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Research Article

Differential Cytokine Secretion In Differently Processed Platelet Rich Plasma And Its Temporal Secretory Pattern

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ABSTRACT

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Platelet Secretome – Differential Secretion -RANTES(CCL5)- IL-8-TNF α , -TGF β - Regenerative Therapy—Tailor Made Product

Background: Platelets are increasingly used for stem cell research , tissue regeneration , wound healing , immunomodulation , anti-inflammatory, pro inflammatory reaction . Methods of preparation , incubation and activation may produce different products.

Materials and Methods: Supernatant of platelet concentrates produced by various methods were used to measure RANTES(CCL5), IL-8,TNF α , TGF β , by ELISA test at different time periods.

Results: Platelets produced by different techniques has different levels of white cell contamination & was significantly more in standard PRP. Cytokines in all the platelet preparations rose significantly over 5 days ($p < 0.01$) . The rate of rise was different for different cytokines and varied with the preparation and degree of contamination with leucocytes. Platelet irradiation inhibited this secretion.

Conclusion : Secretory behaviour of platelets depends on type of preparation , duration of incubation and leucocyte contamination. Understanding differential platelet secretion behavior will allow ones to produce tailor made products.

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INTRODUCTION

Platelet rich plasma or platelet concentrates can be prepared its different ways. Random donor platelets can be prepared from individual blood donation either by classical double centrifugation technique (PRP) or by combining collection in plastic bags with top and bottom opening¹ combined with centrifugation i.e. Buffy coat poor platelets (BC-PRP). In addition there are several different kind of platelet pheresis machines which are available in the market can produce concentrates using slightly different technology leading to different shear stress during platelet collection^{2,3}. These platelets in addition to individual donation may also undergo leucofiltration during collection of blood or the procedure may be done at bed side. Phersed platelets which are usually leuco-poor and is used within short time after collection is transfused with or rarely without bedside leuco filtration. Platelets are anucleate cells but are metabolically very active. It has pre mRNA, mRNA, SiRNA and more than 800 different proteins stored in various organelles. This complex array of proteins and its post synthetic modifications along with actively absorbed and concentrated proteins and other chemical entities (eg 5HT) are stored in various organelles of platelets. These can be differentially regulated and secreted by different agonists like thrombin , ADP , Thromboxane A2 etc^{4,5}.

As platelet rich plasma or platelet concentrates are normally used to secure haemostasis in severe thrombocytopenic situations^{6,7} for immunomodulation⁸ and for regenerativ and anti inflammatory therapy⁹, it is important to understand that all platelet concentrates and concentrates where platelets are minimally activated with different physical (cold)¹⁰ and chemical agonists^{4,5} and incubated for different time periods produce different kinds

of platelet products with respect to cytokine and growth factor contents. Hence there is no one type of platelet product for different kinds of therapy and in future tailor made platelet product may be available for optimum results in different conditions. In the present study we evaluated platelet concentrates produced by various means and tested at different time provides after maintaining it at 22°C with agitation for a few cytokine secretion.

MATERIAL AND METHODS

15 random donor platelets were obtained by standard technique (PRP)¹¹ Similarly 15 more platelet concentrate were made with top and bottom (Optipress II) bag as buffy coat PRP(BC-PRP). Thirty platelet from platelet pheresis (10 with TrimaAccel, 10 with Amicus and 10 with Cobe spectra machine) and a separate set of random donor platelets (PRP as well as PRP BC 15 each and 30 phersed platelets 10 each in three machines were used to detect radiation effect after 24 hours of radiation)were used for the present study. All donors provided informed consent for the study and the study was sanctioned by Institutional review based.

Leukocyte counts and platelet counts of the sample were done in laboratory counters (Nihon Koden). The platelets were kept at 22°C with horizontal agitation. The 5 ml sample was collected from the bag on different days using aseptic procedure in class II biosafety cabinet. The sample was immediately centrifuged at 3000 rpm for 15 minutes and supernatant was stored at -80°C until tested. Cytokines ie RANTES(CCL5), IL-8, TNF α , TGF β , were assessed by ELISA test (Peprotech , Nj USA & BioLegend , Ca .USA, R&D system, USA and ABCAM , USA respectively). Student's t test was done to assess statistical test of significance for the data so generated.

RESULTS:

Content of various proteins enzymes cytokines , amines , RNA of platelets are given in table

1. Volume, leukocyte count and platelet count of the sample are presented in table – 2 BC – PC and pheresis by various machines produced

significantly higher platelet counts compared to standard PRP. WBC count in PRP unit was significantly higher compared to others. However WBC count of BC-PRP was similar to that of pheresal platelets. Yield of platelets by three different pheresis machine was also significantly different with Trima Accel producing highest yield in shorter period of time and with one venepuncture in contrast to COBE Spectra machine. When asked the donors who has donated platelet on all three machines majority (>80%) donors preferred to donate platelets on Trima-Accel (Terumo – BCT – USA). CCLS (RANTES), TNF α , IL-8 and TGF – β were measured 1 hour after collection of platelet product. Supernatant plasma which was preserved at -70°C were done for estimation of cytokines from irradiated platelets in addition to baseline assay. Levels of various cytokine during various time period obtained from platelet supernatants produced by different techniques are provided in table – 3. Though basal levels of various cytokines varied depending on the mode of production, the subsequent levels of cytokines show a rise with time. Through this rate of rise for different cytokines are different, and the collection day level and day 5 level of these

cytokines were all significantly different ($P < 0.03$ to $P < 0.0001$ student t test). The rise of interleukin 8 level in PRP was significantly higher on day 5 in standard PRP than any other types of platelet concentrates ($P < 0.0001$) Irradiated platelets showed lesser rise or no rise in all the cytokines tested. Rate of rise of each of the cytokine by each processing techniques differed significantly so that rate of rise of RANTES $<$ TGF β $<$ TNF α $<$ IL-8. There were significant differences between individual to individual concentrates on the basal plasma cytokine levels, so that about 10/15 PRP-PC & BC-PC group and 21/30 of platelet pheresis group had cytokine levels below 50 % of the mean levels and the rate of rise of this cytokine on day 5 in 70% of the samples had lower rise of cytokine levels whose basal cytokine level was also low.

Hence these samples which had lower third of the levels of cytokine tended to keep the increment of cytokine on day 5 to lower levels only (65-78% of the group). Only 30% of the platelets showing all the cytokines at lower third level showed in the cytokines to upper third levels of day 5 on 5th day incubation at 22°C in a horizontally shaking incubator.

Table 1. Contents of platelets:

1. **Cytoplasm:** Glycogen, Proteins, Pre –mRNA, mRNA, r ibosomes., TLR2, TLR4.etc.
2. **Mitochondria** and its contents.
3. **Lysosomes:** Acid hydrolases, Glycosidases, Carboxy peptidases, Aryl sulphatases, hexosaminidases, collagenases, Glucuronidases, etc.
4. **Dense Granules :** ADP, ATP, Serotonin, Histamine, GTPases, rab27a, rab27b, CD63, LAMP-1, LAMP-2, P-selectin, Poly phosphate, Pyrophosphate etc.
5. **T granules :** TLR9, PDI, VAMP-8.
6. **Alpha Granules:**
 - i. Adhesion Molecules: α ii β 3, α v β 3, CD9, Fibronectin, GPIb α , multimerin, osteonectin, PECAM, P-selectin, Vitronectin, vWF etc.
 - ii. Cytokines and Chemokines :CCL4, CCL17, ENA-78, Gro α , IL-1, IL-7, IL-8, MCP-1, MCP-3, MIP1 α , NAP2, PF4, RANTES (CCL5), sCD40L, SDF-1.
 - iii. Angiogenic / Growth Factors: ADAM10, ADAMTS13, Angiostatin, Angiopoietin, BDNF, bFGF, BMP-2, BMP-6, CTAP iii, CTGF, EGF, Endostatin, HGF, IGF-1, Kininogens, MMP-1, MMP-2, MMP-9, PDGF, TGF β , thrombospondin, TIMP-1, TIMP-4, VEGF etc.
 - iv. Coagulation Proteins : α 2antiplasmin, α 2antitrypsin, α 2macroglobulin, antithrombin, factors v, viii, xi, xiii, fibrinogen, protein S, nexin 2, prothrombin, TFPI etc.
 - v. Immune Mediators : C1 inhibitor, Complement factors, factor D, Ig G, Ig A, IgM, Platelet factor H, Thymosin B-4 etc.
 - vi. **Others :** Albumin and many other proteins.
7. **Membrane associated proteins :** MyH7, MYH 9, MYH10, tubulin etc.

Table 2: Measurement of various parameters in PRP PC, BC PC and SDP

Sample	Age yrs	Volume ml	pH	WBC /Unit	Platelet /Unit
PRP PC	31.2±7.9	60.4±5.1	7.9±0.31	7.4±3.75x10 ⁷ *	6.05±1.94x10 ¹⁰
BC PC	31.3±7.1	72.0±6.4	7.9±0.29	3.9±2.2x10 ⁷ *	6.54±1.81x10 ¹⁰ §
SDP-Cobe	32.1±8.1	300±42	7.3±0.32	0.44±0.23x10 ⁸	4.1±0.86x10 ¹¹ ‡ §
SDP-Trima	31.7±9.8	318±10	7.4±0.26	0.40±0.21x10 ⁸	4.26±0.88x10 ¹¹ †
SDP-Amicus	32.3±7.6	322±44	7.4±0.19	0.40±0.06x10 ⁸	3.17±0.59x10 ¹¹ †‡

* 't' test between WBC count of PRP PC and BC PC is highly significant (P 0.0043)

† 't' test between platelet count in SDP-Trima and SDP-Amicus is highly significant (P 0.00062)

‡ 't' test between platelet count in SDP Cobe and SDP -Amicus is highly significant (P0.0021)

§ 't' test between platelet count in BC PC and SDP Cobe is highly significant (P 0.00001)

Table 3. CCL5, TNF α , IL-8 and TGF β levels (pg/ml) in platelet supernatants at different periods of Incubation. (Mean +/-1SD).

Products(n)	Time	CCl-5(RANTES)	TNF α	IL-8	TGF β
PRP-PC (15).	1hr.	1210+/- 561.	0.63+/-2.4.	9.26+/- 11.1.	1551+/-465.
	18-24 hr.	1453+/-518.	2.33+/-4.4.	48.7+/-67.8 .	2919+/-567.
	65-72hr.	1599+/-467.	5.65 +/- 9.7 .	307+/_196.	4671+/-468.
	112-120hr.	1617+/-451.	9.3 +/14.9 .	731+/-204.	5128+/-861.
BC-PC(15).	1hr.	1384+/-463.	1.08+/-1.8.	7.9+/-14.1.	1216+/-365.
	18-24hr.	1500+/-426.	0.29+/-0.8 .	14.6+/-22.2.	2165+/-492.
	65-72 hr.	1631+/-389.	6.01+/- 8.3.	58+/-70.	3621 +/-561.
	112-120hr.	1949+/-139.	8.97+/-12.8.	247+/-139.	4671+/-418.
Pheresed Platelets (30) :	1 hr.	1270+/-431.	1.06+/-0.7.	6.3 +/-8.2.	965+/-411.
	18-24Hr.	1717+/-398.	1.7+/-0.9 .	11.4+/-6.8.	1156+/-439.
	65-72 hr.	1884+/-242.	3.2+/-7.6.	30.2+/-7.6.	2179+/-716.
	112-120 hr.	1947+/-133 .	3.8+/-6.9.	116+/-65.	2984+/-649.
Irradiated: PRP-PC(10)	18-24 hr.	1298+/-198.	1.8+/-1.6.	9.1+/-6.3.	1128+/-621.
BC-PC(10)	18-24 hr.	1305+/-430.	0.7+/-0.4.	6.8+/-6.	1321+/-616.
Pheresed(10)	18-24 hr.	1816+/-275.	1.6+/-1.1.	7+/-6.2.	1109+/-328.

DISCUSSION

Traditionally platelet concentrate transfusion was used for bleeding related to non immune are generative thrombocytopenia or in cases of substantial bleeding in patients with platelet function defects or as a prophylactic transfusion in such patients where platelet count fell below $<10000/\mu\text{L}$ or $<20000/\mu\text{L}$ with bleeding. For this kind of therapy major factor was platelet count increment on infusion, relatively low adverse reaction and infection and low level of allosensitization which was dependent on number of contaminating leucocytes. Use of leucocyte filters either during donation or during bed side use has also made substantial difference in platelet alloimmunization in these patients who require frequent platelet transfusion for haemostasis.

However, platelets not only exhibit haemostatic proteins but its more than 800 proteins and peptides are also available during various stages of platelet secretion .Its ability to synthesise new proteins through its pre-mRNA, mRNA platelet provide a thematic portfolio of growth factors, cytokines, chemokines, proteinases, adhesion molecules, Toll like receptors, various prostanoids, nucleotides to suit the context in which platelets are being activated ¹²⁻¹⁴. These cargo of chemicals are compartmentalized very clearly in several granules and vesicular structures and not so clearly packed as microparticles production during diverse agonist associated stimulations ^{15,16}. Even in the granules i.e. alpha granules various compounds are compartmentalized so that various platelet agonists or interaction of various cells with platelets or incubation of these cells at low or high temperatene release / synthesize their cargo in an activation department way and have differential secretary kinetics ¹².

Increasingly autologous or allogenic platelet rich plasma is used for wound healing and for various areas of regenerative medicine ¹⁷. This product is also used to promote growth and differentiation in various stem cell laboratories . Various important growth factors and other peptides which are elaborated by platelet

concentrates are presented in table – 1. This will make it apparent that platelets used for different purpose should have different cytokine/ growth factor cocktails in platelet rich plasma. Our study clearly show that few cytokines we tested showed different rates of secretion at different time periods of incubation at 22⁰ C as well as at the base line levels. There were significant individual variation of the measured cytokines at base line too. However Peterson et al ¹⁸ have shown that at least for angiogenic peptides platelets from individual donors produce remarkably consistent amount of the product at different time periods. This finding when combined with present findings have important implications. Basically it means that there will be some individual whose autologous platelets may not produce enough growth factor for regenerative therapy . Either we have to increase the number of autologous platelets substantially or use good quality allogenic platelets for regenerative therapy. Regarding agonist related differential behavior on platelet secretion it has been shown that ADP tends to produce a secretion rich in VEGF but poor in endostatin and thromboxane A2 has reverse effect ⁴. Similarly thrombin at different concentration targets different platelet granules for secretion⁵

Physical condition like cold can also differentially activate platelets¹⁰.

PRP as therapy is used for reparative, regenerative anti-inflammatory and immunomodulatory purposes. Hence depending on the purpose PRP production and activation needs to be different. Its future applications in clinical medicine is likely to expand further. In addition it has been shown that addition of PRP in a cocktail for invitro expansion of mesenchymal and other stem cells produce better expansion of cells ¹⁹.

The changes in various cytokines secretion on incubation over few days of differently produced platelet concentrate suggest that interaction of platelet secretome with contaminating leukocyte and secretions produced by the leukocytes with platelet may

mutually augment or suppress different cytokine product and secretion¹⁶. Different procedures of platelet rich plasma production may not only produce different levels of leucocyte contamination as shown in table 2 of present study and similar results have been described by others too^{3,20}. Moreover differently produced platelet concentrate may concentrates different class of platelets e.g. large, small dense, light etc which may have different contents ability to synthesis and senate

different cytokine under different circumstances. Hence it is likely that in future PRP will be produced in a different manners and may be activated with different agonists in a more purposive manner. It has rightly been said 'Every PRP gel is not born equal'²¹. Our quest is not for equality but for developing varied protocols of PRP production and activation to suit individual requirements of therapy and we are progressing to that end.

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