www.jmscr.igmpublication.org
Impact Factor (SJIF): 6.379
Index Copernicus Value: 79.54
ISSN (e)-2347-176x ISSN (p) 2455-0450
crossrefDOI: https://dx.doi.org/10.18535/jmscr/v6i9.124

IGM Publication
Journal Of Medical Science And Clinical Research
An Official Publication Of IGM Publication

Anticoagulants Effect on the platelets Rich Plasma Growth Factors Levels

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Abstract
Background: *Platelet-rich plasma (PRP) are platelets concentrates made of autogenous blood with a high number of platelets in a small volume of plasma. The main component that appears to be associated with the therapeutic effect in PRP is the presence of growth factors (GF). Variable techniques for PRP preparation produce variable amounts of growth factors.*
Subjects and Methods: *The study included a total number of 30 healthy males volunteer. We collected blood samples on lithium heparin, EDTA (ethylene diamine tetra acetic acid) and trisodium citrate as anticoagulants and counted platelets in whole blood and PRP which were prepared by single spin and double spin. We measured platelets derived growth factor-BB (PDGF-BB) and transforming growth factor beta-1(TGFB-1) in PRP and platelets poor plasma (PPP).*
Results: *We obtained high significant difference between PRP and PPP were prepared from samples on the different anticoagulants ($p<0.01$) for both TGFB-1 and PDGF-BB. No significant correlation between platelets counts and both TGFB-1 and PDGF-BB in PRP from the different anticoagulants samples ($p>0.05$). High significant difference was found between growth factors in the PRP were prepared by single spin and double spin ($p<0.01$) where TGFB-1 and PDGF-BB were higher in single spin PRP than that prepared by double spin.*
Keywords: *Anticoagulants, Platelets rich plasma, Growth factors.*

Introduction

Platelets are small fragments of megakaryocytes, have important role in clot formation, inflammation and immune response. Platelets contain growth factors in their alpha granules, such as transforming growth factor-beta (TGF-b), fibroblast growth factor-2 (FGF-2), platelet-derived growth factors (PDGF), which are thought to produce beneficial effects on the healing process. The ultimate goal of platelet-rich plasma treatment is to concentrate these growth factors and reintroduce them to a site of injury[1]. Platelet-rich plasma (PRP) is a generic term referring to any sample of autologous plasma with platelets concentrations above baseline blood values. The platelets growth factors content are quite variable among individuals and it is not necessarily proportional to the platelets count. Activation and release of growth factors also occur during platelets processing. Hence, the sole platelets count cannot be predictive of the growth factors content in individual PRP preparations. Platelets are extremely sensitive to any kind of process induced stress, from blood

extraction to PRP gel production. Thus the amount of platelet derived factors available at the end of the manipulation process depends on cumulative effects over platelets, starting from phlebotomy and ending with gel formation[2]. PRP is safe and is free from transmissible diseases such as HIV and hepatitis and delivers high concentrations of growth factors to the surgical area. These are native growth factors in their biologically determined ratios. This is what distinguishes PRP from recombinant growth factors[3]. Variability in the cellular composition of platelet-rich plasma preparations can create methodological challenges for investigators[4].

Aim of the work

Evaluation the effects of anticoagulants and both single and double spin on the platelets rich plasma levels of platelets derived growth factor-BB (PDGF-BB) and transforming growth factor beta-1 (TGFB-1).

Subjects and Methods

This study included 30 healthy males volunteer their age ranged from 18 to 48 years, those volunteers have no relevant diseases and free of any drugs known to affect platelets functions for 7 days before the study. To maintain platelets integrity, we used lithium heparin, EDTA (ethylene diamine tetra acetic acid) and trisodium citrate as anticoagulants. We draw 9 milliliters (mls) blood from each person where they divided into 4 mls were added on tube contains lithium heparin (17 IU/ml), 3 mls blood on K2 EDTA, the concentration of K2EDTA is 1.5±0.25 mg/ml blood and 1.8 mls blood on 0.2 ml trisodium citrate concentration, 32.0 g/l Na3C6H5O7.2H2O.

For every person we prepared: Three PRP were obtained by single spin, three PRP were obtained by double spin and three PPP (from samples were collected on lithium heparin, EDTA and trisodium citrate), For all samples on the different anticoagulants and PRP were obtained by single and double spin we made complete blood counts on Sysmex kx21- Roche diagnostic- for platelets counts. For all prepared PRP and PPP samples we measured TGFB-1 and PDGF-BB by an enzyme linked immunosorbent assay (ELISA).

Method: Single spin separation was made by centrifugation for all samples for 8 minutes at 1000 rpm under constant temperature conditions, the whole blood was separated into three layers: The upper layer is plasma which is consists of two layers; the upper is PPP, and the lower is PRP, the intermediate layer is rich in WBCs and the bottom layer is consisting mostly of RBCs. We collected PPP and preserve at -20 for growth factors quantification and platelets were counted in PRP, then PRP is divided into two tubes; one of them centrifuged at 3500 rpm for 15 minutes under similar temperature conditions and again platelets counts was made from the pellet. the first tube and the collected pellet from the second tube undergo gel formation by addition of CaCl2 (5%) by percent 20% from the whole PPP and PRP (were obtained by single and double spin). After a firm clot formation, the samples were centrifuged for 5 minutes at 3200 rpm and the supernatant was frozen at -20 for growth factors quantification by ELISA.

Growth factors quantification: Quantification of TGFB-1[5] and PDGFBB[6] by ELISA technique using Assypro LLC reagents from affymetrix eBioscience, Bender MedSystems GmbH, Vienna (Austria). The assay employs quantitative sandwich immunoassay technique. The growth factors levels were determined using the ELISA assay method. All kits were tested according to the manufacturer's instructions. In general, the test procedure included adding standards and samples to a microplate precoated with an antibody against each growth factor. Any growth factor present was bound by the immobilized receptor, after any unbound substances were rinsed away, an enzyme-linked polyclonal antibody specific for each growth factor was added to the wells. After a second wash, a substrate solution was added, and color developed in proportion to the amount of bound growth factor in the first step. The color development was stopped, and the intensity of the color was measured using ELISA reader.

Statistical analysis; mean, standard deviation, ANOVA, least significant difference (LSD), paired t test and correlation studies were performed using SPSS software version 14 (SPSS Inc, Chicago, ILL Company).

Results

This study was conducted on 30 healthy males of matched age. TGFB1 mean using single spin method in heparinized PRP was (152.03±31.26) and PPP was (20.46±6.8), EDTA PRP was (80.9±17.8) and PPP was (17.7±5.45), sodium citrate PRP was (77.13±23.4) and PPP was (17.5±10.15). ANOVA denoted that there was a highly significant difference between groups ($p<0.01$), table (1), figure (1). LSD showed high significant value for TGFB1 between heparinized samples and all other anticoagulants ($p<0.01$) while there was a non-significant difference ($p>0.05$) between TGFB1 was measured in EDTA and trisodium citrate samples PRP, table (2).

Regarding PDGFBB using single spin method; ANOVA showed that there was a highly significant difference ($p<0.01$) between all PRP and PPP, mean of heparinized PRP was (25.73±7.09) and (1.8200±1.416) for PPP, EDTA PRP was (17.70±3.706) and (1.407±0.912) for PPP and trisodium citrate PRP was (13.83±3.374) and (1.355±1.05) for PPP, table (3), Figure (2). LSD for PDGFBB denoting high significant difference between all PRP from the different anticoagulants ($p <0.01$), table (4).

Pearson correlations between platelets counts and both TGFB1 and PDGFBB in PRP collected on the different anticoagulant denoting no significant difference between all anticoagulants ($p>0.05$), table (5) and (6) respectively.

The comparison between single and double spin methods for PRP separation by paired t test for both TGFB1 and PDGFBB revealed high significant difference between single and double spin methods for all the different anticoagulants ($p<0.01$), table (7) and (8) respectively.

Table (1): Simple analysis of variance (ANOVA) for TGFB1; mean, standard deviation (SD), F and P value

	Heparin PRP Single spin TGFB1 (ng/ml)	Edta PRP Single spin TGFB1 (ng/ml)	Citrate PRP Single spin TGFB1 (ng/ml)	Heparin PPP Single spin TGFB1 (ng/ml)	Edta PPP Single spin TGFB1 (ng/ml)	Citrate PPP Single spin TGFB1 (ng/ml)	F	P
Mean	152.03	80.90	77.13	20.46	17.73	17.53	181.4	< 0.01**
SD±	±31.26	17.88±	23.42±	6.80±	5.45±	10.15±		

P<0.01 (Highly Significant) = **

Table (2): Least significance difference (LSD) for TGFB1 in the different anticoagulants

	Heparin TGFB1 PRP P	Edta TGFB1 PRP P	Citrate TGFB1 PRP p	Heparin TGFB1 PPP P	Edta TGFB1 PPP P
Edta, TGFB1 (ng/ml) PRP	<0.01**	-	-	-	-
Citrate, TGFB1 (ng/ml) PRP	<0.01**	>0.05^	-	-	-
Heparin, TGFB1 (ng/ml) PPP	<0.01**	<0.01**	<0.01**	-	-
Edta, TGFB1 (ng/ml) PPP	<0.01**	<0.01**	<0.01**	<0.01**	-
Citrate, TGFB1 (ng/ml) PPP	<0.01**	<0.01**	<0.01**	<0.01**	>0.05^

P>0.05 (Non Significant) = ^

P<0.01 (Highly Significant) = **

Table (3): Simple analysis of variance (ANOVA) for PDGFBB; mean, standard deviation (SD), F and P value

	Heparin PRP Single spin PDGFBB Pg/ml)(	Edta PRP Single spin PDGFBB Pg/ml)(	Citrate PRP Single spin PDGFBB Pg/ml)(	Heparin PPP Single spin PDGFBB Pg/ml)(	Edta PPP Single spin PDGFBB Pg/ml)(	Citrate PPP Single spin PDGFBB Pg/ml)(	F	P
Mean	25.7333	17.7000	13.8333	1.8200	1.407	1.355	236.4	<0.01**
SD±	±7.09	3.706±	3.374±	1.416±	0.912±	1.05±		

P <0.01 (Highly Significant) = **

Table (4): Least significance difference (LSD) for PDGFBB of the different anticoagulants

	Heparin PDGFBB Pg/ml)(PRP P	Edta PDGFBB Pg/ml)(PRP P	Citrate PDGFBB Pg/ml)(PRP P	Heparin PDGFBB Pg/ml)(PPP P	Edta PDGFBB Pg/ml)(PPP P
Edta, PDGFBB (Pg/ml)PRP	<0.01**	-	-	-	-
Citrate, PDGFBB (Pg/ml)PRP	<0.01**	<0.01**	-	-	-
Heparin,PDGFBB(Pg/ml)PPP	<0.01**	<0.01**	<0.01**	-	-
Edta , PDGFBB (Pg/ml)PPP	<0.01**	<0.01**	<0.01**	>0.05^	-
Citrate, PDGFBB (Pg/ml)PPP	<0.01**	<0.01**	<0.01**	>0.05^	>0.05^

P>0.05 (Non Significant) = ^
P<0.01 (Highly Significant) = **

Table (5): Pearson correlations between platelets counts and TGFB1 in PRP collected on the different anticoagulant

	TGFB1 in PRP on heparin		TGFB1 in PRP EDTA		TGFB1 in PRP trisodium citrate	
	R	P	R	P	R	P
Platelets counts	0.316	>0.05^	-0.182	>0.05^	0.119	>0.05^

P>0.05 (Non Significant) = ^

Table (6): Pearson correlations between platelets counts and PDGFBB in PRP collected on the different anticoagulant

	PDGFBB in PRP on heparin		PDGFBB in PRP EDTA		PDGFBB in PRP on trisodium citrate	
	R	P	R	P	R	P
Platelets counts	-0.067	>0.05^	-0.221	>0.05^	0.346	>0.05^

P>0.05 (Non Significant) = ^

Table (7): Paired t test for TGFB1 first and second spin

	Single Spin mean±SD	Double Spin mean±SD	Paired t test	P
TGFB1(ng/ml) Lithium heparin	152.03±31.26	131.03±33.35	5.213	<0.01**
TGFB1(ng/ml) Edta	80.90±17.88	63.26±17.57	8.116	<0.01**
TGFB1(ng/ml) Trisodium citrate	77.13±23.42	65.60±19.95	4.44	<0.01**

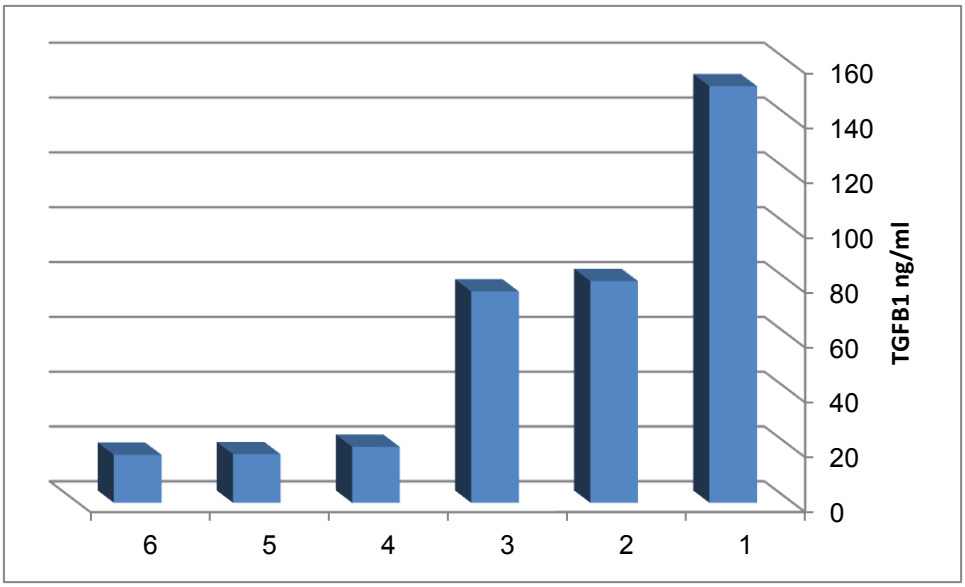
P<0.01 (Highly Significant) = **

Table (8): Paired t test for PDGFBB first and second spin

	Single Spin mean±SD	Double Spin mean±SD	Paired t test	P
PDGFBB (pg/ml) Lithium heparin	25.73±7.09	21.83±3.98	3.135	<0.01**
PDGFBB (pg/ml) Edta	17.7±3.7	15.46±4.19	4.489	<0.01**
PDGFBB (pg/ml) Trisodium citrate	13.83±3.37	12.23±2.84	2.876	<0.01**

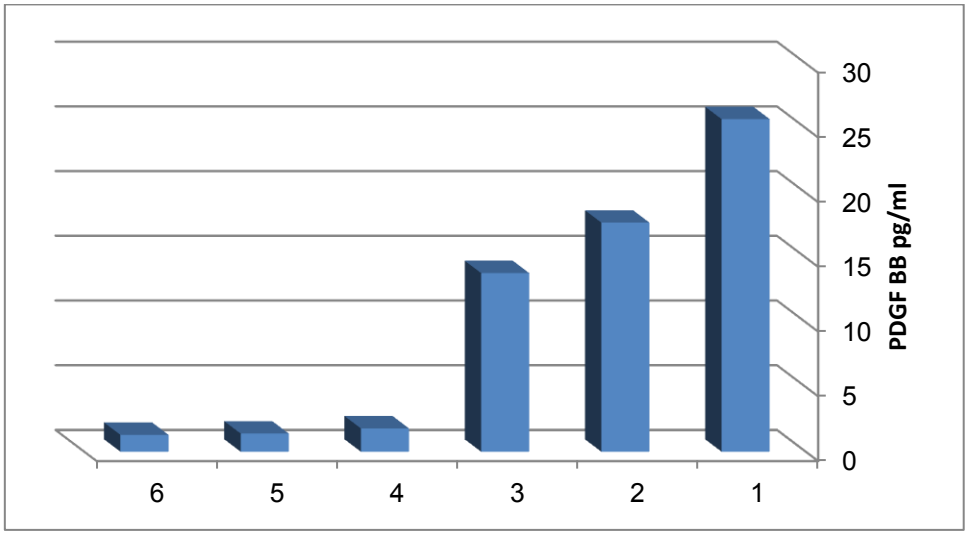
P<0.01 (Highly Significant) = **

Figure (1): Mean of TGFB1 (ng/ml) were collected by single spin.



1=Heparinized PRP, 2= EDTA PRP, 3=Trisodium citrate PRP, 4=Heparinized PPP, 5=EDTA PPP, 6= Trisodium citrate PPP.

Figure (2): Mean of PDGFBB (pg/ml) were collected by single spin



1= Heparinized PRP, 2= EDTA PRP, 3=Trisodium citrate PRP, 4=Heparinized PPP, 5=EDTA PPP, 6= Trisodium citrate PPP.

Discussion

Platelets, an important reservoir of growth factors in the body, play an important role in many processes such as coagulation, immune response, angiogenesis and the healing of damaged tissues. Numerous proteins are contained in the platelets granules. High concentrations of growth factors, in a form of sterile mass that can be used immediately for clinical

purposes. The clinical use of PRP for a wide variety of applications has been reported in oral and maxillo-facial surgery, orthopedic surgery, treatment of soft tissue diseases and injuries, treatment of burns, tissue engineering and implantology [7]. PRP has been utilized and studied since the 1970s and used clinically in humans for its healing properties, and this attributed to the increased concentrations of growth factors and secretory proteins that may enhance the healing process on a cellular level. The hope is that PRP enhances the recruitment, proliferation, and differentiation of cells involved in tissue regeneration[1]. Therefore PRP is a concentrate with the 'right' factors in the 'right' proportions necessary for the healing process. However, many protocols available induce high methodology variability[2].

In our study we found platelets counts mean in heparinized, EDTA and trisodium citrate (after addition 10% which is the dilution factor) samples were 238.16±59, 246 ± 31 and 249± 42 ($x10^3$ cells/µl) increased to 726±181, 734±190 and 740±176 ($x10^3$ cells/µl) in PRP respectively when collected by single spin and with no significant difference between groups ($p>0.05$). While platelets counts increased in the second spin to 923±156, 946±166 and 972±163 ($x10^3$ cells/µl) in lithum heparin, EDTA and trisodium citrate samples PRP but also with no significant difference between different anticoagulants. Higher results for platelets counts were obtained by Queiroz da Silva et al.,[8] where they found the mean of platelets concentration in PRP was 1622 x 10^3 cells/µl, which represents 5.3 folds higher than the basal number (303 x 10^3 cells/µl). Cho et al., reported that the platelets amount should be three to seven times higher than the basal value[9].

We found in our study a single spin centrifugation of 1000 rpm for 8 minutes yields higher growth factors level in PRP than levels were obtained by double spin although higher platelets counts were obtained in the double spin. This can be explained by detoriation of the platelets function due to excessive centrifugation. These results are consistent with Landesberg et al., where they evaluated the effect of different centrifugation forces and showed that > 800-g spin may reduce the amount of growth factor released by PRP[10].

Interestingly, in our study no significant correlation between either TGFB1or PDGFBB and platelets counts in PRP samples were obtained from sample collected on heparin, EDTA or trisodium citrate ($p>0.05$), tables (5) &(6) respectively. These results is consistent with observations reported by Weibrich et al., which indicating that the platelet count may not be a sufficient marker to predict biological activity[11], while Bausset O. et al., reported that correlation between platelets counts and growth factors release could be observed for PDGF-AB, but not for TGF-B1[12].

In our study TGFB-1 in heparinized sample was higher than that in samples collected on EDTA and trisodium citrate with high significant difference ($p<0.01$), table (2). These results are consistent with Bocci V. et al., [13] and Valacchi G. et al., [14] where they reported that the release of growth factors from platelets take place more efficiently in presence of heparin when they utilized heparin normally used for therapeutic purposes and ACD (Citric acid, Na citrate, Glucose) for isolation of PRP[13,14].

The actual challenge for PRP optimization is to determine the main bioactive components responsible for the clinical effects. We should not consider the platelet itself nor its growth factors, but certainly the synergy of both. Thus, the capacity of the preparation method to preserve maximal amount of resting and activable platelets may be a specific advantage[15]. in our study we activate platelets in PRP by CaCl2 for gel formation and growth factors release which gave results consistently with Bausset O. et al., where they found that the method for PRP preparation proposed in their study allows high levels of PDGF-AB, and TGF-b1 release after platelets activation by CaCl2 and glass tubes[12]. Notably, these results were obtained with a lower centrifugation speed presumed to ensure better platelets functionality[16]. In addition, it has been demonstrated that platelets activation in presence of heparin, is significantly produce high amount of PDGF and TGF-b1in heparinized PRP samples, in

contrast with the procedure that use ACD[14]. Moreover, numerous extracellular proteins, growth factors, chemokines, cytokines, enzymes, lipoproteins, involved in a variety of biological processes, interact with heparin and/or heparin sulfate at the cell surface and in the extracellular matrix. The interaction of some important growth factors derived from platelets, as the Fibroblast growth factor (FGF) with its receptor: Fibroblast growth factor receptor (FGFR), is more efficient in presence of heparin[16] . The heparin-binding protein site near to the FGFR regulates the angiogenesis, then the presence of heparin induce an enhancement of the mitogenic activity of FGF[17].

In our study LSD shows no significance difference between TGFB-1 was measured in sample collected on EDTA and trisodium citrate ($p>0.05$), table (2), similar results were obtained by Ronaldo JF et al., where they could not find differences in TGFβ-1 and VEGF concentration among the EDTA and citrate anticoagulants and explained that by similar platelets concentration between groups[18].

Fukaya M et al., considered heparin treated platelets more easily discharge α-granules, which contains PDGF than ACD-A–treated ones as they found the concentration of PDGF decreases in ACD-A while increases in heparin [19], which is consistent with our findings where we found PDGF-BB was the highest in PRP were prepared from heparinized samples than EDTA or citrate samples, with high significant difference between groups ($p <0.01$), table (3).

We agree with Jing Qiao et al., that we need more data to find the proper therapeutic doses for platelets concentrates suitable for different clinical applications [20].

Source of grants: General Organization for Teaching Hospitals and Institutes

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