ORIGINAL ARTICLE

Effect of Freeze-dried Homologous Platelet-rich Plasma Preparation and Sterilization Methods on Levels TGF-β

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ABSTRACT

Introduction: Studies showed that Homologous platelet-rich plasma (H-PRP) is superior to Autologous PRP (A-PRP) which contained larger amount of Transforming growth factor-β (TGF-β) which applicable to numbers of clinical application, but PRP in general had a disadvantage of a short shelf life and impracticality . Our study uses H-PRP, as a by-product from transfusion processes conducted in the Indonesian Red Cross (PMI). The H-PRP had passed transfusion-related infection screening and were rarely used. Sedimentation (S) and single centrifugation (SC) methods were used to produce H-PRP. The H-PRP were freeze-dried (FD) to maintain the Transforming growth factor-β (TGF-β) content more durable and made it easier to apply. FD-H-PRP underwent ɣ-irradiation Sterilization process. TGF-β plays an important role in the wound healing process and is the most abundant growth factor in platelets. This study explored the effects of FD and ɣ-irradiation sterilized H-PRP on TGF-β1 levels. **Methods:** Total of 40 FD-H-PRP samples were used, which were divided into 10 FD-H-PRP(S), 10 FD-H-PRP(S), 10 FD-H-PRP(SC), and 10 FD-H-PRP(SC) that were exposed to 20, and 25 KGy ɣ-irradiation doses, respectively. Enzyme-linked immunoassay test were used to measure TGF-β levels. A nonparametric test was used for statistical analyses. **Results:** TGF-β levels are significantly higher in H-PRP samples generated using the S method than those using the SC method and in samples with a ɣ-irradiation dose of 25-KGy than those with a 20-KGy ɣ-irradiation dose. **Conclusion:** Lowercentrifugation force increases the platelet count and its growth factors, which proved that the S method is superior to the SC method. In contrast, higher ɣ-irradiation dose administered to platelets produce higher TGF-β levels.

Keywords: Sedimentation, Single centrifugation, FD-H-PRP, ɣ-irradiation, TGF-β

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INTRODUCTION

Platelet-rich plasma (PRP) therapy currently uses A-PRP, which causes the bioactivity and consistency of the PRP growth factor to vary, making it challenging to use (1). Although PRP contains several growth factors, such as insulin-like growth factor (IGF), platelet-derived angiogenesis factor, platelet-derived growth factor (PDGF), vascular endothelial growth factor (VEGF), fibroblast growth factor, and transforming growth factor-β (TGF-β), A-PRP is challenging to use in emergency care and its manufacturing process is complex and takes a long time (2). These growth factors can stimulate cell formation, matrix remodeling, and formation of new blood vessels (3); accelerate wound healing of the tissue; and stimulate bone growth (4) so that development carried out for this therapy, in the form of homologous PRP (H-PRP). There are situations, such as in patients with abnormalities, who lack platelet function, and who are unwilling to have their blood drawn, where A-PRP is not the correct choice of treatment. In such cases, homologous PRP (H-PRP) can be used as a treatment option (5). According to Ince's study (6), H-PRP therapy in patients with androgenic alopecia (AA) had outstanding results compared with A-PRP therapy. In H-PRP therapy, patients with AA were checked the hair growth increased and the area of baldness of patients receiving H-PRP therapy was less compared with that of patients receiving A-PRP therapy. This statement is also supported by Prabhu (7), who treated patients with chronic nonhealing ulcers using H-PRP that resulted in patients receiving H-PRP having good wound healing. The success of this therapy depends on the

state and levels of growth factors in H-PRP. H-PRP used in this study is PRP obtained from filtered donor blood to obtain excellent quality PRP and growth factors.

The H-PRP is a by-product from the Indonesian Red Cross (PMI), that is not further processed into more beneficial therapeutic products. H-PRP preparation at PMI uses SC and S methods, which refers to the production of PRP without centrifugation or filtration (8). High-speed centrifugation process that uses electricity is not required to separate whole blood into blood components because the passive S method can compete and in some ways better than the conventional method, which are more inefficient in terms of fund, storage, time, and quality components (9). According to Schmolz, the supernatant produced from the S method contains concentrated platelets, which activates growth factors such as IGF-1, PDGF, TGF-β, and other growth factors, which are primarily active in the wound healing process (10).

H-PRP could be Freeze Dried to have a longer storage time and maintain its growth factor levels equivalent to those of fresh PRP (11), resulting in freeze-Dried homologous platelet-Rich plasma (FD-H-PRP), which can be used in emergency cases. To ensure that FD-H-PRP can be maintained for a longer time, it is necessary to use the sterilization method to make sure the samples are sterile for a prolonged time while its growth factors remain stable. There are various types of sterilization methods, such as ethylene oxide, critical CO2, and ɣ-irradiation. In this study, ɣ-irradiation was used due to its advantages over other sterilization methods, both in terms of penetration and the sterilization results (12). The ɣ-irradiation sterilization does not cause a significant temperature increase, time efficient, does not require post radiation quarantine, and produce no residues. The process can be performed in a high level of precision due to the radiation time could be variably controlled (13). Based on the research of Kusumadewi (14), ɣ-irradiation exposure increases the TGF-β level in FD-H-PRP, with the optimal dose of 20–25 KGy, and using the γ-irradiation sterilization method with a dose of 25 KGy guarantees sterile PRP for 6 months if stored at −20°C (15).

Blakytny (16) stated that the most abundant growth factor in platelets is TGF-β1, which plays a major role in the wound healing process. Additionally, TGF-β1 protects cells from gamma irradiation exposure, reduces DNA damage, and improves cell survival (17).

MATERIALS AND METHODS

This particular experiment studied the difference of TGF-B level in Homologous Platelet-rich plasma (H-PRP) produced by Sedimention (S) compared to Centrifugation (SC) method that were then sterilized by several doses of ɣ-irradiation (20 and 25 KGy). This research was ethically approved by the Faculty of Dentistry's Ethics Committee of Gadjah Mada University, Indonesia with registration number No.00588/KKEP/FKG-UGM/EC/2021.

Blood Samples

The H-PRP samples were O+ type blood taken from the Indonesian Red Cross (PMI) that had passed transfusion-transmissible infection screening, such as hepatitis B, hepatitis C, HIV and syphilis, were then divided into 4groups; FD-H-PRP(S), FD-H-PRP(S), FD-H-PRP(SC), and FD-H-PRP(SC) that were exposed to 20, and 25 KGy ɣ-irradiation doses respectively with each group comprised of ten samples.

H-PRP production

H-PRP production using the passive S method involves separation of the red blood cells from plasma by placing the blood bag in a standing position within blood refrigerator for 8 hours while allowing the cells to precipitate by gravitational effect. Separated blood components, achieved by placing the blood bag in the plasma extractor, are transferred to the spliced satellite pouch and then drained the plasma into the satellite pocket until approximately the plasma level in the main pocket are about 3 cm from the upper layer of the concentrated red blood cells. The connecting hose between the main and satellite bags were sealed with an electric sealer and then the connecting hose were cut.

Centrifugation method of H-PRP production is by placing the whole blood in the centrifuge machine (RC-Kubota). Firstly, the blood samples were balanced in the centrifuge bowl. Samples were centrifuge to speed of 3000 × g at 4°C for 10 min. Afterward, the centrifuge bowl is carefully lifted to avoid the remixing of blood components, pinch and open the connecting tube between the main and satellite bags. Plasma were drained into the satellite bag until it reach the level ±3 cm from the surface of the upper layer of concentrated red blood cells then the connecting hose were sealed with an electric sealer. Afterward the connecting hose were cut and the volume were documented on the label. Plasma are then stored in a blood refrigerator at 2°C–6°C.

Freeze-drying Process

After PRP samples are obtained; the samples were frozen at −40°C for 7 days. The samples were then underwent freeze-drying process that lasted for 3 days using a freeze-drying machine to produce Frezee Dried-PRP (FD-PRP) which afterwards continued with smoothing procedure (mesh) by grounding the FD-PRP using a mortar in a laminar flow hood station. The smoothed FD-PRP were then filtered with a 60-mesh sieve and were placed into microtubes.

Sterilization process and analysis

The packaged samples were then sterilized by γ irradiation by exposing the samples to γ-ray radiation of 20 and 25 KGy in increments of 5 starting from 5 to 25 KGy.

Analysis of TGF-β level using the enzyme-linked immunosorbent assay (ELISA) method were performed according to the manufacturer's instructions included in the kit.

RESULTS

This study was conducted using two blood group O blood bags taken from PMI, of which one blood bag was obtained using the S method and the other blood bag was obtained using the SC method. FD-H-PRP samples were further divided into four groups, with each group consisting of 10 samples. The first group of FD-H-PRP samples was obtained using the SC method and given γ-irradiation of 20 KGy, the second group of FD-H-PRP samples was obtained using the SC method and given γ-irradiation of 25 KGy, the third group of FDH-PRP samples was obtained using the S method and given γ-irradiation of 20 KGy, and the fourth group of FD-H-PRP amples was obtained using the S method and given γ-irradiation of 25.

In Figure I, the data showed that most of the mean values of TGF-β levels have increased with the increasing dose of γ-ray radiation, and the TGF-β level of FD-H-PRP using the S method is higher than that using the SC method. The highest TGF-β level was 9.66 ng/mL at a dose of 25 KGy using the S method.

Figure 1. Chart of the average levels of TGF-β1 compare to γ-irradiation dose of 20.25 KGy for both manufacturing methods (ng/mL)

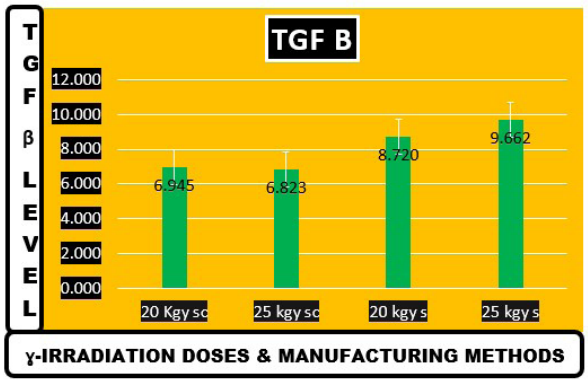


Figure 1 : The data showed that most of the mean values of TGF-β levels have increased with the increasing dose of γ-ray radiation, and the TGF-β level of FD-H-PRP using the S method is higher than that using the SC method. The highest TGF-β level was 9.66 ng/mL at a dose of 25 KGy using the S method.

Table I shows that TGF-β1 levels increased with increasing doses of γ-irradiation and FD-H-PRP with higher TGF-β levels using the S method than those using the SC method. Followed by the test of normality with the Shapiro–Wilk test, which was used because the sample size is <50. Data are considered normally distributed if the p value is > 0.05. Table II show the results of the normality test for γ-irradiation dose data.

Table I : Average and standard deviation of TGF-β1 levels by method group and γ-irradiation dose (ng/ml)

γ-Irradiation dose group	Sample (n)	Mean and Standard Deviation $\bar{X}$ ±SB
SC 20 KGy	10	6,95 ± 0,29
SC 25 KGy	10	6,82 ± 0,31
S 20 KGy	10	8,72 ± 0,80
S 25 KGY	10	9.66 ± 0,90

Table II : The results of the normality test for TGF-β1 levels on the method and dose of-ray radiation using the Shapiro-Wilk test.

	Shapiro Wilk			Note
	Statistic	df	Sig.	
Gamma-ray Dose	0,882	40	0,001	Not normally distributed

The normality test results in Table II show a significance value of 0.001 (p < 0.05), which showed that the data are not normally distributed, which indicates that further homogeneity test is needed. Levene's test was used for the homogeneity test.

The results of the Levene test in Table III showed a significance value of 0.001 (p < 0.05). This indicates that the data distribution in the group is not homogeneous. The data results show that it is not normally distributed and not homogeneous, then it is followed by a nonparametric test using the Kruskal–Wallis method. The TGF-β levels at each dose of γ-irradiation were compared using the Kruskal–Wallis test.

In Table IV, p = 0.000 (p < 0.05) indicates a significant difference in TGF-β levels in the FD-H-PRP sterilization in each group of the preparation method and the

ɣ-irradiation doses, the SC 20 KGy, SC 25 KGy, S 20 KGy, and S 25 KGy. The statistical test was continued using the Mann–Whitney test to determine the difference between each ɣ-irradiation dose.

Table III : The results of the homogeneity test of TGF-β1 levels on the method and dose of-ray radiation with the Levene test

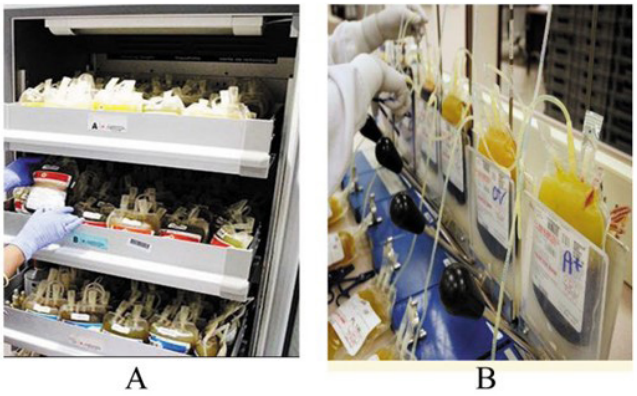
Test of Homogeneity of Variances					
	Levene Statistic	**df1**	**df2**	**Sig.**	**Note**
TGF-β Doses	6,351	3	36	0,001	Not Homogeneus (Heterogen)

Table IV : Kruskal Wallis test results TGF-β1 levels on the method and dose of-ray radiation

	Gamma-ray Doses	**Sig.**	**Note**
TGF-β	20 KGy (SC)	0,000	Significant
	25 KGy (SC)		
	20 KGy (S)		
	25 KGy (S)		
	Total		

Table V showed that the TGF-β1 levels in almost all groups of preparation methods and-ɣ-irradiation doses were significantly different ($p < 0.05$).

The results of this study showed that FD-H-PRP can be used for a prolonged time and at any time, including in emergency cases, and can accelerate wound healing, tissue regeneration, and bone formation processes.



A B

Figure 2 : The preparation for H-PRP sedimentation method. A) Placing blood bags in standing position in the blood refrigerator for 8 hours allowing the cells to precipitate by gravitational effect. B) Separation of blood components by placing the blood bag carefully in the plasma extractor.

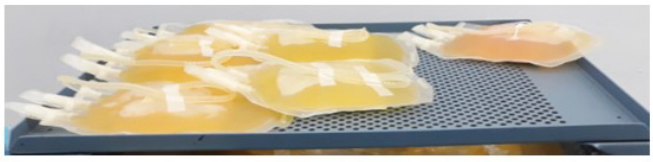
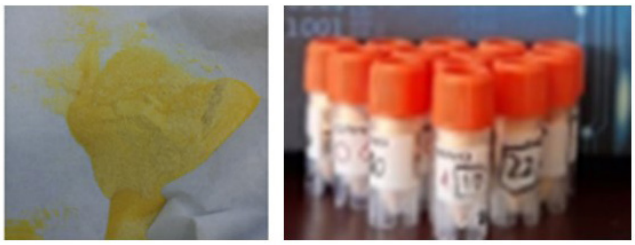


Figure 3 : The Homologous platelet-rich plasma in a the satellite blood bag, that has been cut off and put on a moving tray to prevent PRP damage.



A B

Figure 4 : The Freeze-dried Homologous Platelet-Rich Smoothing and packaging. A) Freeze-dried Homologous Platelet-Rich after mesh process. B) The freeze-dried H- PRP packaged in microtubes.

Table V :The results of the Mann-Whitney test for TGF-β1 levels against ɣirradiation dose

ɣ-ray Radiation	**SC 20 KGy**	**SC 25 KGy**	**S 20 KGy**	**S 25 KGy**
SC 20 KGy		0,070	0,000*	0,000*
SC 25 KGy			0,000*	0,000*
S 20 KGy				0,028
S 25 KGy				

Information : * = significant (p<0,05)

DISCUSSION

The results of this study showed that the TGF-β level of the PRP using the S method is higher than that using the SC method for both 20- and 25-KGy doses. This happened because reducing the relative centrifugation force (RCF) will increase platelet and leucocyte counts and their growth factors, especially TGF-β (18). The centrifugation process is remarkably influential on the cells and growth factors (19). Gifford (9) states that to separate whole blood into high-quality components, high-speed and electric centrifugation is not required because the passive S method can compete with and, in some ways, is superior to the centrifugation method, and platelets free centrifugation, produces a large number of the essential mediators for wound healing such as VEGF, PDG, EGF, TGF-β, with the total levels of the mediators in platelets are significantly higher compare to those found in plasma in the same donor (10).

However, the SC method did not found to have a statistically significant difference between doses of 20 KGy and 25 KGy by the SC method. Kusumadewi (14) stated that the optimal dose for sterilizing FD-H-PRP is 20–25 KGy and showed that the TGF-β levels at doses of 20 and 25 KGy had no significant differences. In terms of sterility and maintaining the optimum condition of the ingredients of materials and health products, the gold standard for sterilization is 25 KGy (20)

At a dose of 25 KGy and 20 KGy with the S method, the TGF-β FD-H-PRP dose is 25 KGy greater than the TGF-β FD-H-PRP level at a dose of 20 KGy, and this is likely due to the greater radiation dose exposure. Exposure to ɣ-ray will cause platelets to produce more TGF-β as a mechanism of trying to protect the cells from more significant DNA damage, which promotes cell survival (17). TGF-β1 plays an important role in protecting cells from damage and stimulating DNA repair activity (21), and according to Blakytny (16), TGF-β level increases because TGF-β is the most abundant growth factor contained in the platelets.

From this study, many benefits can be taken from the FD-H-PRP material using the S method, which can be used as an alternative preparation material during an emergency with an optimal growth factors, incredibly high TGF-β1, where this factor is believed to be very important for tissue regeneration and wound healing (4, 22, 23, 24).

CONCLUSION

Reducing RCF will increase the platelet count and its growth factors. Hence, the S method is superior to the SC method in generating PRP. A 25-KGy irradiation dose resulted in a higher yield of TGF-β than that of 20 KGy. Thus, higher ɣ-ray radiation dose exposure to the platelets upregulates TGF-β1 production to protect cells from DNA damage.

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