Dose dependent effect of γ-irradiation sterilization on transforming growth factor beta levels in freeze-dried homologous platelet-rich plasma

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Abstract
Platelet-rich plasma (PRP) has gained immense importance in past few years. It contains plenty of growth factors, the most abundant of which is transforming growth factor-β (TGF-β). Autologous PRP (APRP) is the most commonly used type of PRP; however, it cannot be given to all patients owing to several limitations like abnormal platelet function. As an alternative, Homologous PRP (HPRP) has been developed and freeze-dried HPRP (FDHPRP) ensures an extended shelf-life. FDHPRP samples are generally sterilized using γ-irradiation, but the use of γ-irradiation breaks the chemical bonds and results in DNA damage. The aim of the present study was to determine the effect of γ-irradiation at different doses on the levels of TGF-β in FDHPRP.

In this study, PRP was developed from blood obtained as by-product from Palang Merah Indonesia(PMI)and used for FDHPRP known to be beneficial for tissue and bone regeneration . The PRP samples were prepared according to the standard PMI procedures. Further, the samples were freeze-dried, meshed, and packaged. Packaged samples were exposed to γ-irradiation at different doses, and TGF-β levels were measured using enzyme linked immunosorbent assay (ELISA). Treatment of FDHPRP samples with γ-irradiation resulted in an increase in the levels of TGF-β A γ-irradiation; dose-dependent variation in TGF-β levels was reported/observed. This might be contributed by the ability of TGF-β to modulate cell sustainability and confer resistance to high-dose of γ-irradiation, thus protecting the cells from DNA damage.

Keywords: Freeze-dried, Homologous, PRP, γ-irradiation, TGF-β, Sterilization dose.

Introduction

Platelet-rich plasma (PRP) is the most current breakthrough in the fields of dentistry and medicine. Many studies have been performed to optimize and enhance its biological properties. PRP is characterized by the presence of numerous growth factors, the most abundant of which is TGF-β. In general, TGF-β cytokine plays several important roles in the human body including wound healing[4], stimulation of cell proliferation and also contributing to an early influx of cells[13]. PRP can stimulate new cell growth and vascular formation, and also enhances matrix modeling[8]. PRP shows several biological properties like enhancement of wound healing in the skin, gingiva, mucosa, connective tissue, and tendon.

Besides these, it can also stimulate bone growth[15]. It finds wide applications in various procedures in medical and dental therapy such as orthopedic and gastrointestinal surgery, plastic surgery, burn therapy, cosmetics, oral and maxillofacial surgery and periodontics[1]. Particularly in dentistry, it is used in periodontal surgery, gingival recession treatment, tissue regeneration and as a bone graft material[11].

Autologous PRP is the most widely used PRP therapy till date. However, plasma sources vary among individuals resulting in variation in the bioactivity of growth factors present in PRP. The production of clinically applicable PRP is a complex and lengthy/tedious process. Therefore, it is quite challenging to keep it handy in case of emergencies[10]. Freeze-dried platelet-rich plasma (FDPRP) has been developed as an alternative to simplify the manufacturing process and shorten the application time. This method offers several advantages over APRP like extended shelf-life and maintenance of growth factors at the levels equivalent to those observed in case of fresh PRP, even after eight weeks of storage[20]. Kieb et al[12] reported that the TGF-β concentration remained unaffected by the use of powdered FDPRP method.

It has been observed that some of the patients cannot use their own blood for APRP. Some patients are not willing to have their blood drawn while some suffer from platelet function anomaly compromising the outcome[18]. These problems can be overcome by the use of HPRP. In order to keep HPRP stable for a longer time, FDPRP is utilized. Since the main purpose of this method is to preserve PRP, a particular sterilization technique is required. Crawford et al[5] reported that the freeze-dried method cannot inactivate retroviruses e.g. Human Immunodeficiency Virus/HIV and Hepatitis C virus.

Sterilization using γ-irradiation is commonly used for terminal sterilization of freeze-dried tissues. This method is characterized by good/better penetration ability, and it works effectively on fully wrapped tissues as well. However, the

use of this method within the standard dose limits is less effective for viruses and bacterial spores. The adverse effects of γ-irradiation include break down of chemical bonds, decrease in the tensile strength of collagen, and shrinkage of irradiated collagen sponge[16]. It has been accepted/shown that the effects of γ-irradiation on soft tissues and bone allografts are dose-dependent, and higher doses of γ-irradiation result in greater losses in the biomechanical integrity of the tissue[3].

Harrel et al[9] found that γ-irradiation could decrease the levels of growth factors depending on the intensity of γ-irradiation applied. In another study by Paolin et al[17], γ-irradiation of human amniotic membrane (HAM) with 10 KGy, 20 KGy, and 30 KGy resulted in reduced levels of tissue inhibitor of metalloproteinase 4 and epidermal growth factor while TGF-β levels remained unaffected. Sterilization using 15 KGy of γ-irradiation did not show any significant decrease in growth factors in HAM cryopreserved in glycerol as compared to fresh HAM.

Based on these results, Muraglia et al[14] concluded that 25 KGy of γ-irradiation could maintain the sterility of PRP for 6 months at −20°C, while 5 KGy γ-irradiation decreased the number of colony forming units. The doses of 0.1 KGy and 1 KGy γ-irradiation did not show any such effect. Another study reported a positive correlation between the γ-irradiation dose (using 10, 20, and 30 KGy γ-irradiation dose) and TGF-β level in freeze-dried HAM[17]. Based on the results of all these studies, we can hypothesize that γ-irradiation of FDHPRP samples might also be associated with changes in the levels of TGF-β.

The γ-irradiation frequently used to sterilize medical equipments and bone allografts is terminally sourced from cobalt-60. The irradiation time and dose required depend on the thickness and volume of the product in question[6]. There are few references (national and international) that can be useful for determining the appropriate dose of γ-irradiation. ISO 11137, 11737-1, 11737-2, 11737-3 and 13409 can be referred to decide upon several aspects such as to determine initial contamination present in a product, dose of γ-irradiation required, and conditions where lower doses of radiation can be possibly used for healthcare products.

As per the recommendations of fourth edition of Farmakope Indonesia, the sterilization dose required for healthcare products is generally 25 KGy. However, in some cases, lower doses can be used depending on the initial concentration of the microorganisms present, type of microorganisms, and various other factors[7]. In cases where the sterilized products are manufactured under conditions suitable enough to minimize microbial contamination, it is possible to use γ-irradiation at a dose lower than 25 KGy.

The present study aimed to evaluate the changes in the levels of TGF-β cytokine in FDHPRP upon sterilization with different doses of γ-irradiation. FDHPRP was prepared using standard procedure and sterilized using γ-irradiation at different doses. TGF-β levels were measured using enzyme linked immunosorbent assay (ELISA) test. The statistical significance of the data obtained was established using several statistical tools including Kolmogorov-Smirnov test, Levene's test, Mann-Whitney test, and Kruskal Wallis test. The results of the study helped to establish the minimum dose of γ-irradiation suitable for sterilization of FDHPRP.

Material and Methods

PRP sample preparation: For PRP preparation, 120 mL of type-O blood was acquired from Palang Merah Indonesia (PMI) and used as a sample source. Since the individuals with blood group O are universal donors, O blood type was a preferred choice for this study. The blood sample used was actually a waste product of PMI. The presence of hepatitis B, hepatitis C, HIV, and syphilis infection in the blood sample was tested using transfusion-transmitted infectious disease test. PRP was prepared by the centrifugation of whole blood using an RC KUBOTA 9942 centrifuge according to the standard PMI procedure. The resulting plasma was stored in blood bank refrigerator at 2°C–6°C. The plasma was further transferred to a cool bag at a temperature of 2°C–4°C for freeze drying.

Freeze drying procedure: The PRP sample was incubated in a freezer at −40°C for 7 days. Following this, the sample was freeze-dried for 3 days using a freeze dryer (Modulyo, Edwards).

Grinding procedure: FDHPRP sample was ground using a mortar and pestle. The powdered sample was filtered by passing it through a 60-mesh filter. Further, the filtered sample was transferred into a microtube and placed in a laminar air flow cabinet.

Sterilization using γ-irradiation: 60 microtubes samples were divided into six groups with 10 microtubes samples per group. Each group was exposed to a different dose of γ-irradiation in a γ-irradiator sourced from cobalt-60 (OB-Servo Ignis type Gamma Irradiator). The doses of γ-irradiation used in the study were 0, 5, 10, 15, 20, and 25 KGy.

Analysis of TGF-β levels: The levels of TGF-β in FDHPRP were analyzed using ELISA kit (Human TGF-β1 Elabscience Biotechnology Inc., USA). The experiment was performed as per the manufacturer's instructions.

Results and Discussion

The levels of TGF-β (represented as mean and standard deviation) observed in all the sample groups are summarized in table 1. TGF-β levels showed a positive correlation with the dose of γ-irradiation up to the standard dose recommended for healthcare products (25 KGy). The normality of the data was analyzed using the Kolmogorov-Smirnov test. The results of the normality test are summarized in table 2. The significance level of < 0.05 indicated that the data lacked normal distribution. For

evaluating the homogeneity of variances, Levene's test was performed. As shown in table 3, P value of < 0.05 was observed for Levene's test suggesting that the variances of the analyzed data were not homogenous.

Based on these results, the best statistical analysis suitable for the present data was Kruskal Wallis test. As shown in table 4, significant differences were observed among the different tested groups. To further assess the differences among the groups, Mann-Whitney test was performed.

Table 1
Mean and Standard Deviation of TGF- β Levels

Group	γ-Irradiation Dose	Mean and Standard Deviation
I	0 KGy	0.432 ± 0.068232
II	5 KGy	0.516 ± 0.029424
III	10 KGy	0.423 ± 0.05554
IV	15 KGy	0.572 ± 0.018421
V	20 KGy	0.624 ± 0.019365
VI	25 KGy	0.616 ± 0.020469

Table 2
Kolmogorov-Smirnov Test for Normality

	Statistic	df	Sig.	Note
TGF-β level	0.135	60	0.008	Not normal

Table 3
Homogeneity Test

	Levene's statistic	df1	df2	Sig.	Note
TGF-β level	14.687	5	54	0.000	Not homogeneous

Table 4
Kruskal Wallis Test

	γ-irradiation Dose	N	Mean	P sig.	Note
TGF- β level	0 KGy	10	0.432	0.00	Significant
	5 KGy	10	0.516		
	10 KGy	10	0.423		
	15 KGy	10	0.572		
	20 KGy	10	0.624		
	25 KGy	10	0.616		
	Total	60			

Table 5
Mann-Whitney Test

	Dose of γ-irradiation	Dose of γ-irradiation	Sig.
TGF- β level	0 KGy	5 KGy	0.013
		10 KGy	0.838
		15 KGy	0.001
		20 KGy	0.000
		25 KGy	0.000
	5 KGy	10 KGy	0.001
		15 KGy	0.000
		20 KGy	0.000
		25 KGy	0.000
	10 KGy	15 KGy	0.000
		20 KGy	0.000
		25 KGy	0.000
	15 KGy	20 KGy	0.001
		25 KGy	0.000
	20 KGy	25 KGy	0.070

As shown in table 5, significant differences were observed in the levels of TGF-β in almost all the comparisons made between γ-irradiation doses ($P < 0.05$). However, the differences were not statistically significant for the comparisons made between 0 KGy and 10 KGy doses and between 20 KGy and 25 KGy doses.

The results of this study showed that the levels of TGF-β increased with the increase in the dose of γ-irradiation. This was supported by Kruskal Wallis test and Mann-Whitney test results. This finding is in agreement with the proposed theory which stated that increase in the dose of γ-irradiation would result in an increase in the TGF-β levels in FHDPRP. TGF-β is known to play multiple functions in the regulation of various biological processes in the human body such as cell growth and differentiation, bone remodeling, angiogenesis, and maintenance of homeostasis.

It is also involved in the stimulation and inhibition of apoptosis via specific genes and facilitates cell sustainability[16]. TGF-β has been reported to be essential for effective osteoinduction. Thus, the changes observed in the levels of TGF-β in FDHPRP upon γ-irradiation could be attributed to its role in cell sustainability and prevention of DNA fragmentation[2]. TGF-β is also known to confer resistance to high doses of γ-irradiation[19]. Thus, upon exposure to γ-irradiation, the levels of TGF-β would increase depending upon the dose of γ-irradiation to protect the cells from DNA fragmentation. In the present study, the best dose of γ-irradiation suitable for FDHPRP sterilization was found to be 20 KGy, which was lower than the generally accepted gold standard for medical devices and materials.

Conclusion

In this study, the effect of γ-irradiation on TGF-β levels in FDHPRP samples was studied using ELISA test. The levels of TGF-β showed a γ-irradiation dose-dependent increase up to a dose of 20KGy. The best suitable dose of γ-irradiation to ensure complete sterilization of FDHPRP samples was 20 KGy.

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