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Various growth factor yields in various platelet rich plasma processing methods

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ABSTRACT

Aim: To compare the growth factor contents of the various PRP processing methods to get an insight on the difference in growth factor yield from the various processing methods. **Experimental:** PRP before and after expiry dates was collected from Indonesian Red Cross. PRP was processed by various methods to release the growth factors, and growth factor levels were measured. Growth factor levels between various kinds of processing were compared using ANOVA and those from before and after expiry date samples were compared using t test. **Results:** Comparison of thrombin activation, one, two, and three freeze thaw cycle(s) of all samples revealed no significant difference in PDGF-AB, EGF, and IGF levels ($p > 0.05$). However, there were significant differences in TGFβ and VEGF levels between thrombin activation and freeze thaw cycles. Comparisons between samples of before and after expiry date revealed no significant differences in all growth factor levels. **Conclusion:** One, two, and three freeze thaw cycle(s) yielded no significant difference in most growth factor levels.

KEYWORDS

Thrombocyte concentrate;
Platelet rich plasma;
Growth factors;
Freeze-thaw;
Thrombin activated.

INTRODUCTION

Processed trombocyte concentrate or platelet rich plasma (PRP) have been used as fetal bovine serum (FBS) substitute in various stem cell culture studies, and have shown good results using various PRP concentrations[1-10]. There are various methods to get the PRP and also various processing methods to release the growth factors from the PRP. Some studies used various freeze-thaw cycles[1-4, 6,9], while others used thrombin[3- 5,8] or thrombin/Ca Cl2 activated methods[7, 9,10]. A systematic review on growth factor measurements of

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the various processing methods showed wide ranges of growth factor levels. Moreover, platelet counts of the various growth factor measurements were very variable, ranging from 188 to 15,000 x 10^6 platelets/mL that were reflected by the use of various concentrations of PRP in culture medium in various studies[11].

Therefore, this study compared the growth factor contents of the various PRP processing methods to get an insight on the difference in growth factor yield from the various processing methods.

EXPERIMENTAL

This study was done in the Department of Biology, Faculty of Medicine, Universitas Indonesia, in November 2013. Ethical clearance for this study was obtained from the Ethical Committee, Faculty of Medicine, Universitas Indonesia. Human AB PRP was obtained from Indonesian Red Cross. PRPs before and after expiry dates were collected. Further, the PRPs were processed by various methods to release the growth factors, and growth factors were measured. Before processing, platelet counts for each sample were measured, and all data were noted and analyzed.

Platelet count

An aliquot of each sample was sent to Pramita Laboratory to analyze the platelet count in each sample. The platelet counts were done using Sysmec XN1000, and the results for each sample were noted and presented as a table.

Processing of PRP and growth factor measurements

Processing of the PRPs were done by one, two, and three freeze-thaw cycles at -20°C, and CaCl2/ thrombin activation (2.5% CaCl2 and 3 IU human thrombin: PRP= 1:1). The measured growth factors were transforming growth factor β (TGF β) using human TGF β ELISA kit (Sigma RAB0460-IKT), platelet derived growth factor AB (PDGF-AB) using human PDGF-AB ELISA kit (Sigma RAB0396-IKT), vascular endothelial growth factor (VEGF) using human VEGF ELISA kit (Sigma RAB0508-IKT), epidermal growth factor (EGF) using human EGF ELISA kit (Sigma RAB0149-IKT), and insulin-like growth factor 1 (IGF-1) using human IGF-1 ELISA kit (Sigma RAB0228-IKT). Growth factor measurements were done in triplicate according to the manufacturer's instruction manual. Measurements were done on all processed PRPs.

Data collection and analysis

Data of growth factor levels before processing and for each processing were noted, tabulated, and the means and standard deviations were computed. Growth factor levels were compared to platelet count of each sample. The difference in growth factor levels between the various processing were analyzed using ANOVA or Kruskal Wallis test, when the data were not appropriate for ANOVA. Further, for each processing method, the differences in growth factor levels between over and before expiry date samples were analyzed by independent sample t test or Mann-Whitney test, when the data were not appropriate for t test.

RESULTS

We processed six packs (samples) of PRP, four were after expiry date, and two were before expiry date. Platelet counts for each sample can be seen in TABLE 1, and the mean ± standard deviation was 582,833 ± 295,764. We did the processing and measurements for pack one and two, followed by pack three and four, and finally pack five, and six on three successive days. For each measurement, a standard curve for each growth factor was made, and growth factor concentrations were interpolated on the respective standard curves.

TABLE 1 : Platelet count in each sample

Sample	Platelet/µl
S-1	283,000
S-2	610,000
S-3	1,013,000
S-4	836,000
S-5	296,000
S-6	459,000

s-= sample-

For freeze thaw processing, growth factors of most packs were measured, but for CaCl2/thrombin activation, only two to four packs were measured, and were not done for PDGF-AB. Moreover, TGF β processing

and growth factor measurements were only done on sample one to four, due to insufficient well number after optimization. Overall growth factor measurements (replication) that were done on all PRP samples can be seen in TABLE 2.

TABLE 2 : Means and standard deviations of various growth factors in various processing

Growth factor	Processing	n	Mean	SD
TGF-beta-1 (ng/ml)	TA	12	101.02	77.72
	FT-1x	12	25.14	22.72
	FT-2x	12	31.08	19.53
	FT-3x	12	28.32	42.03
PDGF-AB (pg/ml)	FT-1x	10	3,002.68	300.09
	FT-2x	11	2,926.46	259.89
	FT-3x	11	3,206.30	347.51
EGF (pg/ml)	TA	6	93.25	119.91
	FT-1x	16	79.32	35.13
	FT-2x	17	86.57	23.68
	FT-3x	17	70.51	41.13
IGF-1 (ng/ml)	TA	6	0.00	0.00
	FT-1x	18	0.00	0.00
	FT-2x	17	0.05	0.12
	FT-3x	17	0.08	0.23
VEGF (pg/ml)	TA	6	1,489.97	1,197.60
	FT-1x	16	2,671.48	1,001.65
	FT-2x	17	2,282.17	659.09
	FT-3x	17	2,011.94	831.84

TA= thrombin activation, FT-1= one freeze thaw cycle, FT-2= two freeze thaw cycles, FT-3= three freeze thaw cycles, n= replication, SD= standard deviation

Growth factor levels

Means and standard deviations of all growth factor levels for each processing method can be seen in TABLE 2. All data met the requirement for ANOVA, except for TGF β and IGF-1 data. Square root transformation of TGF β data showed normal distribution, and ANOVA test was performed. However, most of IGF-1 data showed zero value, and only data from sample 3 and 4 showed a value between zero and one. Therefore, the data had no normal distribution, and comparison of IGF levels between various processing was done by Kruskal Wallis test. The lowest IGF level above zero was 0.1796, and the highest was 0.9527ng/ml.

Comparison of thrombin activation, one, two, and three freeze thaw cycle(s) of all samples revealed no significant difference in PDGF-AB, EGF, and IGF-1 levels ($p > 0.05$). However, there were significant differences in TGF β and VEGF levels (TABLE 3). Further, comparisons between samples of before and after expiry date for all kinds of processing revealed no significant differences in PDGF-AB, VEGF, and EGF levels using independent t test, and no significant difference in IGF level using Mann-Whitney test ($p > 0.05$).

TABLE 3 : p values of TGF β and VEGF level comparison between various processing

TGFβ **				
	TA	**FT-1x**	**FT-2x**	**FT-3x**
TA		0.000*	0.003*	0.000*
FT-1x	0.000*		0.476	0.992
FT-2x	0.003*	0.476		0.470
FT-3x	0.000*	0.992	0.470	
VEGF				
	TA	**FT-1x**	**FT-2x**	**FT-3x**
TA		0.007*	0.006*	0.218
FT-1x	0.007*		0.204	0.034*
FT-2x	0.006*	0.204		0.376
FT-3x	0.218	0.034*	0.376	

TA= thrombin activation, FT-1= one freeze thaw cycle, FT-2= two freeze thaw cycles, FT-3= three freeze thaw cycles, *= significant difference ($p < 0.05$), **= analyzed on transformed data (square root)

DISCUSSIONS

For thrombin activation, TGF β and VEGF level in our study was a little higher compared to the study of Cho et al (2011), which measured TGF β, PDGF-AB (that we did not measure), IGF-1 and VEGF, and found that TGF β and VEGF level was 89.8 and 1 ng/ml respectively, from a platelet count of 1,666,000 ± 402,000/ µl. However, our IGF-1 level was zero, compared to 87ng/ml[5]. This discrepancy might be due to the thrombin activation in Cho et al study that used a final concentration of 100 U of bovine thrombin/ml, while we used a combination of human thrombin/CaCl2.

Rauch et al (2011) used three freeze thaw cycles at -20°C and found that TGF β, PDGF-AB, VEGF, EGF, and IGF-1 level was 768.9 ±395.0, 84.19 ±34.86, 7.04 ±6.72, 18.34 ±6.76, and 15.20 ±5.34, respectively from up to 15,000,000 platelets/µl[2], which was

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comparable to our results for TGF β, PDGF-AB, and EGF level that was computed at the same platelet counts.

Kakudo et al (2008) used thrombin activation using 0.5M CaCl2/thrombin: PRP= 1:1 and found that TGF β level was 96.38 ± 16.77[10], compared to 101.2 ± 77.72 in our study.

Kurita et al (2008) used 200 U thrombin to activate 188,000 platelets /µl from 100 ml whole blood, and found that EGF level was 265 pg/ml[8], much higher than our result that was measured from more platelet count. Comparing studies might show variable results, as the used procedures were not exactly the same. Therefore, there were discrepancies between the results.

In our study, most IGF level data showed zero values, except for data from sample 3 and 4, and might be due to the platelet counts in sample 3 and 4 were much more higher compared to other samples (TABLE1). We concluded that IGF levels in sample 1, 2, 5, and 6 were below the detection limit of the kit, which was used in this study (human IGF-1 ELISA kit [Sigma RAB0228-IKT]), and thus showed zero levels. The minimum detectable dose of IGF-I that can be detected using the ELISA kit is around 0.2ng/ml[12], which was corroborated by the lowest detectable level that was 0.1796.

In this study, TGF β level in thrombin activation was significantly higher, while VEGF level was significantly lower compared to freeze thaw cycles. Most growth factor levels showed no significant difference between the three kinds of freeze thaw cycles, except in VEGF levels, which showed a significant lower level in three freeze thaw cycle compared to one freeze thaw cycle (TABLE 2 and 3). We assumed that the first, second, and even the third freeze thaw did not lyse all, but only part of the platelets, while freezing and thawing might cause damage to the growth factors. Damaged growth factors were replaced by release of fresh growth factors from the lysed platelets at subsequent freeze thaw cycle, which make the growth factor levels to be relatively constant, though there were a wide range in platelet counts. Further, some growth factors might be more resistant to freezing and thawing, thus the variation in the various results in growth factor levels.

For culture medium supplement, as FBS substitute, we suggest to use froze thawed PRP, as one, two or three cycles showed no significant difference in most growth factor levels. Moreover, the freezing can be used as a means of storage.

CONCLUSION

One, two, and three freeze thaw cycle(s) yielded no significant difference in most growth factor levels.

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