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Effect of Lysed Platelet Count in Platelet Concentrates on Various Growth Factor Levels after Freeze Thaw Cycles

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Abstract : Platelet concentrate (PC) has been used as substitute to the use of fetal bovine serum (FBS) in cell culture media. However, it's use as additive in cell culture media showed inconsistent results on cell proliferation, and the inconsistent results may be due to variability of platelet counts or growth factor content. Standard protocols are lacking for the preparation of PC before it is applied as additive in cell culture media. The growth factor content of PC can be released by freeze-thaw cycles, which range from one to three cycles before use for cell culture media. This study aimed to measure base-line platelet counts and growth factor levels and compare to platelet counts and growth factor levels after one, two and three freeze-thaw cycles.

In this study, we obtained PC from Indonesian Red Cross. The PCs were aliquoted and stored at -20°C and then subjected to 1 to 3 freeze-thaw cycles. The number of platelet before and after freeze-thaw cycles were measured using Sysmex XN 2000 (Pramita Lab). ELISA method was performed to measure the growth factors' (TGF-β1, PDGF, EGF, IGF-1, VEGF) levels. Anova one-way, Spearman rank and a linear regression analysis were performed to analyze the data.

The average of platelet counts before and after 1 to 3 freeze-thaw cycle(s) are $582,833 \pm 295,764$ platelets/μL, $411,611 \pm 329,078$ platelets/μL, $273,417 \pm 351,967$ platelets/μL, and $179,167 \pm 216,904$ platelets/μL respectively. The platelet counts were decreased gradually and no significant difference among lysed platelet counts after 1 to 3 freeze-thaw cycle(s) ($p > 0.05$). Moreover, there were no correlation between platelet counts and growth factor levels ($p > 0.05$).

In conclusion, fresh and outdated PC from Indonesian Red Cross can be stored at -20°C followed by 1 to 3 freeze-thaw cycle(s) to release its growth factor contents to be used as FBS substitute in cell culture media.
Key words: platelet-rich plasma, platelet count, freeze-thaw cycle, growth factor.

Introduction

Platelet/thrombocyte concentrate (PC) in Indonesia is provided by the Blood Transfusion Unit of the Indonesian Red Cross (UTD-PMI). In clinical use, PC is expired after five days.[1] In addition to clinical purposes, PC can also be used as Fetal Bovine Serum (FBS) substitute in cell culture medium. Fetal bovine serum contains xeno-proteins, for example N-Glycolylneuraminic Acid (Neu5Gc), which can lead to immune

reactions in patients, such as chronic inflammatory reactions and anaphylactic reactions. It is also suggested that the immunogenic contamination due the the presence of FBS in cell culture medium would be difficult to be completely eliminated from the cultured cells and thus negatively influence their use in stem cell based therapy.[2,3]

Platelet concentrates are rich in growth factors such as TGF-β1, PDGF, EGF, IGF-1, VEGF, cytokines, chemokines, etc.[4] Those growth factors are required for cell culture growth and expansion. A standardized technique to release the growth factors from platelets is highly required in using PC as additive in cell culture medium. However, standard protocols are lacking for the preparation of PC before it is applied as additive in cell culture medium.

Several studies processed PC by thrombin activation,[5-7] whereas other studies used a combination of thrombin and $CaCl_2$ to activate the platelets.[8-12] In addition, PC is usually stored frozen before usage, and some studies used treatment by freezing and thawing at certain temperature to release the growth factors.[13-16] Platelet concentrate storage is most easily done at $-20^{o}C$.[17]

The growth factor content of PC can be released by mechanical stress. Mechanical stress by freeze-thaw process can lead to platelet membrane rupture and the lysed platelets then release their contents.[18] In this study, freeze-thaw process was done in $-20^{o}C$ and thawing in room temperature (RT), and the procedure ranged from one to three times before the PC was use for cell culture. The number of freeze-thaw cycles may affect the number of lysed platelets, which in turn may influence the growth factors' levels.

Platelet concentrate can be used and still rich in growth factors until over three weeks after its expiration date.[15] However, it's use as additive in cell culture medium showed inconsistent results on cell proliferation, and the inconsistent results may be due to variability of platelet counts or growth factor content.[17] Thus, it is important to determine the effect of freezing on growth factor levels and lysed platelet counts in fresh and expired UTD PMI PC.

Methods

This study was done in the Department of Biology, Faculty of Medicine, Universitas Indonesia. All procedures in this study have been approved by the Ethical Committee, Faculty of Medicine, Universitas Indonesia. Outdated and fresh human AB PC was provided by the blood bank of Indonesian Red Cross (UTD-PMI). Outdated PC was determined after its expiry date as marked on the label. PCs were aliquoted 1 ml into each cryopreservation tube (Biologix, China) then subjected to 1 to 3 freeze-thaw cycles, by freezing at $-20^{o}C$ for 30 minutes and thawing at room temperature (RT) for 15 minutes. Before and after 1 to 3 freeze-thaw cycles, platelet counts for each sample were performed. Concentrations of growth factors were determined using enzyme linked immunosorbent assay (ELISA), then all data were noted and analyzed.

Platelet count and growth factor measurements

An aliquot of sample from before and after each freeze-thaw cycles was sent to Pramita Laboratory to analyze the platelet count. The platelet counts were performed using Sysmex XN 2000 hematology analyzer, and the results are presented as Mean ± SD in a Table. Lysed platelet counts were calculated as interval between platelet counts of consecutive freeze-thaw cycles from each sample. Platelet lysis was presented as counts and percentages of lysed platelets compared to platelet counts before lysis. Growth factors (TGF-β1, PDGF-AB, EGF, IGF-1, and VEGF) were determined by commercially available ELISA kit from Sigma – USA according to the manufacturer's instructions. The absorbance was measured using Multiskan (Thermo-Scientific-USA) and Accureader Elisa reader (Metertech-Taiwan).

Statistical analysis

Statistical analysis was performed using SPSS program version 20.0 for windows and Microsoft Excel 2007. Parametric statistics (ANOVA) were used to compare platelet counts and growth factor levels between base-line, after one, two and three freeze-thaw cycles, but when the data were not appropriate for parametric test, non-parametric statistics were used. The same analysis was done to compare lysed platelet counts after one, two and three freeze-thaw cycles. Further, LSD (Least Significant Difference) was used as a post-hoc multiple comparison test. Normal distribution was confirmed using Shapiro-Wilk test. $P < 0.05$ was considered statistically significant. Spearman rank and linear regression analysis was performed to evaluate correlation between lysed platelet counts from all samples (outdated and fresh) and growth factor levels.

Results

Effect of freeze-thaw cycles on platelet counts

There were six samples of PC, four were outdated PC and two were fresh PC. Platelet counts were done to all samples before and after each freeze-thaw process. The data were presented as mean ± SD. Platelet lysis (lysed platelet count) was presented as a percentage of the interval between consecutive freeze-thaw cycles toward the previous platelet count. Base-line or before freeze-thaw cycle platelet counts showed significant difference to the platelet counts after 2 and 3 times freeze-thaw cycles ($P < 0.05$). The lysed platelet counts were 29% after one freeze-thaw cycle, 34% after two freeze-thaw cycles, and 34% after three freeze-thaw cycles (Figure 1).
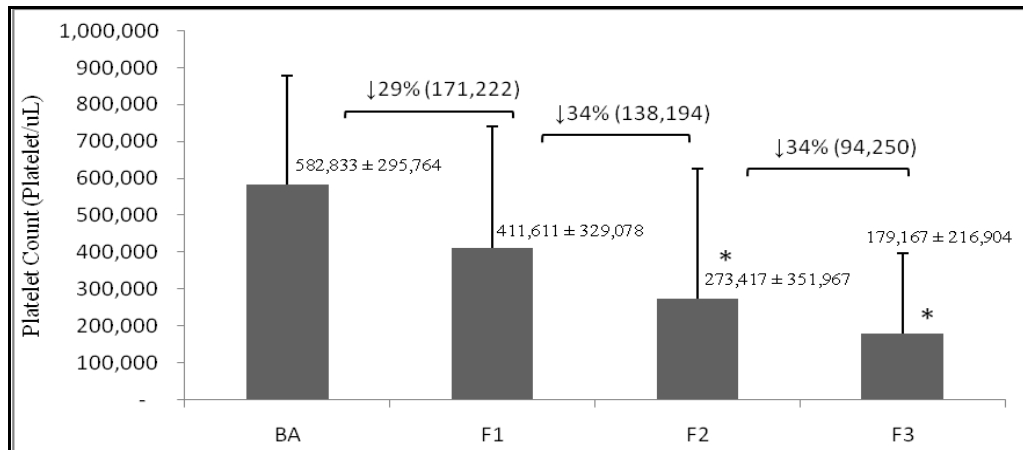

Figure 1. Percentage of lysed platelet counts (BA – F1; F1 – F2; F2 – F3).
No significant difference between lysed platelet counts after one, two and three freeze-thaw cycles (ANOVA on ln transformed data). *Platelet counts after two and three freeze-thaw cycles showed significant difference compared to baseline (ANOVA on ln transformed data) ($P < 0.05$).
Abbreviation: BA, before freeze-thaw cycles; F1, F2, F3, one, two, and three freeze-thaw cycles.

Concentration of growth factor

Growth factor levels of all samples (outdated and fresh PC) were analyzed. Measurement of growth factor level after freeze-thaw cycles were done for all samples. TGF-β1 measurement was not done for fresh PC and PDGF AB measurement before freeze-thaw process was not done for outdated PC due to inadequate well number after optimization. There were no statistically differences in PDGF, EGF, IGF-1, and VEGF levels between fresh and outdated PC ($P > 0.05$). There were no differences in TGF-β1, PDGF AB, EGF, and IGF-1 level between before freeze-thaw process and after 1 to 3 times freeze-thaw cycles (P > 0.05). However, freeze-thaw process significantly increased the level of VEGF (P < 0.05) especially one time freeze-thaw cycle, which gave the highest result of VEGF level from both outdated and fresh PC (2537 ± 1114 pg/ml and 3109 ± 241 pg/ml). Two times freeze-thaw cycles (F2) showed the highest TGF-β1 from outdated PC(31.08 ± 19.53 ng/ml), PDGF AB from both outdated and fresh PC (3254 ± 461 pg/ml and 3149 ± 170 pg/ml), and EGF level (88 ± 28 pg/ml) from outdated PC, while levels of EGF (88 ± 28 pg/ml) from fresh PC and level of IGF-1 (0.11 ± 0.27 ng/ml) from outdated PC was highest after three freeze-thaw cycles.

Correlation between lysed platelet counts and growth factor level

A linear regression analysis was used to determine whether a correlation existed between lysed platelet counts and growth factor levels. Results are given in Figure 2. There were no significant correlations between lysed platelet counts and growth factor levels (P > 0.05). IGF-1 showed the highest correlation, which was 12.4%. Thus, growth factor levels tended to be the same for all freeze thaw cycles.
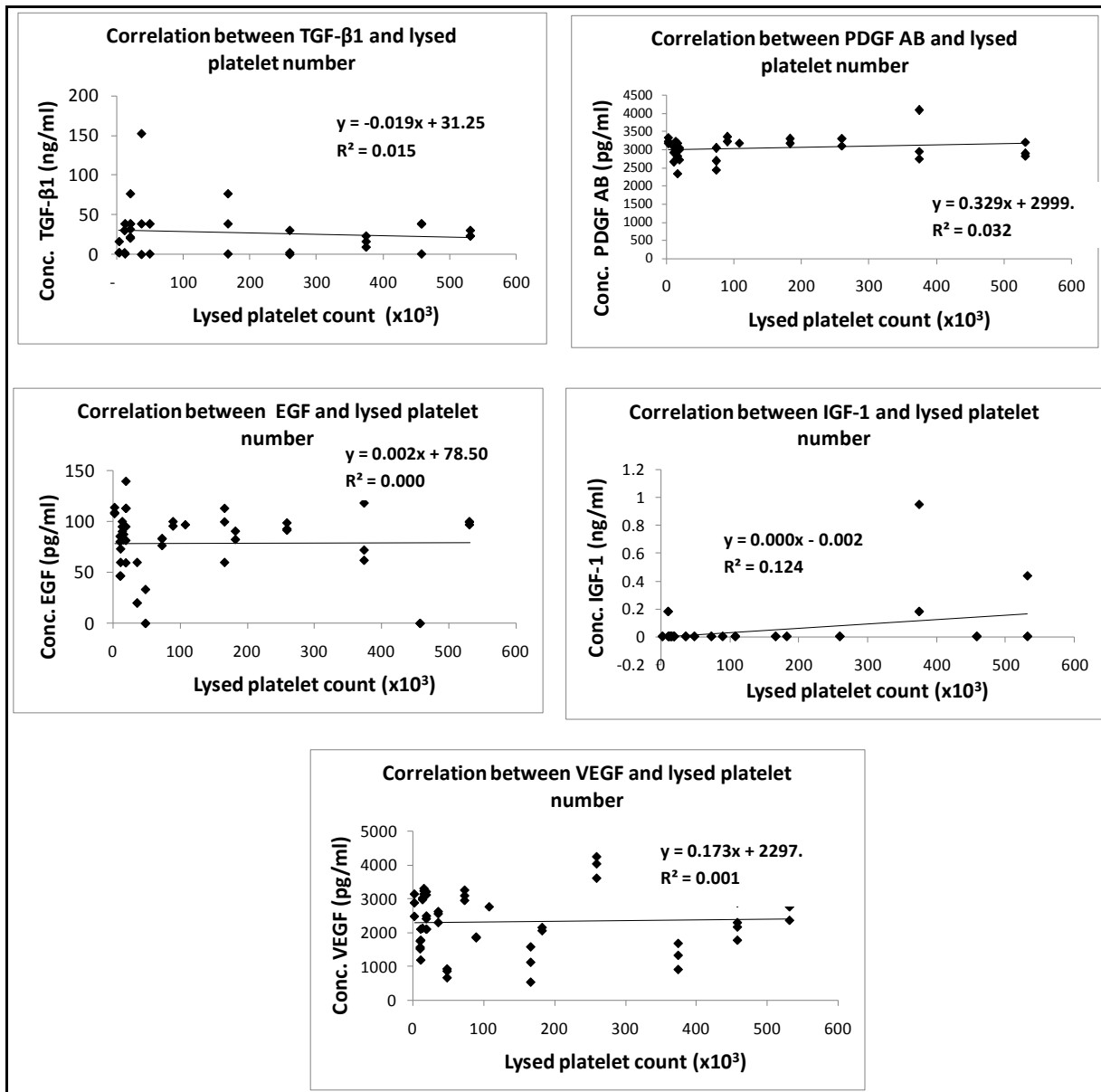
Figure 2. A linear regression analysis between lysed platelet counts and growth factor levels. The Spearman Rank test was performed and there were no significant correlation between lysed platelet counts and growth factor levels ($P > 0.05$). Almost all the values of coefficient determination (R) are below 10% which indicated that there was no correlation.

Discussion

Platelets are fragments of cytoplasm of megakaryocytes that contains mitochondria, smooth endoplasmic reticulum and granules. Granules in the cytoplasm of platelets are divided into three types, i.e. electron dense granules, α-granules and lysosomal granules. The α-granules contain a wide variety of growth factors.[19-22]

In this study, the freeze-thaw process ranged from one to three times and was intended as a method to release the growth factors from fresh and outdated PC through platelet lysis due to physical stress induced membrane plasma rupture. Platelet lysis may occur due to lost of its membrane integrity by freeze-thaw process,[18] which was preceded by changes in the components of membrane plasma such as cholesterol, triglycerides, and lipoproteins. [23] There were some phenomenas of physical behavior changes of membrane-solute-water systems during freezing.[24] The freezing process induced large mechanical stresses in the membrane and those stresses produced a range of physical deformations due to intracellular ice crystallization ("mechanical damage") so that the plasma membrane had lost the availability to sustain its function.[18, 23-30]

Based on this work, the freeze-thaw process did not lysed all of the platelets at once. So that, in the use of PC as an additive in cell culture medium and as FBS substitute it can be stored frozen and then thawed when

it is needed, and the freeze-thaw cycles can be done for several times until all the platelets are lysed. In this study, freeze-thaw cycles can be repeated until 1 to 3 times.

This study observed that two and three freeze-thaw cycles significantly reduced the number of platelet count ($P < 0.05$ (figure 1). We showed that first and second freeze-thaw cycles leaved enough platelets to be lysed in the third cycle. Our result confirmed the results of the study conducted by Hwei et al,[31] which stated that platelet count was significantly reduced by 40-50% by one freezing and thawing cycle at -20°C. Further, Baik et al,[32] stated that not all platelets were lysed by one to three freeze-thaw cycles at -70°C compared to the base-line platelet count before freeze-thaw process. The number of freeze thaw cycles that can be performed was supposed to be determined by the baseline platelet count. The more was the base-line platelet count, the more freeze-thaw cycles could be conducted.

All measurements of five growth factors (TGF-β1, PDGF AB, EGF, IGF-1 and VEGF) were performed in triplicate using commercially ELISA kits, and we found that there were no significant difference in growth factor level between in fresh and outdated PC ($P > 0.05$). Outdated platelets are still rich in growth factors and thus can be used as FBS substitute in cell culture medium and its growth factor contents were shown to be stable up to five months over expiry date at -20°C.[15, 33-34] The most important factor of platelet as supplement in cell culture medium is its growth factor contents.[35] The limitation of this study was that only two samples of fresh PC were evaluated, due to inadequate well after optimization. Repeated freeze-thaw induced degranulation of platelets, thereby releasing their growth factor content. However, the number of freeze-thaw cycles showed random results and no statistical difference between one, two, and three freeze-thaw cycles (data not shown). A previous study showed that increased number of freeze-thaw cycles could result in elevated level of some growth factors (VEGF and PDGF),[32] in contrast, another study showed significant decreased of some growth factors in platelet after freezing.[32] Growth factors are proteins, and protein stability may differ between proteins, and certain proteins can be damaged by freezing and thawing process that was proven by unstable result of some protein contents in serum or plasma sample.[23, 27]

This study showed that there was no significant relationship between lysed platelet counts and growth factor levels ($P > 0.05$). The use of one to three times freeze-thaw cycle method to release growth factor content of platelet may cause some growth factor denaturation.[27, 36, 37] In the first freeze-thaw cycle, some levels of growth factors was released and measured, and they will be denatured after second freeze-thaw cycle, but they were they were replaces by new growth factor release. Further, growth factor released in second freeze-thaw cycle might be denatured in third freeze-thaw cycle. This result was relevant because in our experiment, we found that platelets did not lysed completely after one, two and three freeze-thaw cycles (figure 1). Baik et al[32] stated that there was positive correlation between platelet count and levels of growth factors VEGF, PDGF, and EGF. In addition, there was also a positive correlation between the number of HaCaT cells in *in vitro* culture with high levels of growth factors VEGF and PDGF. However, there was no correlation between platelet number and the number of HaCaT cells in *in vitro* culture.[32] The results of this study is in line with the research conducted by Eppley et al,[38] which showed that there was no correlation between the levels of most growth factors and lysed platelet number, although there was a correlation of TGF-β1, which was at 60.41%. In our study, the highest number of coefficient determination was in IGF-1 at 12.4% (figure 2). We assumed that the absence of correlation between lysed platelet counts and growth factors levels was due to non significant difference between platelet counts after one, two and three freeze-thaw cycles. Another possibility might be due to the presence of variation in platelet counts between patients or donors.[38] The difference in platelet count of patients or donors may be affected by gender.[39] Further, the method of PC preparation and other biological contents in PC may influence the platelet count and growth factor contents and level.[32, 40, 41]

The study by Weibrich et al[42] showed that the correlation between number of platelet and growth factor levels was very weak or almost absent with coefficient correlation ≤ 0.35. In another study, Weibrich et al[39] found that there was relevant correlation among growth factors type, there was correlation between level of PDGF AB and PDGF BB and TGF-β1, PDGF BB correlated with TGF-β1. Another factor that can affect the levels of the growth factor is the age of the patient or donor, IGF-1 levels decreased by increasing age.[42] This weak correlation between the number of platelet lysis and growth factor levels also may be caused by the production of cytokines or growth factors at the cellular level that varies between individual, thus affecting the growth factor levels in each PC sample.[41, 42] In this study, platelet concentrates that were obtained from collection of some donors might have variations in the cellular levels of growth factors. It seems that the levels of growth factors in each individual can be influenced by unknown biological factors.[37, 39, 40, 42]

Conclusion

Outdated PC released comparable levels of growth factors as fresh PC. Fresh and outdated PC from Indonesian Red Cross can be stored at -20°C followed by 1 to 3 freeze-thaw cycle(s) to release its growth factor contents to be used as FBS substitute in cell culture media. Further analysis needs to be performed to confirm the use of outdated PC before it is applied as supplement in cell culture medium.

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