Quantification of Platelets and Platelet Derived Growth Factors from “Platelet-Rich-Plasma” (PRP) Prepared at Different Centrifugal Force (g) and Time

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• Platelet derived Biomaterials
  – Gained a lot of attention
  – Progressive increase in scientific data to support it

• “Characterization” of these products (eg PRP) is important before analyzing their clinical efficiency.

• Centrifugation is one of the most important step for preparing PRP in lab as it determines both the cellular as well as plasma component of the final product.
RCF = 28.38 \times R \times \left(\frac{RPM}{1000}\right)^2

RCF = \text{relative centrifugal force (x g)}; R = \text{radius in inches}; RPM = \text{revolutions per minute}
Aim & Objectives

• To highlight the “heterogeneity” of PRP products prepared at different centrifugal force and time and analyse:

  – Variation in “cellular” composition of PRP prepared

  – Quantify the “Concentration of growth factors” released
Material and Method

• Ethical Approval
  – Study conducted at “Dr Ram Manohar Lohia Hospital, New Delhi”

  – The study was approved by the “institutional ethical committee” based on ICMR (Indian council of Medical Research) guidelines.

  – Proper consents were taken from all the volunteers enrolled in the study.
Material and Method

• Inclusion Criteria
  – Age > 18 yrs
  – Not taking any NSAID’s or Asprin
  – Not Suffering from any bleeding disorders

• Blood Collection
  – 100 mL of whole blood (WB) was collected in a sterile blood collection bag with CPDA as the anticoagulant (AC); (14 mL AC for 100 mL WB ratio).
  – The EDTA anticoagulated blood samples (collected separately) for baseline values
100 mL Whole blood (WB) collected with anticoagulant (AC; CPDA) at [WB: AC; 100 mL: 14 mL]

Nine aliquots of 10 mL each prepared

Set of three aliquots allotted a group of centrifugal force (A: 110g, B: 208g & C: 440g)

Group A

Group B

Group C

5 min (A1/B1/C1), 10 min (A2,B2,C2), 20 min (A3,B3,C3)

Supernatant (PRP) is separated

Activated with equal volume of Calcified Thromboplastin (Activator)

Centrifugation at 1000 rpm for 3 min (Clot Formation)

Blood Counts

Overnight Incubation at 4-8 °C (Clot Retraction)

Centrifugation at 3000 rpm for 3 min

Supernatant storage (-70°C)

Sample (0.5 mL) Centrifuged & Supernatant stored (-70°C)

Growth factor quantification

P-Selectin quantification

EDTA Sample (Baseline)

Baseline Blood Counts
Material and Method

• Centrifuge:
  – Table top lab centrifuge (Remi 8M, Rotor R81)

• Activation of PRP
  – **Activator** – “Calcified thromboplastin”
    • [Uniplastin; Tulip Diagnostics (P) Ltd, India]
  – *Equal volume of pre warmed* (37°C)
Material and Method

• P-Selectin and Growth Factor quantification
  – Commercial “Sandwich ELISA kits” were used to quantify
    • CD62P - Diaclone SAS; France
    • PDGF-AB - Qayee-Bio, China
    • TGF-β1 - DRG; Germany
    • VEGF - Boster Immuno Leader, Boster Biological Technology, USA
  – Performed as per the manufacturer’s instructions.
Material and Method

• Blood Counts testing
  – Automated cell counter (Sysmex KX-21, Japan)
  – All samples were tested in duplication
  – Diluted with PBS where ever needed.

• Statistical Analysis
  – Results are presented as mean ± standard deviation (SD).
  – Two-way analysis of variance was used to determine significant differences between means.
  – Student’s t-test was applied to compare means of growth factor levels.
a. **Platelet Capture Efficiency / Platelet Yield (PY; %)** =
\[
\frac{\text{Volume of Product (ml)} \times \text{Platelet Concentration in the Product (x10}^{9} / \text{L})}{\text{Volume of WB Collected (ml)} \times \text{Platelet Concentration in WB (x10}^{9} / \text{L})} \times 100
\]

b. **WBC Capture Efficiency / WBC Yield (WY; %)** =
\[
\frac{\text{Volume of Product (ml)} \times \text{WBC Concentration in the Product (x10}^{9} / \text{L})}{\text{Volume of WB Collected (ml)} \times \text{WBC Concentration in WB (x10}^{9} / \text{L})} \times 100
\]

c. **Volume Harvested / Volume Yield (VY; %)** =
\[
\frac{\text{Volume of Product (ml)}}{\text{Volume of WB Processed (ml)}} \times 100
\]

d. **Relative Concentration of Platelet (RCP; %)** =
\[
\frac{\text{Platelet Concentration in the Product (x10}^{9} / \text{L})}{\text{Platelet Conc (x10}^{9} / \text{L}) + \text{WBC Conc (x10}^{9} / \text{L}) + \text{RBC Conc (x10}^{9} / \text{L})} \times 100
\]

e. **Relative Concentration of Platelet Increment**
\[
\text{RCP of the product (\%)} - \text{Baseline RCP of the whole blood (\%)}
\]

f. **Fraction increase in Platelet/ WBC concentration (FPW)** =
\[
\frac{\text{Platelet / WBC Concentration in the Product (x10}^{9} / \text{L})}{\text{Platelet / WBC Concentration in WB (x10}^{9} / \text{L})}
\]

g. **Growth Factors released by 10^6 platelets** =
\[
\frac{\text{Concentration of GF (pg/mL)}}{\text{Platelet count in the PRP (/mL)}}
\]
**Demographic Profile** of Volunteers

- A total of 8 volunteers were enrolled
- Age distribution - 24-32 years

- **Baseline blood counts** *Comparable (p ≥ 0.05)*
  - WBC count - $6.38 \pm 1 \times 10^3 / \mu\text{L}$,
  - RBC count - $4.23 \pm 0.7 \times 10^6 / \mu\text{L}$
  - Platelet counts - $218 \pm 72.4 \times 10^3 / \mu\text{L}$.
# Cellular Variation

<table>
<thead>
<tr>
<th>Groups</th>
<th>Volume (Mean ± SD; ml)</th>
<th>Volume Yield (Mean ± SD; %)</th>
<th>WBC Yield (Mean ± SD; %)</th>
<th>Platelets Yield (Mean ± SD; %)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A1</td>
<td>0.85 ± 0.3</td>
<td>8.5 ± 3.2</td>
<td>7.3 ± 4.2</td>
<td>17.2 ± 4.2</td>
</tr>
<tr>
<td>A2</td>
<td>2.25 ± 0.8</td>
<td>22.5 ± 8.4</td>
<td>15.6 ± 4.5</td>
<td>44.1 ± 10.3</td>
</tr>
<tr>
<td>A3</td>
<td>3.75 ± 0.7</td>
<td>37.5 ± 7.4</td>
<td>18.3 ± 4.8</td>
<td>70.1 ± 4.6</td>
</tr>
<tr>
<td>B1</td>
<td>1.62 ± 1.1</td>
<td>16.25 ± 11.8</td>
<td>8.8 ± 6.4</td>
<td>31.4 ± 15.9</td>
</tr>
<tr>
<td>B2</td>
<td>3 ± 0.8</td>
<td>30.6 ± 8.6</td>
<td>15.4 ± 4.7</td>
<td>58.5 ± 8.6</td>
</tr>
<tr>
<td>B3</td>
<td>4.4 ± 0.3</td>
<td>44.3 ± 7.2</td>
<td>13.5 ± 4.2</td>
<td>75.9 ± 5.9</td>
</tr>
<tr>
<td>C1</td>
<td>4.4 ± 0.3</td>
<td>26.8 ± 7.9</td>
<td>14.3 ± 5.8</td>
<td>51.5 ± 8.2</td>
</tr>
<tr>
<td>C2</td>
<td>2.6 ± 0.3</td>
<td>44.3 ± 6.2</td>
<td>15.9 ± 5.7</td>
<td>78.7 ± 5.7</td>
</tr>
<tr>
<td>C3</td>
<td>5.1 ± 0.5</td>
<td>51.8 ± 5.3</td>
<td>3.3 ± 2</td>
<td>56.3 ± 15.9</td>
</tr>
<tr>
<td>Groups</td>
<td>Relative Concentration of Platelets (Mean ± SD; %)</td>
<td>Relative Concentration of platelet Increment (Mean ± SD)</td>
<td>Fractional Increment in PLT/WBC (Mean ± SD)</td>
<td></td>
</tr>
<tr>
<td>--------</td>
<td>--------------------------------------------------</td>
<td>----------------------------------------------------------</td>
<td>--------------------------------------------</td>
<td></td>
</tr>
<tr>
<td>A1</td>
<td>73 ± 1.3</td>
<td>15.7 ± 3.5</td>
<td>2.8 ± 1.1</td>
<td></td>
</tr>
<tr>
<td>A2</td>
<td>78 ± 1.6</td>
<td>16.4 ± 1.3</td>
<td>2.9 ± 0.7</td>
<td></td>
</tr>
<tr>
<td>A3</td>
<td>84 ± 1.8</td>
<td>17.9 ± 3</td>
<td>4.1 ± 1.2</td>
<td></td>
</tr>
<tr>
<td>B1</td>
<td>78 ± 1.5</td>
<td>16.5 ± 2.1</td>
<td>4 ± 1.1</td>
<td></td>
</tr>
<tr>
<td>B2</td>
<td>80 ± 1.5</td>
<td>17.1 ± 2.6</td>
<td>4 ± 0.9</td>
<td></td>
</tr>
<tr>
<td>B3</td>
<td>87 ± 1.6</td>
<td>18.6 ± 2.7</td>
<td>6.1 ± 1.9</td>
<td></td>
</tr>
<tr>
<td>C1</td>
<td>77 ± 1.5</td>
<td>16.4 ± 2</td>
<td>4 ± 1.4</td>
<td></td>
</tr>
<tr>
<td>C2</td>
<td>85 ± 1.2</td>
<td>18 ± 2.6</td>
<td>5.5 ± 2.1</td>
<td></td>
</tr>
<tr>
<td>C3</td>
<td>93 ± 8</td>
<td>20 ± 14.7</td>
<td>23.6 ± 16.2</td>
<td></td>
</tr>
</tbody>
</table>
a. Platelet Capture Efficiency / Platelet Yield (PY; %) =
   \[ \frac{\text{Volume of Product (ml)} \times \text{Platelet Concentration in the Product} \times 10^9/L \times 100}{\text{Volume of WB Collected (ml)} \times \text{Platelet Concentration in WB} \times 10^9/L} \]

b. WBC Capture Efficiency / WBC Yield (WY; %) =
   \[ \frac{\text{Volume of Product (ml)} \times \text{WBC Concentration in the Product} \times 10^9/L \times 100}{\text{Volume of WB Collected (ml)} \times \text{WBC Concentration in WB} \times 10^9/L} \]

c. Volume Harvested / Volume Yield (VY; %) =
   \[ \frac{\text{Volume of Product (ml)} \times 100}{\text{Volume of WB Processed (ml)}} \]

d. Relative Concentration of Platelet (RCP; %) =
   \[ \frac{\text{Platelet Concentration in the Product} \times 10^9/L \times 100}{\text{Platelet Conc} \times 10^9/L + \text{WBC Conc} \times 10^9/L + \text{RBC Conc} \times 10^9/L} \]

e. Relative Concentration of Platelet Increment
   \[ \text{RCP of the product} \% - \text{Baseline RCP of the whole blood} \% \]

f. Fraction increase in Platelet/ WBC concentration (FPW) =
   \[ \frac{\text{Platelet} / \text{WBC Concentration in the Product} \times 10^9/L}{\text{Platelet} / \text{WBC Concentration in WB} \times 10^9/L} \]

g. Growth Factors released by 10^6 platelets =
   \[ \frac{\text{Concentration of GF (pg/mL)}}{\text{Platelet count in the PRP} (/mL)}} \]
P-Selectin & Growth Factors

**X Axis** - Groups representing Centrifugal force (g) and Time

**Y Axis** - Concentration (ng/mL)

- **P-Selectin**
- **PDGF**
- **TGF-β1**
- **VEGF**
# P-Selectin & Growth Factors

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Baseline Concentration</th>
<th>PRP Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>PDGF-AB</td>
<td>10.6 ± 12.7 ng/mL</td>
<td>24.1 ± 37.9 ng/mL (A2)</td>
</tr>
<tr>
<td>TGF-β1</td>
<td>15.1 ± 16.4 ng/mL</td>
<td>46.7 ± 21.8 ng/mL (A2)</td>
</tr>
<tr>
<td>VEGF</td>
<td>664.3 ± 469.7 pg/mL</td>
<td>2757.5 ± 2145.1 pg/mL (C2)</td>
</tr>
<tr>
<td>P-Selectin</td>
<td>5.1 ± 3.6 ng/mL</td>
<td>12.1 ± 3 ng/mL (A2)</td>
</tr>
</tbody>
</table>
Our study highlights the “heterogeneity” of the PRP products prepared at different centrifugal force (g) and time (t).

Both time and centrifugal force influence the final product:
- Higher centrifugation force yields significantly higher platelet yield when prepared at similar time and vice versa.
- Although the higher centrifugal force or time leads to greater plasma recovery, it also leads to more platelets pelleting down out of the plasma.
- Can result in auto-aggregation and consequently reduction in clinical response¹.

P-Selectin \((CD62P)\) Quantification

- Marker of Activation
- No significant change from the baseline
- Also reported by Eppley et al\(^2\)
- Activation is reported \(^3\)
  - On storing these platelet products
  - Preparing them with double spin.

Growth Factors Released

- Growth Factors released per $10^6$ Platelets !!!

<table>
<thead>
<tr>
<th>Variable Centrifugal Force (g) and time</th>
<th>PDGF-AB</th>
<th>TGF-β1</th>
<th>VEGF</th>
</tr>
</thead>
<tbody>
<tr>
<td>A1 110g 5 min</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A2 110g 10 min</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A3 110g 20 min</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>B1 208g 5 min</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>B2 208g 10 min</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>B3 208g 20 min</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C1 440g 5 min</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C2 440g 10 min</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C3 440g 20 min</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
So which is the Best “Force n Time”

<table>
<thead>
<tr>
<th>Parameter</th>
<th>B3 208g x 20 min</th>
<th>C2 440g x 10 min</th>
</tr>
</thead>
<tbody>
<tr>
<td>Platelet Yield</td>
<td>75.9 ± 5.9 %</td>
<td>78.7 ± 5.7 %</td>
</tr>
<tr>
<td>RCP increment</td>
<td>18.6 ± 2.7</td>
<td>18 ± 2.6</td>
</tr>
<tr>
<td>Fractional Increment in Plt/WBC count</td>
<td>6.1 ± 1.9</td>
<td>5.5 ± 2.1</td>
</tr>
<tr>
<td>PDGF-AB (/10^6 plt)</td>
<td>58.8 pg</td>
<td>82.3 pg</td>
</tr>
<tr>
<td>TGF-β1 (/10^6 plt)</td>
<td>92.9 pg</td>
<td>100 pg</td>
</tr>
<tr>
<td>VEGF (/10^6 plt)</td>
<td>5.84 pg</td>
<td>9 pg</td>
</tr>
</tbody>
</table>
Can we predict the *Best Method*?

- This is only an In-Vitro representation
- PRP releases a cocktail of cytokines and growth factors
- Interplay with the targeted tissue still under study
- Provides positive effect but is that appropriate effect is still to be evaluated !!!!
Limitations of the study!!

- Small Sample size
- Centrifuge not temperature controlled
- Limited number of bio-markers studied
- CPDA as “anticoagulant” studied (so results cannot be extrapolated of compared with ACD using devices)
- Other “activation” methods such as Freeze-Thaw/ Only Calcium where not evaluated
- “Sterility” was not commented upon as it was not an interventional study only lab methods were evaluated
Conclusion

• This study was to highlight the variation of PRP prepared by just evaluating one of its preparatory parameter.

• Use standard terminology to uniform the results.

• The results helps to quantify the growth factors released if PRP is prepared by similar fashion.

• This study helps us to explain our clinical counter parts requiring these products that on

  “how a simple lab variation can change the dose of these growth factors and further could effect the clinical outcome.”
"The secret to finding all knowledge is to use exactly the right keywords when you google."
“Cellular Variation”

- “Plt/ WBC conc. “ of PRP has been in debate

<table>
<thead>
<tr>
<th>Constant Force (g)</th>
<th>5 min Vs 10 min</th>
<th>5 min Vs 20 min</th>
<th>10 min Vs 20 min</th>
</tr>
</thead>
<tbody>
<tr>
<td>100 g</td>
<td>0.84</td>
<td>0.52</td>
<td>0.04*</td>
</tr>
<tr>
<td>208 g</td>
<td>0.96</td>
<td>0.02*</td>
<td>0.01*</td>
</tr>
<tr>
<td>400 g</td>
<td>0.12</td>
<td>0.01*</td>
<td>0.01*</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Constant Time (min)</th>
<th>100 g Vs 208 g</th>
<th>100 g Vs 400 g</th>
<th>208 g Vs 400 g</th>
</tr>
</thead>
<tbody>
<tr>
<td>5 min</td>
<td>0.05</td>
<td>0.08</td>
<td>0.97</td>
</tr>
<tr>
<td>10 min</td>
<td>0.02*</td>
<td>0.01*</td>
<td>0.09</td>
</tr>
<tr>
<td>20 min</td>
<td>0.02*</td>
<td>0.01*</td>
<td>0.01*</td>
</tr>
</tbody>
</table>