EFFECT OF FREEZING ON THE CLOTTING AND GROWTH FACTOR PROFILES OF PLATELET-RICH PLASMA VERSUS PLATELET-POOR PLASMA: ITS IMPLICATION IN TISSUE ENGINEERING

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Abstract

Platelet-rich plasma (PRP) and platelet-poor plasma (PPP) have been widely used in regenerative medicine due to their rich growth factor content and ability to clot as fibrin/platelet gel. This study compared the clotting profiles and growth factor concentrations of PRP and PPP, before and after freezing. Forty milliliters of blood was obtained from 30 healthy volunteers, aged 20-35 years and subjected to different centrifugation conditions to produce PRP and PPP. Half of the plasma was analyzed immediately and another half was stored at -20°C for two weeks before analysis. The plasma was analyzed for platelet count, fibrinogen concentration, activated partial thromboplastin time (APTT), prothrombin time (PT) and growth factors concentration (PDGF-BB and TGF-β1). The plasma was then added with cells and clotted to produce tissue engineered constructs, during which construct clotting time and degradation rate were measured. Mean platelet concentration of PRP and PPP were (432 ± 109) x 10^9/L and (81 ± 33) x 10^9/L respectively. Fibrinogen levels was significantly higher in PPP (3.20 ± 0.8 µmol/L) than in PRP (3.15 ± 0.73 µmol/L), (p<0.01). PDGF-BB levels were higher in PRP than in PPP while no difference was observed in TGF-β1 levels in the two plasma fractions. On the other hand, PT was higher for PPP while APTT was higher in PRP and plasma construct clotting time was similar between the two plasma fractions. After freezing, platelet count and TGF-β1 concentration decreased significantly (p<0.01) while PDGF-BB concentration, construct clotting time and degradation time (construct stability) increased significantly (p<0.05). The use of PRP for tissue engineering is recommended as it has higher growth factor content yet maintaining a clotting profile within acceptable range. Although freezing of plasma compromised TGF-β1 levels and construct clotting time, it is still practicable and confers advantage like PDGF-BB activation and construct stability.

1.0 Introduction

Platelet-rich plasma (PRP) and platelet-poor plasma (PPP) have been widely used in regenerative medicine due to their rich growth factor content and ability to clot as fibrin/platelet gel. Plasma constitutes 55% of blood content and can be fractionated into platelet rich plasma (PRP) and platelet poor plasma (PPP) by centrifugation at different speed and duration. PRP and PPP as the names suggested contain enriched or depleted platelets when compared with whole blood. Platelets or thrombocytes are small, oval-shaped enucleated cell fragments derived from fragmentation of megakaryocytes with approximately 2 µm in diameter. Although lacking in nucleic content, they contain organelles and structures such as mitochondria, microtubes and...
granules [1]. Platelet activation in response to blood vessel damage, results in the formation of a platelet plug and blood clot to stop bleeding and provide hemostasis by secreting biologically active proteins [1]. In addition, platelets when activated, release growth factors that increase collagen synthesis, accelerate epithelial and epidermal regeneration, promote angiogenesis, enhance wound strength, hasten hemostasis, improve tissue regeneration, decrease dermal scarring, hasten remodeling, reduce pain and reduce infection. Previous reports showed platelet enrichments of 300% to 700% and a 7-fold increase in TGF and a 30-fold increase of PDGF in PRP compared to whole blood [2,3].

In vitro, when plasma is combined with a thrombin-calcium chloride solution, polymerization of fibrinogen occurs and fibrin clot/glue is produced. Fibrin glue has also been known to be very useful as sealant in a variety of clinical application, including procedures such as colonic anastomosis as well as in seroma prevention following soft tissue dissection [4]. Fibrin glue has a moldable property. It can act both as a 3D cell carrier and also as a source of growth factors for tissue engineering applications [5,6].

Tissue engineering aims to develop biological tissue substitutes to restore, maintain or improve tissue function. In general, there are three key elements that are important to form a tissue engineered construct, namely scaffolds or cell carrier, signaling molecules (growth factors), and cells.

In the natural process of blood clotting, fibrin is converted from fibrinogen, the major clot-forming substrate in blood plasma by the action of the enzyme thrombin. The fibrin molecules then aggregate into a clot and is then strengthened by thrombin-modified factor XIII by creating cross-links between molecules. The tensile strength and adhesive properties of fibrin glue are proportional to the concentration of fibrinogen (normal range is 2–4.5 g/L). Thus, the higher the concentration of fibrinogen, the higher its tensile strength as more cross-links are formed between [7]. There are two clotting pathways, namely the intrinsic and extrinsic pathways which lead to fibrin formation. The extrinsic pathway is measured by prothrombin time (PT), which involves measurement of factors II, V, VII, X and fibrinogen. Meanwhile, the intrinsic pathway is measured by activated partial thromboplastin time (APTT), which measures factors VIII, IX, X, XII and von Willebrand factor (vWF). The normal reference range of PT and APTT in our Medical Centre is 11-13.5 seconds and 22-34 seconds respectively.

Growth factor, one of the three vital elements in tissue engineering, is released by platelets upon activation during coagulation process. Among the growth factors released are platelet derived growth factors (PDGF) and transforming growth factor-beta (TGF-β) which are known to enhance cell proliferation and wound healing. PDGF has been identified as a major mitogen for undifferentiated osteoprogenitor cells, fibroblasts, osteoblasts, smooth muscle cells and glial cells [22]. TGF-β is a potent promoter of ECM synthesis and reinforcement, enhances chemotaxis and mitogenesis of osteoblasts precursors, and upregulates vascular endothelial growth factor (VEGF) that promotes angiogenesis and inflammatory cell recruitment [22]. TGF-β has shown its potential in clinical settings where it is involved in enhancing soft and hard tissue healing, suppressing autoimmune diseases and controlling chronic inflammatory diseases associated with fibrosis. Moreover, when conjugated with a polymer, TGF-β can also increase mechanical integrity of cultured tissue constructs [8]. PRP was defined to contain a platelet concentration of 1×10^12 platelets/L any level below that was found to be unable to enhance wound healing [9].

In the clinical setting, autologous cells and plasma from patients are derived ideally in the same sitting. The plasma will be processed and stored while the cells are culture expanded. The plasma will be thawed and used for tissue construct formation when sufficient cell number has been achieved. In our experience, repeated freeze-thaw cycle eliminates the plasma clotting property. Hence, considerations on aspects such as clotting time, growth factors concentration and construct integrity after freezing should be weighed before deciding between using PRP or PPP for the various tissue engineering applications. Hence, we aimed to determine the clotting properties (APTT, PT and clotting time of tissue engineered construct) and the concentration of the two most abundantly produced growth factors in platelets (PDGF-BB and TGF-β). The integrity of tissue engineered constructs derived from PRP or PPP was inferred by construct degradation rate, in addition, its correlation to fibrinogen and platelet concentration was determined. As plasma is usually stored frozen before usage, the effects of freezing on the above parameters were also investigated.

2.0 Materials and Methods

All procedures in the study have been approved by the Universiti Kebangsaan Malaysia Research Ethics Committee (approval code: FF-007-2010). This study was conducted on 30 healthy volunteers, aged 20-35 years in PPUKM. Our exclusion criteria for a ‘healthy volunteer’ were:

- History of anemia and bleeding disorder.
- Recent history of infection.
- Recent history (within 3 months) of critical blood loss.
- Family history of blood disorder.

All this information was obtained from a simple questionnaire that volunteers need to fill up before proceeding to blood
taking. Informed written consent was taken from all the 30 volunteers.

2.1 Blood Sampling

Aseptic hand washing was practiced prior to blood taking. Gloves were worn and disinfection was applied using alcohol swab at the site of the venepuncture. Using a sterile syringe, 40ml of blood was withdrawn from each donor and placed in five BD Vacutainer® tubes containing sodium citrate as anticoagulant, with a capacity of 9ml each.

2.2 Preparation of PRP and PPP

Blood was divided equally into two 50ml conical tubes. Centrifugation was performed within 1 hour of blood taking (CR3i, Jouan, France). In order to prepare PPP and PRP, centrifugation was set at 3300rpm for 10 minutes for the former and at 1100rpm for 15 minutes for the latter. After centrifugation, the plasma layer was carefully transferred to a new sterile container using a sterile pipette, half of which was stored at -20°C for 2 weeks while the other half was used for immediate analysis.

2.3 Platelet count

Platelet count was determined using plated processor card (Coulter LH 750, Miami, USA) in which pulses of 2-20fl were classified as platelets. The results were cumulated in a form of histogram. Platelet count is defined as the number of thrombocytes obtained from platelet histogram multiplied by a calibration factor.

Platelet count = n x 10^3 cells/µL

2.4 Coagulation profile - Activated partial thromboplastin time (APTT) and prothrombin time (PT)

Coagulation profile was measured using STA compact instrument (Stago Diagnostica, France). The STA compact is a fully automated, bench-top coagulation workstation, capable of performing clotting assay following the principal of viscosity-based detection system and involves an electromagnetic-mechanical system. The oscillation of a steel ball within the cuvette with the sample is monitored by the STA-Compact. In principal, when the plasma starts to clot, its concentration and viscosity will increase and will in turn reduce the movement of the steel ball. When the oscillation of the steel ball is stopped by clot formation, the sensor registers the time in seconds.

2.5 Fibrinogen concentration

Fibrinogen concentration was inferred from plasma clotting time. Plasma was first diluted with Owren-Koller buffer and thrombin was added to initiate plasma clotting. In the presence of excess thrombin, clotting time of the diluted plasma has a direct bearing on level of plasma fibrinogen. Measurement of fibrinogen concentration was calculated from a standard graph.

2.6 Fabrication of Tissue Engineered construct & clotting time (CT) measurement

Cryopreserved NIH 3T3 cell lines were reconstituted and used for the fabrication of tissue engineered constructs. Cells were washed and counted using haemocytometer before used. Approximately 200,000 cells were pelleted in a 15ml conical tube for making each construct. Then, 900μl of PRP or PPP was mixed with 20μl of 0.5M CaCl₂ solution to initiate clotting. Time from mixing to clot formation was recorded.

2.7 Degradation rate

After clotting, 5ml of medium (Ham’s F12-Dulbecco’s modified eagle medium supplemented with fetal bovine serum) was added to the propylethylene conical tube containing the clot. They were then placed in a CO₂ incubator (WTC Binder, Germany) and observed periodically and finally graded at 9th week. Grading was 0 for no changes seen, 1 for 1%- 50% degraded, 2 for 50%-100% degraded.

2.8 Growth factor concentration

TGF-β1 concentration was measured using Quantikine® Human TGF-β1 Immunoassay kit (R&D Systems, Minneapolis, USA) following the manufacturer’s protocol. In brief, plasma was diluted with the ‘Sample Diluent’ provided in the kit (PRP at 60x dilution, while PPP at 6x dilution). Diluted plasma was then activated by adding 1N HCl, incubated for 10 minutes before neutralization with 1.2N NaOH/0.5 M HEPES. Next, assay diluent included in the kit was added into each well of the provided 96-well plate. Activated samples were then added into the wells and incubated for 2 hours. TGF-β1 standard (provided in the kit) and the sample diluent was used reference standard and negative control respectively. After incubation, aspiration and washing, TGF-β1 conjugate was added into each well and incubated for another 2 hours. This is followed by aspiration, washing and addition of substrate solution into each well. The wells were then incubated for another 30 minutes protected from light. Lastly, the ‘Stop Solution’ was added into each well and the plate was then read at a wavelength of 570 nm using a spectrophotometer within 30 minutes upon addition of the ‘Stop Solution’.

PDGF-BB concentration was measured using Quantikine® Human PDGF-BB Immunoassay (R&D Systems, Minneapolis, USA). Briefly, ‘Sample Diluent’ was added into
each well of the provided 96-well plate. Samples were then added and incubated for another 2 hours. The wells were then washed with buffer, followed by addition of PDGF-BB conjugate and incubation for another 2 hours. The wells were washed with buffer and ‘Substrate Solution’ was added into each well and incubated for another 30 minutes protected from light. Finally, ‘Stop Solution’ was added into each well and the plate was then read at a wavelength of 570 nm using a spectrophotometer within the next 30 minutes.

2.9 Statistical Analysis

Measurements were expressed as mean ± standard error of mean. Paired t-test and Pearson’s correlation test was performed using SPSS 12.0.1 for Windows. Statistical confidence was set at P< 0.05.

Fig.1 Mean platelet concentration in PRP and PPP, before and after freezing.

PRP contains higher concentration of platelet compared to PPP, before freezing (p<0.001) and also after freezing p=0.002. After freezing, both PRP and PPP had significantly reduced platelet concentration (p<0.001).

3.0 Results

3.1 Platelet and fibrinogen concentration

Mean platelet concentration of PRP was \((432 ± 20) \times 10^9/L\), a 4-5 fold higher concentration compared to PPP \((81 ± 6) \times 10^9/L\) (p<0.01). Freezing significantly decreased the platelet concentration by 40-50% resulted in mean concentration of \((223 ± 49) \times 10^9/L\) and \((50 ± 4) \times 10^9/L\) in PRP and PPP respectively (Figure 1). Fibrinogen concentration was not significantly different between PRP \((3.15 ± 0.13 \mu\text{mol/L})\) and PPP \((3.20 ± 0.14 \mu\text{mol/L})\), p>0.05 (Figure 2). After freezing, there was statistically significant increase in fibrinogen content in PPP \((3.50 ± 0.20\mu\text{mol/L}), (p=0.04)\) but not in PRP \((3.20 ± 0.20\mu\text{mol/L}, (P>0.05)\).

3.2 Clotting profile

Mean APTT was 42.7 ± 0.6s for PRP and 40.9 ± 0.6s for PPP before freezing. After freezing, mean APTT was 43.6 ± 1.1s for PRP and 42.4 ± 0.7s for PPP (Figure 3). On the other hand, mean PT for PRP was 13.3 ± 0.1s and 13.2 ± 0.1 for PPP. Post-freezing, PT was 14.3 ± 0.4 for PRP and 13.3 ± 0.1 for PPP (Figure 4). In short, there is statistically significant increment of APTT and PT for both plasma fractions after freezing (p<0.05).

Figure 5 shows the photo of a tissue engineered construct using plasma clot as the scaffold material. CT for PRP was 356 ± 65s and 259 ± 37s for PPP before freezing. After freezing, CT for PRP was 945 ± 156s and 1390 ± 204s for
PPP (Figure 6). Freezing significantly increased CT for PRP (by approximately 9 minutes, p=0.004) and for PPP (by approximately 18.5 minutes, p<0.001).

Photos of tissue engineered constructs at different stages or degree of degradation after 9 weeks of incubation are shown in Figure 7. As shown in Figure 8, degradation rate of PRP (1.27±0.172; before freezing) reduced significantly after freezing (0.2±0.04) (p=0.009) but not significant for PPP (0.6±0.17; before freezing to 0.5±0.145; after freezing) (p>0.05). Before freezing, PPP degrades slower than PRP (p=0.006), while after freezing, PRP degrades slower than PPP (p<0.001).

### 3.3 Growth factor profile

Data in Figure 9 indicates that no difference in TGF-β1 concentration was noted in PRP (561 ± 101ng/ml) and PPP (568 ± 62ng/ml) before freezing (p=0.957). Freezing reduced TGF-β1 concentration in both PRP (215 ± 29ng/ml, p<0.001) and PPP (64 ± 16ng/ml, p<0.001). After freezing, TGF-β1 concentration in PPP was significantly lower than PRP (approximately 3 fold reduction, p=0.001).

PDGF-BB concentration was not detectable in PPP before freezing. In comparison, PDGF-BB concentration was significantly higher in PRP (0.37±0.16ng/ml, p<0.001) before freezing as shown in Figure 10. Interestingly, freezing significantly increased PDGF-BB concentration in both PRP (5.12±0.26ng/ml, p<0.001) and PPP (1.57± 0.43 ng/ml, p<0.05). Nonetheless, PDGF-BB concentration in PRP was still significantly higher than that in PPP after freezing (p<0.001).

### 4.0 Discussion

Fibrin clot has been widely used in regenerative medicine. Commercial fibrin glue is derived from allogenic human PPP whereby fibrinogen and thrombin are mixed with the addition of CaCl₂ to form a fibrin clot. Autologous fibrin clot have also gained popularity in periodontal and bone regeneration, in which bovine thrombin is used to initiate the clotting. In tissue engineering, fibrin clot acts as a cell carrier, a source of growth factor and a scaffold for making three dimensional tissue constructs.

It is well known that tissue factors, through the extrinsic pathway initiates the cascade of coagulation and were reported to be present in most cells albeit higher in endothelial cells [4]. In our Centre, we have made used of tissue factors secreted by cells instead of thrombin to initiate clotting in the fabrication of tissue engineered constructs. This has the advantage of being able to create a totally autologous construct.
Routinely, PPP is used with the notion that it clots faster. However, due to our interest to also enhance growth factor content of our constructs, differential clotting and growth factor profiles of PRP and PPP was investigated. In addition, for tissue engineering purposes, patient’s tissue specimen for cell expansion is usually harvested along with his/her blood for autologous serum and fibrin preparation in the same seating. Hence, a common practice in the Centre is to store the isolated plasma in a freezer until sufficient cell expansion has been achieved and ready for tissue engineered construct fabrication. Therefore, the effect of freezing on the clotting and growth factor profiles was of paramount concern.

In this study, PRP and PPP are prepared using a single step differential centrifugation protocol. At low centrifugation speed, the heavier molecular weight blood cells get precipitated leaving a platelet-enriched plasma fraction, PRP. By increasing the centrifugation speed, the platelets too get precipitated hence leaving a platelet-deprived plasma fraction, PPP. In practice, PPP can also be obtained by a simple filtration of PRP with 0.45 or 0.22 micrometer pore size filter.

In our study, the platelet enrichment in PRP was approximately 400-500% higher than that in PPP via the single centrifugation protocol. Rick G Smith et al. reported a normal concentration of fibrinogen in PRP and a higher concentration in PPP [10]. However, difference in fibrinogen concentration between PRP and PPP fractionated using the simple protocol was unremarkable.

Coagulation profile in terms of APTT and PT was significantly different in PRP and PPP. These findings showed that APTT and PT assays are platelet count dependent. While clotting time for plasma construct is platelet count independent as no significant difference in plasma construct clotting time was noted between PRP and PPP.

Clotting of tissue engineered construct is similar to primary plug formation in vivo. Theoretically, platelet plays an important role during the initiation phase of clotting by contact activation. In this phase, factor XII gets in contact with negatively charge surface such as phospholipids, lipoprotein and platelet, gets activated and in turn activates a cascade coagulation factors and conversion of prothrombin to thrombin [11]. In this study, no significant difference was noted between clotting time of PRP and PPP although the number of platelets significantly differs in the two. Hence, we concluded that platelet activation may not be significant event in the clotting of tissue engineered constructs. We postulate that the addition of living cells could provide the necessary tissue factors and phospholipid surface that triggered the coagulation cascade.

PRP constructs significantly degraded faster than PPP constructs. Degradation of constructs can be associated with an increase in protease production and in turn indicates greater cellular activity during tissue remodeling. In the case of tissue engineered bone or cartilage constructs using fibrin clot, osteoprogenitor and cartilage precursor cells would inevitably produce bone and cartilaginous matrix that enhances construct stability while fibrin degradation takes place [12]. In this study however, fibroblasts cell line was used and thus did not mimic the desired tissue regeneration in a tissue engineered construct.

In our findings, PRP presented similar TGF-β1 and higher PDGF-BB levels to PPP. Normal TGF-β1 and PDGF-BB concentration in whole blood was reported to range from 20ng/ml to 30ng/ml and 1.5ng/ml to 2.5ng/ml respectively, and PRP preparation from whole blood would enrich the concentration of these growth factors to about 7-30 folds [2, 3, 13]. In our study, TGF-β1 concentration in PRP (561 ±
101 ng/ml) was approximately 20-30 folds higher than that in whole blood while PDGF-BB concentration in PRP (0.37 ± 0.16 ng/ml) was 5-8 folds lower than whole blood. As PDGF-BB is a product of platelet activation, we postulate that platelet activation was minimal in our plasma preparation procedure. This was further confirmed by our findings that freezing (a process which activates the platelets) increased PDGF-BB levels in both PRP (5.12 ± 0.26 ng/ml, p<0.001) and PPP (1.57 ± 0.43 ng/ml).

In a study by Estebanell et al., centrifugation and storage process resulted in certain degree of platelet activation [17]. When platelets get activated, various changes such as platelet morphology, increase in the number of negatively charged phospholipid and secretion of granules content may lead to modification of integrin membrane glycoprotein GbIIb/IIIa at the platelet surface. This in turn leads to binding of prothrombin to the glycoprotein which facilitates its conversion to thrombin. Besides that, platelet activation also increases the expression of thrombin receptor on platelet, leading to more binding of thrombin to its receptors. These glycoproteins have also enhanced affinity to interact with fibrinogen [10]. This explains our observation whereby PRP which has a greater amount of activated platelet than PPP clotted faster after freezing.

After freezing, constructs degraded significantly slower both for PRP and PPP, and PRP constructs degraded significantly slower than PPP constructs. From this observation, we postulate that platelet activation confers some form of stabilization effect on the constructs. Consistent with this postulation, PRP constructs which have a higher amount of activated platelet degraded slower than PPP constructs after freezing.

Effect of freezing and storage

Platelet count in both PRP and PPP was significantly reduced after freezing, probably due to structural damage on the platelet after freeze-thaw cycle as reported previously by other researcher [14,15]. In our study, freezing had statistically significant but minute effect on fibrinogen concentration. This confirms the findings of Rick G. Smith et al. that the effect of freezing and storage to the fibrinogen concentration was minimal and thus irrelevant for clinical interpretation [10].

Our study illustrates a statistically significant increment of APTT and PT for both PRP and PPP, similar to the findings of Casella et al. and Alesci et al. [16,23] Casella et al. reported that increment in APTT and PT of plasma samples became statistically significant when the samples are stored at -20°C for more than 48 hours [23], whereas Alesci et al.’s study showed that the longer the plasma samples were stored frozen (for up to 4 months), the higher the value of PT and APTT would be [16]. However, in practical, an increment of less than 2 seconds for both APTT and PT during tissue construction after being in storage for merely 2 weeks, is considered negligible. Conversely, freezing and storage significantly increased clotting time for PRP and PPP. From the standpoint of construct fabrication, a shorter clotting time is preferred. It would be interesting to investigate the clotting factors that are negatively affected by freezing.

A previous study suggested that repeated freeze-thaw cycles will damage platelet membranes and thus released growth factors efficiently into the plasma [18]. This so called “cold induction activation” of platelets produces a magnitude of growth factor release statistically comparable to those pre-
treated with thrombin [21]. Though it may decrease the platelet ability to provide optimal hemostatic effectiveness, this proves to be most useful in regenerative medicine to promote healing and cell growth [21]. In our study, concentration of PDGF-BB increased significantly after freezing in both PRP and PPP, and this effect was more significant in PRP. Researchers had reported an approximate accumulation of three to five times the levels of PDGF in the supernatants of platelet components during storage [19]. In the contrary, TGF-β1 concentration decreased significantly after freezing, and the effect was more dramatic in PPP. Although not statistically significant, Gau et al reported a similar trend of decrement [21]. It was previously reported that prolonged bench set time (>3 hours) after centrifugation resulted in decreased concentration of TGF-β1 but not PDGF-BB, VEGF, or EGF [20]. However, no studies to date reported the effect of freezing on TGF-β1. Rate of freezing and thawing the plasma also affects the recovery of proteins. It was reported that when freezing is slow and thawing is fast, the activity recovery is fast. On the contrary, fast freezing and slow thawing results in slower activity recovery due to greater damage to the proteins [14]. In our study, thawing was done in room temperature and freezing in a -20°C freezer. Further optimization on the freezing and thawing conditions shall be performed in future to enhance or preserve growth factor activity.

4.0 Conclusion

The use of PRP for tissue engineering is recommended as it has higher growth factor content that is desirable for tissue regeneration while maintaining similar clotting profile as PPP. Freezing although compromises the TGF-β1 activity, confers other benefits such as construct stability and PDGF-BB release. Comparatively, post-frozen PRP is superior to post-frozen PPP because greater TGF-β1 activity is retained, more PDGF-BB is released and construct stability is greatly enhanced. The ability to freeze plasma for tissue engineering applications helps resolve many logistic issues in the clinical setting and is practicable in clinical setting.

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