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"Growth Factors Release on P-PRP Activated with Thrombin Assessment Assay"

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Abstract

The development of platelet concentrates has generated great expectations in bone surgery. These compounds provides autologous growth factors for tissue healing and regeneration, but disparity of methods hinders the patterns of use. The objective of our study is proposing a standard model for Platelet Rich Plasma preparation, involving growth factors release. Sheep peripheral blood were obtained with anticoagulant citrate dextrose 50 N (Citra), three different speed centrifugation methods and plasma activation (1000 units of thrombin and 10% CaCl2 per ml were performed. The release of growth factors were determined by ELISA (R & D systems). The higher platelets concentration was obtained with two centrifugations, first 500 g 10 min, followed by 1900g for 8 min. After activation clotting immediate release of growth factors by platelet degranulation were achieved. The PDGF increased more than 1.9 fold till 537.9 pg/ml and TGF-β1 increases almost 8 times till 7150 pgr/ml in 180 min. Our standard method proposed, triggers the release of growth factors, immediately after activation with a dose-response relationship between platelets and growth factors. This means that the PRP prepared in our experimental conditions is really a plasma rich in growth factors ready for clinical use.

Keywords: Platelet-rich plasma pure (P-PRP); Growth factors release; TGF-β1, PDGF

1. Background

The development of platelet concentrates has generated great expectations in bone surgery.

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These surgical use concentrates, given different names depending on the composition both in cell series and growth factors. The P-PRP, platelet-rich plasma pure, also called PRGF or PRF, the L-PRP leukocytes platelet rich plasma, P-PRF, plasma fibrin-rich and L-PRF rich plasma both in fibrin and lecucocitos. (Dohan Ehrenfest D.M.,et al 2012 &,Schär MO., Diaz-Romero J., Kohl S., Zumstein MA., Nesic D. 2015)

These compounds are designed to deliver growth factors and provides an easy and low cost high concentration of autologous growth factors for the tissue healing and regeneration (Cho, Kim, Kwon, Kim, Choi& Kim2014; Shen, Gao, Zhang & He 2015). But how the PRP is prepared? How the activation and as the release of growth factors is produced? Today it remains misunderstand and no consensus achieved. Several methods of obtaining platelet concentrates has been published and has determined the amount of growth factors, but such is the number of different methods, which actually we are not talking about the same compound, and therefore it is not possible to compare to one another. The autologous PRP is the combination of seven natural growth factors within a normal clot who acts as carrier. (Lane, Tomin, & Bostrom 1999; Gil Albarova, Garrido Lahiguera, Gil Albarova & Melgosa Gil 2003; Sanchez et al. 2009).

These growth factors such as TGF-β, PDGF, VEGF etc. are tissue promotors, and healing by acting on cell chemotaxis, proliferation and differentiation, tissue debris removal, angiogenesis and matrix extracelular fixation (Torrella, Toledo, Hondares &, Calañas 2003). Even act as cell signal transducers and as a cellular communications network (Landesberg, Roy & Glickman2000; Eppley, Woodell & Higgins J. 2004; Sánchez Martin2005).Different platelet concentrates have been used in both animal and human models in the treatment of musculoskeletal injuries (Soomekh2011 & Hapa O. et al. 2012), dental implants evaluation (De Obarrio, Arausz-Dutari & Chamberlain 2000; Preeti & Anisha2013), as a biological glue (Findikcioglu, Findikcioglu, Yavuzer, Elmas & Atabay 2009; Dong et al. 2012; Dragoo 2012; Lee 2012) with completely contradictory results., It has beneficial clinical effect in patients with tendopathies but otherwise the role of PRP in orthopedic surgery remains unknown (Bonete 2007; Bonete, Carrasco&Gomar 2010).

In the use of PRP the more growth factors released and located in the wound, more stem cells are stimulated to produce new tissue, therefore, it allow probably the body heal faster and more efficiently (Zechner et al. 2003; Kawasumi, Kitoh, Siwicka & Ishiguro 2008). But there are still many questions to be answered about PRP preparation, risks and clinical use. At present there is no consensus on the use, production and characterization of PRP and to a pattern establishment we need more clinical studies (Carrasco, Bonete & Gomar 2009; Malhotra, Pelletier, Yu & Walsh 2013).

Landesberg[21] demonstrated that platelet activation is affected by various factors such as the release of growth factors, the type of coagulant used, the rate and duration of the centrifugation, and gel preparation (Landesberg, Roy & Glickman2000; Eppley, Woodell & Higgins 2004; Gandhi, Bibbo, Pinzur & Lin 2005; Everts, Knape & Weibrich et al.2006). Some researchers have relied on manual protocols developed for concentrating platelets in humans (Ohba et al.2012), which use the double centrifugation to increase the number and time of centrifugation speed (Carrasco, Bonete & Gomar 2011; López, Giraldo & Carmona 2012, Bausset O. et al 2014). The leukocytes presence, type and amount in the PRP concentrate has been controversial (Carmona, López & Giraldo 2011; Giovanini et al.2012) it can benefit the tissue regeneration processes but leukocytes are involved in the formation of fibrosis and increased inflammation (Chizzolini, Brembilla, Montanari & Truchetet2011). The choice of platelet activator can significantly influence the release kinetics. Collagen activation shows a sustained release pattern for 30 days, while the pattern of release of growth factors following activation by thrombin without leukocytes remains unknown.

The studies and results disparity provides very little clinical evidence with great variability in the preparation of concentrates and in the number of platelets and growth factors which hinders the patterns of use (Carrasco, Bonete & Gomar 2009; Preeti & Anisha 2013).

The main objective of our study is to propose a model using standardized application of P-PRP, we have carried out an experimental study involving the growth factors PDGF and TGF-β1 identification and quantification. its correlation with the number of platelets and release kinetics after activation with thrombin providing the general use conditions.

2. Materials and Methods

2.1 Blood Sample Preparation

Blood samples were obtained from peripheral blood 10 merino sheep extracted into 4 ml tubes with 0.32 ml of ADC A (anticoagulant citrate dextrose solution 50 N Citra clot MA), at pH 7.46 (physiological conditions) The tubes were stirred and kept on ice The blood thus obtained was separated into two sample groups, one with whole blood as a control and the other to obtain PRP in different concentration methods and platelet separation.

2.2 Platelet Counting

The counting was performed by automated cell counter Abbot, model Celldyn-3700. Platelet rich plasma was prepared from peripheral blood collected with citrate dextrose, the tubes were centrifuged at 500, 600 and 750 g for 10 minutes, whereby the platelet phase were separated and leukocytes and erythrocytes were discarded. Then a second centrifugation at 1900 g was carried out for 8 minutes to concentrate the platelets. Samples were used for counting platelets and activated by the addition of 1000 units of thrombin (Sigma Co., St. Louis Mo.) and 10% $CaCl_2$ per ml for clot formation (Figure 1). Samples without addition were used as control.

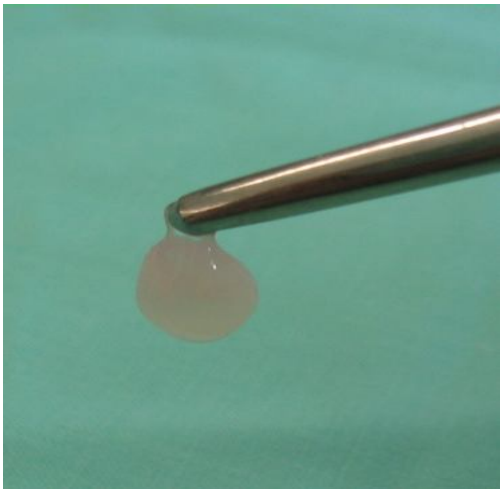

Figure 1: Clot Formation

2.3 Growth Factor Quantification

Each sample clotted was used to determine levels of PDGF and TGF β1 by ELISA (R & D system, Minneapolis Minn.) according to the manufacturer's instructions. At least 5 determinations were carried out for each sample.

2.4 PDGF and TGF-β**1** Basal Levels

To determine the basal values of PDGF and TGF-β1 by ELISA, plasma was extracted from peripheral blood kept on ice with EDTA as anticoagulant. The samples were centrifuged at 1000 g for 30 minutes and then a second centrifugation at 10,000 g was performed for 10 minutes at 2-8 ° C in an Eppendorf 5415 Centrifuge.

2.5 Growth Factors P-PRP Levels

P-PRP growth factors from peripheral blood with anticoagulant ACD A, were determined after activation with 1000 units of thrombin and 10% CaCl2 per ml. Once the gel has formed, it takes less than 5 minutes, we proceeded to growth factors collection by centrifugation at 1500 g for 5 min, and supernatant sample aliquots were taken at 10 ', 60' and 180 ' and were frozen at -80 ° C. (). Determination of TGF-β1 activation in ELISA required acid medium (1N HCl) and incubation for 24 hours at 2-8 ° C. The linear regression of the calibration curves for both growth factors were calculated. Correlation coefficient spractically equivalent to unit. Table I

Table I: Linear Regresion. ELISA Growth Factor Determination		
PDGF	Abs 504-570nm=0.0011* pgr PDGF -0.0182	R =0.999
TGF β1	Abs 450-570nm=0.0007*pgr TGF B1 – 0.0563	R = 0.9929

2.6 Statistical Analysis

All values are expressed as mean ± std with vc (variation coefficient) and comparison between groups was performed using the Student t test. In cases where a comparison involving more than two groups the analysis of variance (ANOVA) was used to assess significant differences between groups. If significant differences were confirmed each difference was examined using the Pearson coefficient. Correlation studies were made between the concentration of platelets in the gel and the release of growth factors. The probability was considered statistically significant if $P < .05$

3. Results

3.1 Counting Blood Cells

The platelet count were obtained for peripheral blood (basal value428750 ±41719, 3) Table II. The platelet-rich plasma for each sample method, one centrifugation (500g, 123000, 600 g81000, 750 g36500) and after a second centrifugation of 1900g (500 g, 167,5 $\cdot 10^6$, 600 g 149, $5 \cdot 10^6$, 750 g $142 \cdot 10^6$) (Tables III and IV).

Table II. Average Value of the Number of Platelets in Peripheral Blood Platelets / ml

Samples of blood N=5	
mean	**428750**
std	**41719,3**
v.c.	**0,0947**

Table III: Average Value of the Number of Platelets Determinated in PRP by one Centrifugation. Platelets / ml

Centrifuged 10 min	**A. 500 g**	**B. 600 g**	**C. 750 g**
mean	**123000**	**81000**	**36500**
std	**12727.9**	**2828.42**	**4949.74**
v.c.	**0.1034**	**0.0349**	**0,135**

We have determined the platelets number in three different PRP methods of preparation. The results show that only one centrifugation method, the number of platelets is smaller, due to a significant drag effect or cell washing. On the other hand the number of platelets is enhanced when the samples have been centrifuged twice (Table IV). reaching an increase of three orders of magnitude (10^3) under these conditions.

Table IV: Value of the Number of Platelets Determinated in PRP by a 2nd Centrifugation 1900 g. Platelets / ml			
	A. 500 g	**B.600 g**	**C.750 g**
Mean	**$167,5 \cdot 10^6$**	**$149,5 \cdot 10^6$**	**$142 \cdot 10^6$**
std	**$30,4,10^6$**	**$24,7,10^6$**	**$19,79,10^6$**
v. c.	**0,181**	**0,165**	**0,139**

3.2 Growth Factors Determination

We have observed that growth factors baseline values for PDGF is 281.31 pgr/ml and for TGF β1 is 785.48 pgr/ml (Table V). with a variation coefficient less than 15 %. (4.5 and 12.3 respectively)

Table V: Basal Level of growth Factors in Peripheral Blood			
	mean	**std**	**v. c.**
PDGF pgr/ml	**281.31**	**12.79**	**0.045**
TGF β1 pgr/ml	**785.48**	**96.94**	**0.123**

The effect of the activation with thrombin and calcium chloride after clotting is the immediate release of growth factors by platelet degranulation in all centrifuging methods. After activation the PDGF increased more than 1.5 fold the maximum obtained was 1.9 fold **537.9**pg/ml (Table VI).

Table VI: PDGF Released

A.500 g	mean	std	v.c..	pgr/ml released	%
10 min	505.0	27.0	0.0549	256.0	93.88
60 min	518.4	27.0	0.053		96.37
180 min	537.9	4.3	0.008	1.9 times	100.0
B.600 g					
10 min	470.7	2.01	0.0042	189.39	100.0
60 min	454.06	19.7	0.043		96.46
180 min	427.82	21.69	0.0506	1.5 times	91.02
C.750 g					
10 min	449.77	30.5	0.0678	209.58	91.62
60 min	489.88	22.7	0.0463		99.79
180 min	490.89	11.09	0.0226	1.7 times	100.0

In the case of TGF-β1 increases more than 4 fold, even, in the case of 500 g is almost 8 times till **7150 pgr/ml**. That means a huge increase of growth factors release (Table VII).

Table VII: TGF β1 Released

A.500 g	mean	std	v.c.	Pgr/ml Released	%
10 min	4697	900	0.19	6365	65.69
60 min	4944	49	0.009	8 times	69.14
180 min	7150	762	0.100		100.0
B.600 g					
10 min	3724	200.0	0.053	3754	82.04
60 min	3935	65.5	0.016	4.7 times	86.69
180 min	4539	150	0.033		100.0
C.750 g					
10 min	4324	198.0	0.045	3886	92.57
60 min	4772	250.0	0.052	4.9 times	102.1
180 min	4671	368	0.078		100.0

3.3 Kinetics of Growth Factors release

The release of the growth factors was monitored for 250 minutes. PDGF was released by 93.88% in the first 10 ', while TGF β1 was released only 65% in the first 10'. (Figure II). The release of TGF β1 is slower, peaking after 3 hours. Not significantly increasing from this time (data not shown).

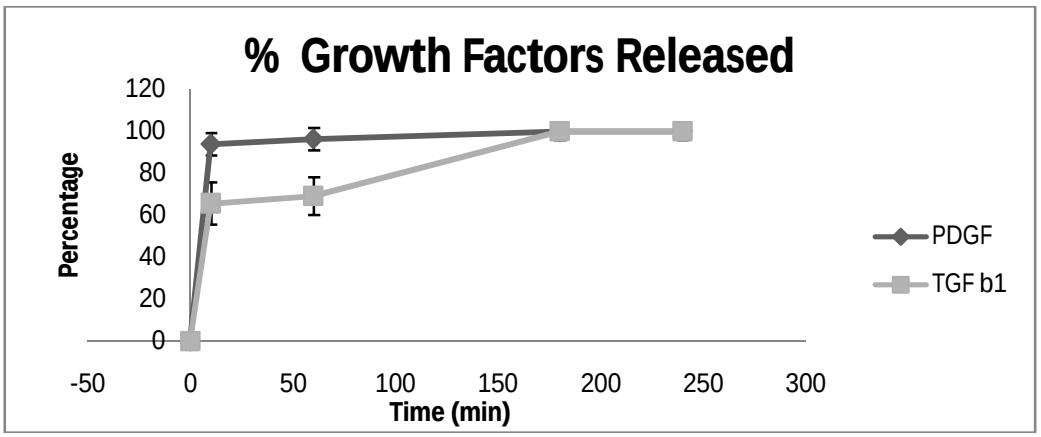

Figure 2: PDGF and TGF β1 Kinetic Release

The release rate for groth factors peaks in the first 10 min, reaching maximum for TGF β1 almost 450 pg/min and for PDGF, 50 pg/min, these values are not sustained in time.

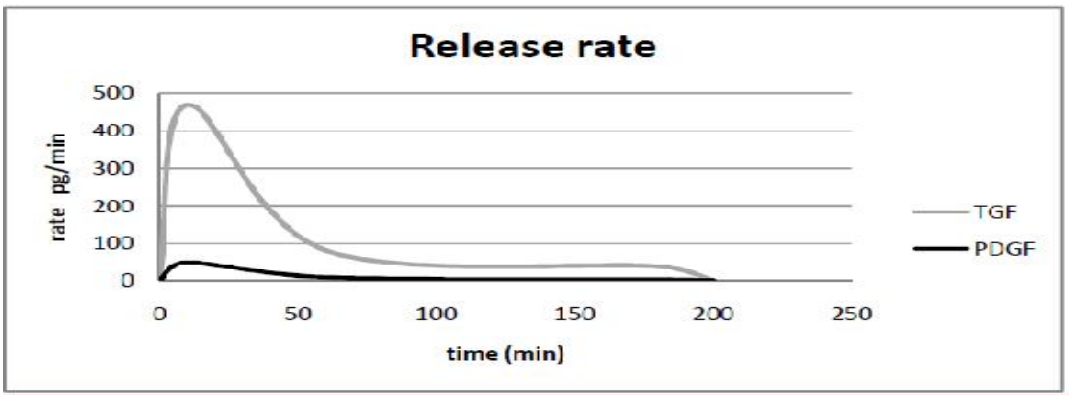

Figure 3: Growth Factor Release Rate. Máximum Releases at 10 Minutes, not Sustained in Time

The ratio between TGF β1 /PDGF increases constantly in the first 180 min with a polynomial regression of . $r^2$0.947. TGF β1 is released 11 times more. Fig 4

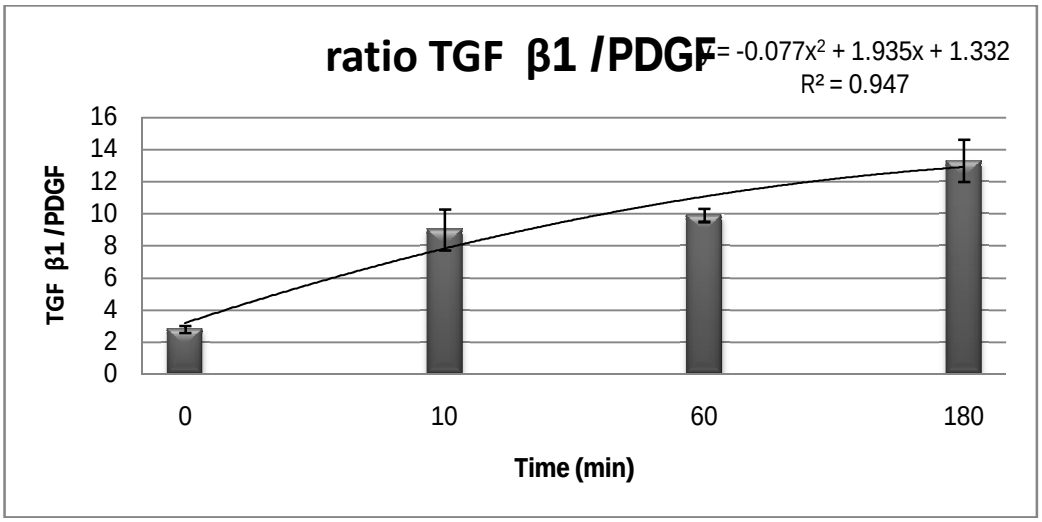

Figure 4: Estechiometry TGF β1 /PDGF (pgr). (1º centrifugation 500 g/2º 190 g)

3.4 Statistical Correlation

Growth factors release are related to platelets number, while PDGF release is linked to the release of TGF-β1-dependent manner (Pearson coeff. R= 0.93, 0.96 in each case). The linear relationship between platelets number versus growth factors liberated is close to unity (r2 = 0.913) (Pearson coef. R = 0.92) (Table VIII).

TABLA VIII: Pearson Correlation. * Closed to Unity

	X(platelets)	Y1(PDGF)	Y2(TGFb1)
	Rx,y1	**Rx,y2**	**Ry1,y2**
Pearson coeficient	***0.9244**	***0.933**	***0.9605**
Significance T student test P<0.05	0.26	0.2535	0.1959
Significance T student test P<0.01	0.33	0.3145	0.2431
Linear regression		R^2=0.855	R^2=0.847

4. Discussion

The use of platelet rich plasma (PRP) or plasma rich in growth factors PRF has been a breakthrough in the stimulation and acceleration of the consolidation of bone and soft tissue. This technology has the risk that is not understood exactly so handling and misuse (Bonete Lluch, 2007) is performed, given the great variability of methods of preparation and use different application conditions (Carrasco, Bonete & Gomar 2009; Malhotra, Pelletier, Yu & Walsh 2013)The application of proteins released by platelets is particularly important in pathological states such as fracture healing compromised as a result of inadequate biological environment with very low concentrations of TGF, IGF and PDGF (Roldan et al. 2004).Based on the different methods of PRF preparation, our first objective was to quantify the platelets basal level in the peripheral blood (Table I). Also the platelets was quantified in terms of the method of centrifugation. For one single centrifugation Anitua's method (Anituaet al. 2004) concentration is low because a lot of platelets carried by the red and white cell layers is lost (Table II). Therefore, it is necessary to centrifuge the sample twice, then platelets concentrate much more. In our study three different centrifugation speeds in response to the literature was performed. Thus the three methods used the higher concentration of platelets was obtained with two centrifugations first 500 g for 10 ' discarding supernatant and followed by 1900g for 8' (Table III).

We have analyzed the values of TGF β1 and PDGF, as they are considered two of the most important growth factors and they are found in highest concentration in platelets. Thus, TGF β1 is in the fracture hematoma within 24 hours after injury and is the major growth factor involved in the regulation of bone and cartilage formation after injury and in new bone growth remodeling (Laurencin 2003; Cho et al 2014). Moreover PDGF synthesis increases in response to external stimuli such as low oxygen tension and or thrombin stimulation of other growth factors. PDGF is secreted by platelets in the fracture callus factor which can stimulate recruitment of reparative cells and other mesenchymal cell types (Yuniko Abe, Asanura, Tonomura, Kimura &, Masuda2007; Schär, Diaz-Romero, Kohl, Zumstein & Nesic 2015). In our research group, in a previous experimental study in rabbits in which a cortical defect was created in the tibia and fibula, no significant correlation was found between platelet count and geometric and densitometric variables analyzed following the use of PRP or PRF (BoneteLluch, 2007). PRP use does not alter the repair and healing processes in the control group (Bonete, Carrasco & Gomar 2010).

The levels of TGF β1 and PDGF in our samples obtained agree with the experimental work of Eppley et al (Eppley, Woodell & Higgins 2004) to yield a positive relationship in the number of growth factors released and the number of platelets in contrast to reports by other authors (Weibrich, Kleis, Hafner & Hitzler 2002; Mazzucco, Balbo, Cattana, Guaschino, Borzini 2009). (Table VIII)

Platelet activation by calcium chloride gel is based on the participation of calcium in platelet activation (Gruber, Varga, Fisher & Watzek2002; Yuniko Abe, Asanura, Tonomura, Kimura& Masuda 2007). By contrast, in vitro, it was found that the addition of $CaCl_2$ did not activate platelets, consequently no degranulation was produced and therefore no growth factors were released (Bielecki & Dohan Ehrenfest 2012). For this reason the use of $CaCl_2$ is necessary but with other activators. For this study we used bovinethrombin.

In our study the use of exogenous thrombin provides a quick release in the first 10 'reaching more than 90% of total release. Figure 2. These results are similar to those published by Harrison et al 2011, where thrombin activation generates the immediate release of growth factors and thrombin active exogenous TGF β1 from the latent form to the active form. The release of TGF β1 is much more slow and cumulative over time (Figure 2, Table VI and VII) as occurs in collagen activation where the release of TGF β1 is also slower than that for PDGF. However, as shown in Figure 3 the discharge of TGF β1 speed release (> 450 pgr / min) is much greater than that generated in the PDGF (50 pgr / min) due to the activation mechanism of thrombin by reaction platelet enzymatic cleavage. Thrombin excess can lead to irreversible platelet degranulation with the loss of integrity of the platelets and subsequent release of growth factors (Ranly, Lohmann, Andreacchio, Boyan & Schwartz 2007).

The estechiometry between TGF β1/PDGF in basal levels is almost 2.8, after activationit increases till 11 fold. The requeriments of TGF β1 in the cell seems to be higher with a polinomialincrease tripling initial values in the first 3 hours after activation. r^2 0.947 in the polinomial regression.(Fig 4), The TGF group of cytokines is necessary for initiate signaling via receptors for the post injury histogenesis (Villapol, Logan, &Symes 2013).The relation between TGF β1 and PDGF release is maintained for at least 3 hours to afford a greater release of TGF β1 from around 11 times.

The release of specific growth factors in our samples agrees those ofFufa group (Fufa, Shealy, Jacobson, Kevy & Murray 2008) that observed release growth factors at significant levels in both the collagen- like activation with thrombin (Harrison et al 2011). These studies seem to suggest that the PRP can have beneficial effects on the proliferation of bone marrow stem cells (Wen et al 2014) without promoting osteoblast differentiation. It should be noted that this factor release occurs in the absence of erythrocytes and leukocytes so we can rule out any possible interference that may distort the composition of our PRP in clinical use. The main limitation of our study is precisely the determination of only two growth factors, the most commonly used in published papers, but do not know what the answer of other cytokines . So VEGF levels could be altered by not sampled levels of lymphocytes and neutrophils as in many platelet concentrates.

The lack of consensus on the composition and production of plasma concentrates makes it impossible to establish a rule covering all published research.

5. Conclusions

1. Activation of platelets with thrombin and CaCl2 exogenous triggers the release of growth factors, immediately after activation. In the case of PDGF especially in the first 10 minutes in the case of TGF β1 release is slower and this continued up to 3 hours after application. This means that the PRP prepared in our experimental conditions is really a plasma rich in growth factors ready for clinical use.
2. Appears to be a dose-response relationship between the number of platelets and growth factors released as well as between growth factors together (statistically significant).
3. The best method of obtaining PRP for use in studies of bone healing in the presence of growth factors would be by two centrifugations, the first of 500 g for 10 'and the second of 1900 g for 8'. Under these conditions an average of 537.9 pgr PDGF / ml and 7.150 pgr TGF β1/ml are released in situ and are maintained for at least 3 hours.

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