the platelets, assume responsibility for wound-healing regulation by secreting their own factors. Thus, the platelets at the repair site ultimately set the pace for wound repair.

Platelet concentrates (PC) [platelet-rich plasma (PRP) and platelet-rich fibrin (PRF)] are frequently used for surgical procedures in many medical fields^[1], particularly in oral and maxillofacial surgery^[2,3], plastic surgery^[4] and sports medicine^[5,6]. The objective of all these technologies is to extract (through centrifugation) all the elements from a blood sample that could be useful to improve healing and promote tissue regeneration^[7], particularly: The platelets (rich in growth factors)^[8], the fibrin (supporting matrix)^[9] and in some cases the cell content (mostly leukocytes)^[9]. A natural blood clot contains 95% red blood cells, 5% platelets, less than 1% white blood cells, and numerous amounts of fibrin strands. A PRP blood clot contains 4% red blood cells, 95% platelets, and 1% white blood cells. The literature on these products is quite confusing and controversial due to the lack of proper characterization of these many different products^[10,11]. Compared to application of single, supra-physiological concentrations of recombinant growth factors, PC has the

advantage of offering multiple, synergistically working growth factors at the wound site and in concentrations that are physiologically and biologically more relevant. But the question is whether it is only the platelet in PC's that plays lead role or are the non-platelet components equally important when considering the clinical applications. Some authors have in-fact suggested that RBC's and WBC's could be pernicious as they may contribute in inflammatory reactions leading to damage of the treated tissues^[12-14]. Until these controversies are resolved in clinical literature, a big question still persists whether the non-platelet cellular components of PC have any role in their biological activities such as platelet activation and subsequent release of growth factors?

The natural healing process in any wound starts as blood coagulation leading to fibrin/platelet clot and matrix. PC's were introduced to reinforce this natural wound healing process. For example fibrin glues which are being used as surgical adjuvants since > 40 years. Over the period, this idea evolved to a more refined concept of tissue regeneration which was enhanced by the cells and the growth factors contained in these preparations. Initially used as surgical adjuvant, the PRP/PRF became the new glorified regenerative medicine approach. Platelets, leukocytes, fibrin, growth factors and other cells are the primary active players in the physiological wound healing process. Combined together they form a kind of engineered tissue which is derived from the blood circulation. However, this complex combination is ultimately decisive for the optimal performance. Therefore, the L-PRF clot, i.e., Leukocyte-and PRF, was commonly known as an "optimized blood clot".

EVOLUTION OF PC

1954

Kingsley^[15] first used the term PRP to earmark thrombocyte concentrate during experiments related to blood coagulation.

1970

"Fibrin glue" was introduced by Matras^[16] which improved healing of skin wounds in rat models. Fibrin glue was made by polymerizing fibrinogen with thrombin and calcium. However, due to low concentration of fibrinogen in donor plasma, the quality and stability of fibrin glue was suboptimal.

1975-1978

Numerous research works suggested an enhanced concept for using blood extracts and designated them as "platelet-fibrinogen-thrombin mixtures"^[17].

1979

Another author called it "gelatin platelet - gel foam". This new proposition asserted the performance of platelets, and demonstrated exquisite preliminary results in general surgery, neurosurgery and ophthalmology. However till then all these products were used primarily for their "gluey

effect", without consideration of effects of growth factors or their healing properties.

1986

Knighton *et al*^[18] first demonstrated that PC successfully promote healing and they termed it as "platelet-derived wound healing factors (PDWHF)", which was successfully tested for the management of skin ulcers.

1988, 1990

Kingsley *et al*^[15] and Knighton *et al*^[19] used a slightly different term "platelet-derived wound healing formula (PDWHF)".

1997

Whitman *et al*^[20] named their product PRP during preparation but when the end product had a consistency of a fibrin gel and therefore labeled it as "platelet gel".

1998

The development of these techniques continued slowly until the article of Marx *et al*^[21], which started the craze for these techniques. However, all these products were designated as PRP without deliberation of their content or architecture, and this paucity of terminology continued for many years. Some commercial companies, in lieu of better visibility, started labeling their products with distinct commercial names.

1999

One of the popular methods advertised on large scale to prepare pure platelet rich plasma was commercialized as plasma rich in growth factors (PRGF) or also called as preparation rich in growth factors (Endoret, Victoria, Biotechnology Institute BTI, Spain). However, because of lack of specific pipetting steps and also lack of ergonomics, there were significant issues with this technique^[11]. Another widely promoted technique for P-PRP was commercialized by the name Vivostat PRF (Alleroed, Denmark). However, as the name implies it is not a PRF but produces a PRP product.

2000

Simultaneously, Choukroun *et al*^[22] developed another form of PC in France which was labeled as PRF, based on the strong fibrin gel polymerization found in this preparation. It was stamped as a "second-generation" platelet concentrate because it was obviously different from other PRPs. This proved an important milestone in the evolution of terminology.

2006

Bielecki *et al*^[23] and Cieslik-Bielecka *et al*^[24,25] proposed to define PRP as inactive substance, while PRG (Platelet Rich Gel) was a more biologically activated fibrin matrix rich in platelets, leukocytes and relative active molecule.

Sacco^[26] introduced a new concept of CGF (concentrated growth factors). For making CGF from venous blood, rpm in range of 2400-2700 was used to separate

cells. The fibrin rich blocks that were obtained were much larger, richer and denser.

2008

Everts *et al*^[27,28] focused on the leukocyte component of the platelet concentrate and the two forms, i.e., non-activated and activated. The inactivated/non-activated product was called "platelet-leukocyte rich plasma (P-LRP) and activated gel was labeled platelet-leukocyte-gel" (PLG).

2009

The first classification about platelet concentrate was proposed by Dohan Ehrenfest *et al*^[11]. This classification defined 4 main families based on separation of the products using 2 key parameters: The cellular content (primarily leukocytes) and the fibrin architecture: (1) Pure platelet-rich plasma (P-PRP) - or leukocyte-poor platelet-rich plasma (LP-PRP); (2) Leukocyte- and platelet-rich plasma (L-PRP); (3) Pure PRF (P-PRF) - or leukocyte-poor PRF; and (4) Leukocyte- and platelet-rich fibrin (L-PRF).

2010

Concept of sticky bone (autologous fibrin glue mixed with bone graft) was introduced by Sohn^[29] in 2010.

2012

Mishra *et al*^[30] proposed another classification which was limited to PRP and applicable to sports medicine only. They stated 4 types of PRP based on presence or absence of leukocytes and whether or not the PRP is activated and all types can fall into 2 sub-types: A: Platelets > 5 × baseline or B: Platelets < 5 × baseline. In all the following types "solution" means non-activated PRP and gel means activated PRP. Type 1: L-PRP solution; Type 2: L-PRP gel; Type 3: P-PRP solution; Type 4: P-PRP gel.

At about the same time DeLong *et al*^[31] introduced another classification system called PAW (Platelets quantity, Activation mode, White cells presence). However it again was only restricted to PRP families and was similar to classification by Mishra *et al*^[30].

2014

Choukroun^[32] introduced an advanced PRF called A-PRF (claimed to contain more monocytes). Tunalı *et al*^[33] introduced a new product called T-PRF (Titanium-prepared PRF).

2015

Mourão *et al*^[34] gave detailed technical note on preparation of i-PRF.

EVOLUTION IN PREPARATION TECHNIQUES

Fibrin glues, fibrin sealants or fibrin tissue adhesives are derivatives of human plasma that resemble the final stages

of blood coagulation wherein a fibrin clot is formed, available commercially in Europe since late 1970's. There are two types of fibrin sealants: Homologous and autologous. Homologous/commercial variant was prepared by mixing 2 components, *i.e.*, fibrinogen component containing factor XIII and the thrombin component containing calcium ions. Homologous fibrinogen concentrates were prepared from plasma cryoprecipitate or from Cohn fraction I. However, due to the risk of transmitting infections, later fibrin sealants were prepared from autogenous whole plasma and polymerization was instituted using bovine thrombin.

True concentrate of platelets, was termed PRP, which can be manufactured by using two techniques. Both these techniques differ in their technical aspects: (1) General-purpose cell separators; and (2) Platelet-concentrating cell separators.

The former technique (general-purpose cell separators) requires about 450 mL of blood and also usually requires a hospital setting. Blood is drawn into a citrate-phosphate-dextrose anticoagulant containing collection bag. In the first cycle it is centrifuged at 5600 rpm to separate RBCs, platelet poor plasma (PPP) and PRP. Subsequently the speed of the centrifuge is reduced to 2400 rpm to get a final separation of about 30 mL of PRP from the RBCs. A major advantage of this technique is that the remaining PPP and RBCs can be restituted to the patient's circulation or can be discarded. The ELMD-500 (Medtronic Electromedic, Auto Transfusion System, Parker, CO, United States) cell separator is widely used for this technique. The second technique, Platelet-concentrating cell separators, is more widely used since this equipment can be accommodated in a dental clinic setup. This technology permits the procurement of PRP using smaller quantities of blood. Currently, two such systems are approved by FDA and commercially available: Harvest SmartPrep Platelet Concentrate System (HSPCS; Harvest Technologies, Plymouth, MA, United States) and the 3i Platelet Concentrate Collection System (3i PCCS; 3i Implant Innovations, Palm Beach Gardens, FL, United States). Several studies have been performed to compare the efficacy of these systems^[6-8]. Although, traditionally a double-spin technique has been used, authors such as Eby^[35] have proposed the use of a single spin technique. The preparation and processing of PRP is quite similar in most of the platelet-concentrating systems, however the anticoagulant used and the speed and duration of centrifugation may differ.

An important evolution of terminology appeared when several authors, particularly the groups of Dohan Ehrenfest *et al*^[8,36] pointed out that the PC were also associated with various forms of circulating cells, particularly leukocytes, and labeled it as L-PRP (Leukocyte rich platelet rich plasma). Large number of commercial or experimental systems exists for the preparation of L-PRP. In past years many automated protocols have been developed that require minimum handling of blood products, for example Biomet GPS III (Biomet Inc., Warsaw, IN, United States) and Harvest Smart-PreP (Harvest Technologies, Plymouth, MA, United States). There are also other kits which require

more handling of blood products, for example Regen PRP (RegenLab, Le Mont-sur-Lausanne, Switzerland) or Plateltex (Prague, Czech Republic). Rutkowski *et al*^[37] (2008) demonstrated single spin centrifugation for 10 min at 1350 g for preparation of PRP and they reported six-times enrichment of platelet concentrate. Interestingly they also reported that platelet morphology changes over a period of 6 h bench set time. In fact, even after 2 h the platelets in PRP started to appear less normal. They concluded that PRP bench set times should not exceed 2 h to maintain maximal levels of growth factors, TGFβ1 and of platelet morphology. Akhundov *et al*^[38] (2012) claimed to introduce a cost effective technique for procuring PRP. They harvested patient's blood using syringe/Vacutainer tubes containing 10 mmol/L 3.8% citrate. This citrate treated blood was transferred to 50 mL Falcon centrifuge tube and centrifuged for 15 min at 280 g at room temperature. After centrifuge, platelets and plasma were removed using 5 mL pipette and transferred to a new 50 mL Falcon centrifuge tube and centrifuged again for 15 min at 280 g at room temperature. The pellet with 1-2 mL of plasma was transferred to new syringe for use in patient for injection or topical application.

Fukaya *et al*^[39] (2014) reported an innovative yet economic technique for preparing PRP which consisted of a modification of a (disposable) 5-mL syringe that was inserted into a regular centrifuge. The syringe was positioned in the centrifuge in such a manner that the platelet rich plasma separated adjacent to the tip of the syringe. They also highlighted that instead of heparin or EDTA (ethylene diamine tetra acetic acid), majority of commercial kits adopt dextrose solution A (ACD-A) as an anticoagulant. Even though coagulation and platelet aggregation are very different and anticoagulants never suppress platelet aggregation, no commercial kits consider adding platelet aggregation inhibitor. It's known that aggregated platelets attach to the wall of syringes and are unable to detach from them easily. However their primary aggregation is reversible and the platelets detach from the syringe wall and float in the plasma again after many hours. But in routine clinical practice we cannot wait so long. Therefore authors have suggested addition of platelet aggregation inhibitor "prostaglandin E1 (PGE1)" to anticoagulant ACD-A for preparation of PRP with dense PDGF-BB.

The sole product in the family of P-PRF is the fibrinet PRFM (Platelet-Rich Fibrin Matrix, Cascade Medical, NJ, United States). These are high-density fibrin network preparation with poor leukocyte content. They exist purely in a strongly activated gel form that cannot be injected or used like conventional fibrin glues but instead can be manipulated like a real solid material for other applications. However an important disadvantage of this technique is its high cost and relative complexity of the procedure as compared to the other forms of PRF available such as the L-PRF. The L-PRF was developed and evaluated as a one-step centrifugation without anti-coagulation or blood activator^[40]. However, currently the sole commercially available, FDA approved system for making L-PRF, is the

Intra-Spin L-PRF (Intra-Lock Inc., FL, United States). It has something called "Xpression preparation box", which allows the production of generous quantities of membranes and fibrin in relatively small time. Mazzucco *et al*^[41] (2016) compared the mechanical properties of PRF against PRGF and found that the former was stronger. It should be noted that the early protocol to produce L-PRF was 3000 rpm/10 min, while since many years the 2700 rpm/12 min protocol is mostly used that gives much better polymerized L-PRF and therefore stronger membranes than the 3000 rpm/10 min protocol. The original L-PRF system now exists only in one CE/FDA cleared form that is termed Intra-Spin L-PRF as stated above. A brief compilation of different types and techniques of platelet concentrate is presented in Table 1^[22,26,29,32-34,41-50].

RECENT ADVANCES

After PRF a concept of "Concentrated Growth Factors (CGF)" was introduced in 2006 by Sacco^[26]. A special centrifuge called Medifuge (Italy), is used to prepare CGF, similar to PRF, but with a different centrifugation speed which allows the separation of a fibrin matrix which is much denser, larger and richer in growth factors. CGF has been shown to have a greater versatility and better regenerative capacity, as reported for alveolar ridge and sinus augmentation (Sohn *et al*^[51], 2009). In a study, Rodella *et al*^[52] could demonstrate the presence of VEGF and TGF- β 1 in RBC and CGF layers. This suggests that improved CGF procedure could enhance the quantity of growth factors in the CGF layer or, alternatively, a possible use of RBC layer in clinical applications. In addition to this, the existence of CD34 positive cells, within the CGF network, could lead to investigation of their clinical implications in future.

Ample evidence has emerged recently on the role of monocytes on the vessels growth and bone regeneration. Monocytes play an important role in vascularization, bone growth and production of VEGF. Monocytes are known to have BMP receptors and recently it was discovered that they produce BMP-2. In an attempt to incorporate the monocytes within the PRF, Choukroun^[32] introduced an advanced PRF called A-PRFTM. They have discovered earlier soft tissue growth, more release of BMPs, greater and faster vascularization and more cytokine release than conventional PRF.

A concept of fabricating growth factors-enriched bone graft matrix (also known as "sticky bone") using autologous fibrin glue has been demonstrated since 2010^[29]. Sticky bone provides stabilization of bone graft in the defect, and therefore, accelerates tissue healing and minimizes bone loss during healing period. To obtain autologous fibrin glue, 20-60 CC of venous blood is centrifuged at 2400-2700 rpm using a specific centrifuge (Medifuge, Silfradent srl, Sofia, Italy) for 2 min. Out of the two layers obtained, the deeper layer is RBC's and the superficial layer is AFG. This AFG is then extracted using a syringe and mixed with particulate bone powder and allowed to rest for 5-10 min for polymerization, which results in a yellow colored mass

called "sticky bone"^[53]. Sohn *et al*^[53] also noted that the polymerization can be accelerated by adding the exudates obtained after compression that they used to make CGF membrane. These exudates contained growth factors and autologous thrombin in RBC layer due to which the auto-polymerization completed faster^[53]. The resultant sticky bone is moldable, prevents micro and macro movement of grafted bone, entraps platelets and leukocytes in its fibrin network, is natural and prevents ingrowth of soft tissues in graft.

Mourão *et al*^[34] (2015) described a technique to obtain an injectable form of PRF called i-PRF. In this technique a short centrifuge for 2 min at 3300 rpm gave an orange color fluid which can be injected or mixed with bone graft to give a well agglutinated "steak" for bone grafting.

Although successful procedures have been reported extensively using Choukran's L-PRF, physicians such as O'Connell^[54] had raised concern regarding possible health hazard with the particles of silica in the glass tubes. In spite of the fact that the silica particles are sufficiently dense so as to sediment along with the RBC's, they are small enough so that a fraction of them will remain colloiddally suspended in the platelet-poor plasma layers, buffy coat and fibrin and might eventually reach the patient during treatment. In this context a study was done by Dohan Ehrenfest *et al*^[9] in 2010 evaluating the cell composition and 3D organization of L-PRF persuaded by different types of collection tubes (such as glass-coated plastic tubes or dry glass) and compression techniques (soft or forcible) on the final L-PRF-membrane architecture. Authors demonstrated that there was no influence of the type of tested tube on the architecture of this second generation PC. However Tunali *et al*^[33] in 2014, introduced a new product called T-PRF (Titanium-prepared PRF). The use of titanium tubes for collection and centrifugation instead of glass tubes was established on the hypothesis that titanium may be a more efficient platelet activator than silica, for preparing L-PRF. Based on light, scanning electron and fluorescence microscopy analysis, Tunali *et al*^[33] concluded that T-PRF has immensely organized network along with a continuous integrity and even the fibrin network was thicker and also it covered larger area.

Anitua *et al*^[55] (2015) in an *in-vitro* study, evaluated the outcome of different ozone treatments on biologic properties of PRGF. They found that using "continuous flow protocol" of ozone treatment of PRGF, fibrin scaffold formation, growth factor levels along with proliferative potential was drastically reduced. In contrast, ozone treatment using "syringe method" had no effect on the biological outcomes of this autologous therapy, so ozone therapy in combination with PRGF can be effectively used.

APPLICATION OF PC IN PERIODONTICS AND IMPLANT DENTISTRY

Various *in vitro* studies have demonstrated that PRP exerts

Table 1 Compilation of different platelet concentrates, their discovery and different protocols available

Platelet concentrate type	Method (automated/manual)	Highlights
P-PRP	Cell separator PRP (Automated)	PRP collected by discontinuous method where patient is connected to machine continuously, around 300 mL PRP can be collected. When PRP is obtained from a blood bag of 450 mL, 40 mL of PRP can be obtained per bag. Differential ultracentrifugation employed (3000 g) Type of advanced cell separator designed to produce fibrin sealant It is cumbersome, expensive, have low and damaged platelet yielding capacity
	Weibrich <i>et al</i> ^[50]	
	Vivostat PRF (Automated)	
	Leitner <i>et al</i> ^[42]	
	Anitua's PRGF# (Manual)	
L-PRP	Anitua ^[43]	Citrated blood is collected in 5 mL tubes and softly centrifuged for 8 min at 460 g Platelet poor layer (1 mL) is discarded and the PRGF layer above buffy coat layer is pipetted out from all tubes and collected in one tube. Calcium chloride is added for clotting. However there are problem in ergonomics and reproducibility of the procedure
	Nahita PRP (Manual)	Protocol similar to Anitua's PRGF
	Tamimi <i>et al</i> ^[44]	
	PCCS PRP (Automated)	Consists of two compartments, citrated blood is transferred into first compartment and centrifuged for a short time. Using air pressure, upper layer PPP and buffy coat are transferred into second compartment and centrifuged for a longer time. PPP is transferred back to first compartment and final product - leukocyte and PRP is left behind. It is no longer available
	Weibrich <i>et al</i> ^[45]	It also has two compartments, but requires less manipulation
	SmartPreP PRP (Automated)	It is a multifunctional system which can also be used to concentrate stem cells from bone marrow transplant
	Weibrich <i>et al</i> ^[46]	
	Megalian APS PRP (Automated)	This advanced cell separator had optical reader. It has compact size, designed for small blood samples (upto 50 mL). Although, platelet collection efficacy is high but cell preservation is yet to be known
	Christensen <i>et al</i> ^[47]	
	GPS PRP (Automated)	Another variation of 2 chambers, 2 stage centrifuge protocol
	Martovits <i>et al</i> ^[48]	PPP is discarded and second centrifuge is with RBC layer. Final PRP is aspirated from the surface of RBC layer
	Friadent PRP (Manual)	Both these techniques employ classical method of 2 stage centrifuge. First soft spin that gives three layers. PPP and buffy coat transferred to another tube and after hard spin the PPP is discarded leaving behind PRP
	Weibrich <i>et al</i> ^[46]	
	Curasan PRP (Manual)	Depending on technique of collecting buffy coat, one can randomly get either P-PRP or L-PRP
	Weibrich <i>et al</i> ^[50]	
P-PRF	AutoloGel (Automatic)	The final product was called as "autologous platelet-rich plasma gel"
	Driver <i>et al</i> ^[49]	
	Regen PRP (Manual)	Both these techniques uses specific jellifying agents such as calcium gluconate and lyophilized purified batroxobin, an enzyme that cleaves fibrino-peptide to induce fibrin polymerization without bovine thrombin and gelling in about 10 min ^[47]
	Plateltex PRP (Manual)	The Regen method also employs a separator gel within the centrifugation tubes to improve collection of platelets and leucocytes
	Mazzucco <i>et al</i> ^[41]	
	Ace PRP (Manual)	Similar protocol but with variation in centrifugation force and time and types of anticoagulant
	Tamimi <i>et al</i> ^[44]	
	Fibrinet PRFM (Manual)	Consists of two tubes, one for blood collection and another for PRFM clotting. Around 9 mL blood is collected in a tube containing tri-sodium citrate anticoagulant and a separator gel and centrifuged for 6 min at high speed. Buffy coat and PPP are transferred in second tube containing calcium chloride and centrifuged for 15 min and then stable PRFM clot can be collected. Very low amount of leucocytes are obtained due to the specific separator gel used, however the fibrin matrix is more denser and stable than PRP's
	(PRFM Kit, Cascade Medical, New Jersey, United States)	
	Leitner <i>et al</i> ^[42]	
L-PRF	Choukroun's PRF (Manual)	Considered second generation platelet concentrate obtained by natural process without any anticoagulants or jellifying agents
	Choukroun <i>et al</i> ^[22]	Venous blood collected and centrifuged at low speed yielding and RBC layer, PRF clot in middle and acellular plasma top layer
		The PRF clot can be pressed between guage to make a strong membrane
	Intra-Spin ^[9] (Manual)	The only FDA approved kit for PRF. It employs 9 mL glass coated plastic tube, centrifuged at room temperature at 2700 rpm (around 400 g) for 12 min. Contains and Xpression kit to compress the clot to make membranes
	(Intra-lock, United States)	
	Titanium-prepared PRF (experimental)	The platelet activation by using titanium tubes instead of glass tubes seems to offer some high characteristics to T-PRF
	(Manual)	
	Tunali <i>et al</i> ^[33]	The PRF obtained was highly organized and with continuous integrity. The fibrin meshwork is thicker and covers larger area
	Other non FDA cleared centrifuge to produce L-PRF: Salvin 1310 (Salvin Dental) and LW-UPD8 (LW Scientific)	Studies have shown that as compared to Intra-spin, these 2 machines produces more vibration and resonance

CGF	Medifuge, Silfradent srl, Italy	Permits the isolation of a much larger, denser fibrin matrix which is richer in growth factors
	Sacco ^[26]	Demonstrates presence of TGF- β , VEGF and CD34 ⁺
Sticky Bone	Sohn ^[29]	Autologous fibrin glue mixed with bone graft
T-PRF	Tunali <i>et al</i> ^[33]	Titanium tubes were used for collection and centrifugation instead of glass tubes
A-PRF	(Advanced PRF Process, France)	Earlier vascularization, faster soft tissue growth, more cytokines and release of BMPs
	Choukroun ^[32]	
i-PRF	Mourao <i>et al</i> ^[34]	Blood collected in 9 mL tube without any additive, centrifuged for 2 min at 3300 rpm, the resultant orange color fluid in the tube is the i-PRF

PCCS: Platelet concentrate collection system; APS: Autologous platelet separator; PRP: Platelet-rich plasma; PRGF: Plasma rich in growth factors; PRF: Platelet-rich fibrin; TGF: Transforming growth factor; VEGF: Vascular endothelial growth factor; GPS: Gravitational platelet separation system.

positive effects on gingival fibroblasts^[56], oral osteoblasts^[57], and periodontal ligament (PDL) fibroblasts^[58], making it an ideal candidate to facilitate complete periodontal regeneration. PRP may also benefit surgical sites and wound healing *via* its antibacterial properties. This antimicrobial effect has been reported against bacteria such as *Staphylococcus aureus*^[59], *Escherichia coli*^[60], and *Klebsiella pneumoniae*^[61]. PRP was also found to be active against oral microorganisms, including *Enterococcus faecalis*, *Candida albicans*, *Streptococcus agalactiae*, and *Streptococcus oralis*^[62], reinstating that PRP is a potentially useful substance in fighting postoperative infections.

Applications in periodontics

Application of PRP to bone graft material has demonstrated earlier bone regeneration and soft tissue healing^[21]. PRP can also retard epithelial migration by infusing it into resorbable barrier membranes. This will also provide localized source of growth factors that will accelerate soft tissue and hard tissue maturation^[63]. Agrawal and Gupta^[64] (2014) in a split mouth study concluded that a combination of PRP with DFDBA was more efficient than DFDBA with saline for the management of non-contained intrabony defects. In addition to this, a combination of PRP with bovine porous bone mineral and GTR membrane also showed good clinical response^[65]. Combination of PRF and bone graft has also reported exceptional results in periodontic-endodontic furcation defect^[66]. However, Choi *et al*^[67] questioned the benefits of mixing PRP and bone graft material, expressing their concern that it interfere new bone formation. According to the authors, growth factors when present in high concentrations at inappropriate times for prolonged duration can negatively affect the cell behavior. They further affirmed that proliferation and viability of alveolar bone cells are quashed by high PRP concentrations but are accelerated by low PRP concentrations^[68].

PRF is a powerful healing biomaterial with inherent regenerative capacity and can be used in various procedures such as periodontal intrabony defects^[69,70], treatment of furcation^[71], sinus lift procedures^[72] and as application in the field of tissue engineering, it can be used as a scaffold for human periosteal cells *in vitro*^[73]. Eren and Atilla^[74] in 2012 treated bilateral gingival recession with (CAF) coronally advanced flap and (SCTG) subepithelial connective tissue graft on one side and CAF with PRF on other side. They found improvement in all parameters with

both the techniques. Since use of PRF was practical and simple to perform and also eliminates the requirement of donor site wound, they suggested that CAF + PRF as a better alternative to CAF + SCTG. Anilkumar *et al*^[75], reported PRF as a probable but innovative approach for root coverage in treating gingival recession in mandibular anterior region using combination of PRF membrane and laterally positioned flap technique. Aroca *et al*^[76] in a randomized clinical trial concluded that addition of a PRF membrane placed under the MCAF (modified coronally advanced flap) provided additional gain in gingival/mucosal thickness but inferior root coverage over 6 mo follow up period compared to the conventional therapy.

Applications in implantology

Choi *et al*^[77] in 2006 conducted an animal study to compare the sinus lining perforation repair using either the (AFG) autologous fibrin glue or the collagen membrane. Their histological evaluation found that in repaired wounds, where AFG was used, demonstrated newly regenerated continuous epithelium across the original perforation site as compared to collagen membrane treated site where there was no epithelium, inflammatory infiltration was seen along with extensive fibrosis even after 2-wk of healing. Literature reports the applications of PRP in continuity defects^[78], sinus lift augmentation^[79,80], vertical/horizontal ridge augmentations^[81], ridge preservation^[82], periodontal/peri-implant defects^[83]. Several articles have reported the use of L-PRF membranes for the stimulation of bone and gingival healing during sub-antral sinus augmentations^[72] and global rehabilitations using dental implants^[84,85]. The effect of these membranes on soft tissue healing and maturation is particularly significant^[86]. In yet another case report, Del Corso *et al*^[87] in 2012 used L-PRF in immediate implant replacement of maxillary central incisor and reported excellent healing and esthetics. Choukroun *et al*^[88] studied the effect of PRF with (FDBA) freeze-dried bone allograft to augment bone regeneration in direct sinus lifting and found accelerated bone regeneration.

Simonpieri *et al*^[84,85], in a two-part publication, reported an innovative technique for maxillary reconstruction using PRF membranes, FDBA and 0.5% metronidazole solution. A 0.5% metronidazole solution (10 mg) in small quantity provides an effective shielding of the bone graft material against unavoidable bacterial contamination^[89]. The membrane component of PRF was used to guard the surgical site and enhance the soft tissue healing.

However the PRF fragments were blended with the graft particles. They also suggested that the PRF membranes can be trimmed into fragments (millimeter size) and added to graft material, functioning as a “biological connector” between the different elements of the graft, and will form a matrix which will promote the migration of osteoprogenitor cells to the center of the graft, neo-angiogenesis and capture of stem cells^[90,91]. Using the protocol reported in the literature, they frequently observed a greater degree of gingival maturation post-healing. They also noticed thickening of keratinized gingival tissues that eventually enhanced the esthetic integration and final result of their prosthesis. Moreover, all their clinical experiences highlighted that the use of PRF seemed to reduce postoperative edema and pain, and even minor chances of infectious phenomena^[85]. PRF can be condensed to make plugs which can be positioned in the implant osteotomy site to promote sinus floor elevation using a crestal core elevation (CCE) procedure^[92] or osteotome-mediated sinus floor elevation (OMSFE) with simultaneous implant placement^[93]. PRF can not only be used as a substitute for particulate grafting to predictably elevate the sinus floor using a crestal approach, but PRF can also provide protection for the sinus membrane during the use of an osteotome. Even in case of sinus membrane perforation, the fibrin matrix can aid in wound closure^[77,94]. PRF plugs can also be indicated in management of residual extraction sockets^[95]. A technique in which autologous PRF is used in extracted socket after immediate bone augmentation using titanium membranes applied to the socket walls and achieving primary closure, was found to be feasible and safe with adequate bone filling after 8 wk or above for implant fixation^[96]. Hafez *et al*^[97] in 2015 demonstrated that PRF membrane maintains particulate autogenous bone graft and help achieve primary coverage over immediately placed implants. Sohn *et al*^[53] compared CGF membrane and collagen membrane for alveolar ridge augmentation. Their bone biopsy results showed favorable new bone formation along mineral allograft without sign of inflammation. They also evaluated three dimensional ridge augmentation using sticky bone with or without use of titanium mesh, and found favorable augmentation even without the use of titanium mesh^[53].

The use of platelet and immune concentrate during bone grafting offers the following 4 advantages^[85]: Firstly, the fibrin clot plays an important mechanical role, wherein the PRF membrane maintains and protects the bone graft and its fragments, when incorporated in the body of bone graft, serving as biological connectors between bone particles. Secondly, the fibrin network promotes cellular migration, particularly for endothelial cells which are necessary for the neo-angiogenesis^[40], vascularization and survival of the graft. Thirdly, the platelet cytokines (PDGF, TGF-beta, IGF-1) are creating a perpetual process of healing gradually released as the fibrin matrix is resorbed^[84,98]. Lastly, the leukocytes and cytokines in the fibrin network play a significant role in the self-regulation of inflammatory and infectious phenomena within the grafted material^[99].

DISCUSSION

In preparation of PRP, the choice of anticoagulant used is an important parameter in its capability of preserving the platelets’ best possible functionality, integrity and morphology. In particular do Amaral *et al*^[100] (2016) concluded that the use of (EDTA) ethylene-diamine-tetra-acetic acid yielded more platelet in whole blood; however, it increased the mean platelet volume (MPV) following the blood centrifugation steps required for obtaining PRP. Authors also discovered that the use of (ACD) anticoagulant citrate dextrose and sodium citrate (SC) significantly induced mesenchymal cell (MSC) proliferation. Moreover, PRP obtained in sodium citrate anticoagulant not only presented higher platelet recovery after the first centrifugation step but also had a minimal change in MSC gene expression. Citrate seems to be a suitable anticoagulant, because it has been recently shown that thrombin-activated PRP releases all growth factor at the same time in a bolus, while non-activated PRP uses the platelets as a sustained delivery system, exhibiting the best wound healing effects^[101]. PRP is not routinely used nowadays because of complicated preparation techniques, expensive procedure and offer quite mixed clinical results^[2,3]. On the other hand, the L-PRF family has developed very fast over the last years, as the technique is very simple and useful in daily practice, it is user friendly and relatively inexpensive^[11].

One logical question that comes to a clinician is how much rich is PRP or PRF? What is the difference of richness in these PC’s? Literature reports a range of less than 2 fold to around 8.5 fold increase in platelets. In a classification of PRP, Mishra *et al*^[30] suggested a sub-classification of PRP into A and B, where a 5-fold platelet concentrate may be a relevant baseline for definition of PRP (it should also be noted that concentrations greater than 5-fold gave better clinical results). Another aspect of this definition is that this baseline is not universal and may not be valid for all clinical applications. Weibrich *et al*^[102] suggested that different individuals may require different platelet concentration ratios to achieve comparable biological effect.

Although leukocyte rich and leukocyte poor PRP’s have their own place in literature, the importance of non-platelet components in a platelet concentrate remains a mystery. Parrish *et al*^[103] 2016, in an *in-vitro* study demonstrated that leukocyte poor PRP (LP-PRP) showed poor coagulation and poor platelet growth factor release compared to whole blood and leukocyte rich PRP (LR-PRP). They also checked tendon cell proliferation *in-vitro* using serum from LP-PRP and LR-PRP and found greater advantages with the later. LP-PRP was inferior even to whole blood. Thus they concluded that cellular components other than platelet, that are usually eliminated during the course of PRP preparation, are important for efficient functioning of platelets including its thrombin generation, growth factor release and capacity for cell proliferation^[103]. However, these findings need to be confirmed *in-vivo* to make them more justifiable. In addition to this, difference in the

age of patient from who's blood PRF is made also differs structurally and qualitatively. In a recent study, Yajamanya *et al*^[104] (2016) evaluated fibrin network pattern changes of PRF in young and old age groups using a cell-block cytology method. They found that in progressing age groups there was significant decrease in dense and increase in loose fibrin network. They also discovered reduction in the number of platelets and WBC's entrapped within fibrin network with increasing age groups.

It has always been a common thought that L-PRP or L-PRF would give an additional advantage over P-PRP or P-PRF due to the presence of immune cells, *i.e.*, leukocytes. Does that mean that platelets do not have any role to play in immunity? Numerous studies have emphasized that human platelets are a good source of antimicrobial peptides such as: Thymosin β 4, platelet basic protein, platelet factor 4, connective tissue activating peptide III, fibrino-peptides A and B and chemokine (C-C motif) ligand 5^[105]. There are special receptors on the platelets that are known to aggregate with bacteria. Platelets also participate in generating oxygen metabolites, including hydrogen peroxide, superoxide, and hydroxyl free radicals^[106]. Largely, platelets demonstrate impressive activities against the blood-borne pathogens and also play an important role in the innate host defense against the initiation and progression of infections^[106]. In fact Garraud *et al*^[107] in 2015 claimed that "platelets are innate and inflammatory cells and do not only assist immunity but are immune cells themselves". Anitua *et al*^[61] demonstrated that even if an additional dose of leukocytes was present it did not significantly enhance the antimicrobial properties of PRP. Yang *et al*^[108] (2015) in a study evaluated the antimicrobial activity of four plasma preparations: PRP, platelet poor plasma (PPP), platelet depleted plasma (PDP) and PRF. Using haemocytometer, they found leucocytes only in PRP and not in other preparations. However, their results showed that all plasma preparations were efficient enough to inhibit bacterial growth for > 24 h with PRP as the strongest antimicrobial agent. In terms of time-kill assay, authors discovered that PRP, PPP and PDP had similar effect on *F. nucleatum* indicating that it was sensitive to the antibacterial agents in plasma. The poor antimicrobial effect of PRF was attributed to the fact that a mesh of fibrin was formed in PRF, which adsorbed these agents and thus exerted less minimal effect on the growth inhibition of this microorganism. However, one should note that the technique of PRF preparation was not according to the L-PRF protocol given by Choukroun *et al*^[22] in 2000. To make PRF, Yang *et al*^[108] used fraction of PRP and activated it by 23 mmol/L of calcium chloride for 30 min and centrifuged again at 6000 g for 30 min to recover "fibrin-free supernatant" which they labeled as PRF. Hence, although their experiment highlighted the antimicrobial effect of plasma, regardless of platelet and leukocyte concentration, their conclusion of PRF should be read with caution. The basic biological difference between PRP and PRF is that in PRP the polymerization is artificially provoked and there is extrinsic growth

factor enmeshment, whereas in PRF there is natural polymerization with intrinsic growth factors enmeshment. When compared *in-vitro*^[109] studies have revealed that most of the growth factors from P-PRP gel are released in the first hours after preparation and get completely dissolved in the medium after 3-d. In contrast the L-PRF membrane not only remained intact and solid after 7-d but also continuously released large quantities of growth factors. These growth factors are sustainably released for at least 1 wk up to 28 d^[110]. This allows PRF to stimulate the environment for a significant time during wound healing. As a general concern, at the time of any surgery, platelets will start collecting at the surgical site to initiate clotting and healing, which may reduce the whole blood platelet count^[111]. As such, it is recommended that blood should be drawn before the surgery starts because the surgery itself might cause platelet activation and that may eventually interfere with preparation of platelet concentrate^[112,113]. Also the massive release of TSP-1 from PRF membrane has opened up a new range of application for this membrane^[8].

Considering technical aspects for preparation of PRP, for the first centrifuge it is best to keep the speed and time to the shortest that will separate the RBC's and plasma clearly. In the second centrifuge the time and speed should be sufficiently high so that more platelets will precipitate without destroying them^[39]. Ehrenfest *et al*^[114], claimed that for small table centrifuges, the most relevant parameters to be logically evaluated was the vibrations of those centrifuge, the vibration shocks at the time of acceleration and the eventual resonance. All these mechanical properties may impede with the quality and biological signature of the final L-PRF product. The authors tested 4 different centrifuges; *viz*: The original L-PRF centrifuge (Intra-Spin, Intra-Lock) and 3 other laboratory centrifuges: Salvin 1310 (Salvin Dental), LW - UPD8 (LW Scientific) and the A-PRF 12 (Advanced PRF, Process). They demonstrated even if the centrifuges were used in the same conditions and at the same speed there was a significant discrepancy in their vibration levels and 3 out of four quickly reached a threshold of resonance. They found "Intra-Spin" to be the most stable machine tested. At the traditional speed of production of L-PRF, the level of undesirable vibration was between 4.5 and 6 times lower with this machine than with other centrifuges. Moreover, Intra-Spin always stayed under the threshold of resonance, as compared to the other three tested machines^[114].

CONCLUSION

There have already been many technological advancement in preparing and understanding the various types of PC from random single spin centrifugation to fully automated commercially available systems. However, the characterization of such complex products seems to remain incomplete due to the number of parameters involved. Apart from presence or absence of leukocytes, whether or not the activation is carried out, other

parameters that should be taken into consideration are the quantity or rate of platelet collection, the quantity and rate of leukocyte collection, cell composition and preservation during collection, transportation and centrifugation. As discussed earlier, the parameters particular to the centrifuge used are also important such as: Its size, vibration, the duration of centrifugation. Other than that, the cost involved, ergonomics, the form and volume of final product, etc., also need to be taken into consideration while evaluating newer techniques, commercial products, classification systems or indications for their application in medicine and dentistry. With L-PRF being more user friendly and economic, this arsenal is finding wider applications in surgical field. The introduction of i-PRF will also find suitable applications, where injectable form of platelet concentrate is required. Looking at the current trends PRP and L-PRF are most commonly used and have been researched upon. Newer advances such as A-PRF, i-PRF, t-PRF, CGF and sticky bone concept have been reported in single or few cases but no long term or controlled trial have been done to prove the advantage of their advancement over conventional PRP and PRF. So clinicians should use the advancements with caution.

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P- Reviewer: Rattan V, Tatullo M S- Editor: Ji FF L- Editor: A
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