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The efficacy of platelet gel derived from umbilical cord blood on diabetic foot ulcers: A double-blind randomized clinical trial

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ABSTRACT

Introduction: Type 2 diabetes is one of the most prevalent diseases throughout the world. The foot ulcers are severe complications of this disease. Foot ulcers induce the high rate of morbidity, impair quality of life, bring about extreme costs to health service providers, and give rise to waste of time. Recently, platelet-rich plasma (PRP) and platelet gel (PG) have been used for the treatment of chronic wounds. In the present randomized, double-blind, placebo-controlled study, platelet gel derived from umbilical cord blood (UCB) was used to heal the diabetic foot.

Method: The patients were randomly divided into three groups, under the categories of PG, platelet-poor plasma (PPP) and lubricant gel (placebo) (ratio 1:1:1). The processes of gels application were launched for the subject of each group twice per week with 3–4 days' interval. This mechanism protracted for eight weeks. After completion of 8 weeks, the patients were followed up after two weeks and then continued for nine months with one-month interval.

Result: 30 patients underwent statistical analysis. Except for diastolic blood pressure which was significant between groups, there were no statistically significant differences in patients' baseline demographics. No significant differences were detected between groups at baseline of wounds ($P = 0.09$). The results revealed that there is no statistically significant interaction among three groups during follow-up time.

Conclusion: The present study provides evidence that there are no significant differences in the size of wound among PG, PPP, and placebo groups.

1. Introduction

Type 2 diabetes are considered worldwide incidence and prevalence [1]. According to the World Health Organization (WHO), 366 million people will have been affected diabetic by 2030 [2]. Approximately 12–25 % of these afflicted patients are at risk of ulcer particularly in the extremities in their life span [2–4]. The lower limb ulcers are diabetes serious complications [3]. Foot ulcers cause significant morbidity, impair quality of life, incur high costs to health service providers and time-consuming process [4–6]. These wounds can lead to the patients' hospitalization and may result in amputation of limbs [7]. Since, the wound healing is a slow process, it often takes between 2 and 5 months and requires special treatments [8]. Holzer et al. in a retrospective analysis of costs for lower extremity ulcers in people with diabetes come to this conclusion that new treatment strategies should be developed owing to the wound treatment high medical cost [4]. Various interventional approaches have been used for the treatment of diabetes-induced ulcer. One of the treatment methods of the chronic wounds is

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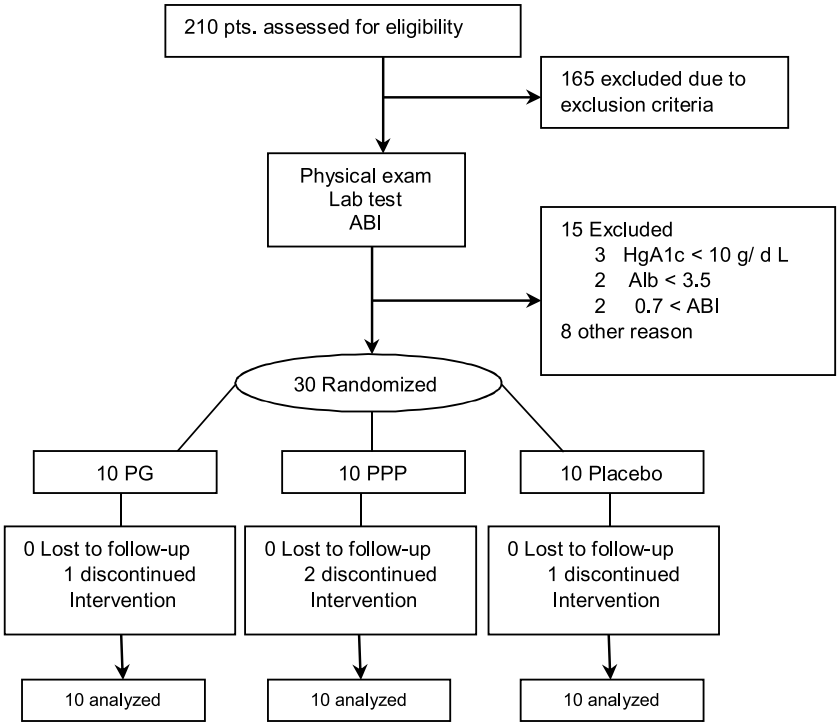

Fig. 1. Trial profile. PG: platelet gel, PPP: platelet poor plasma, placebo: lubricant gel.

Table 1
Demographic details of the study population.

Parameter/group	PG N = 10	PPP N = 10	Placebo N = 10	*P* value
Female sex, n (%)	1 (10)	3 (30)	2 (20)	0.847
Age, years	56.30 ± 9.978	56.33 ± 10.78	58.44 ± 10.23	0.879
FBS	116.13 ± 24.222	149.88 ± 76.59	99.22 ± 17.006	0.099
HbA1C, gm/dL	7.06 ± .96	7.05±.94	6.871 ± 1.49	0.941
Hb	12.28 ± 1.63	12.25 ± 2.28	12.65 ± 2.20	0.904
Chol	159.75 ± 50.95	171.63 ± 25.69	133.50 ± 27.23	0.127
TG	116.88 ± 50.77	209.38 ± 131.83	144.44 ± 67.74	0.128
Total pro, gm/dL	7.11 ± 1.54	7.06 ± .80	6.62 ± 1.12	0.738
BP* systolic	124.44 ± 11.02	141.87 ± 16.46	115.11 ± 39.98	0.126
BP Diastolic	81.66 ± 7.07	89.37 ± 3.20	83.33 ± 7.07	0.043
ABI	1.17 ± .229	1.03 ± .29	1.106±.24	0.637
BMI	30.05 ± 8.63	26.19 ± 2.61	28.108 ± 4.24	0.378
Duration of diabetes, (year)	14.22 ± 6.340	17.13 ± 8.855	12.88 ± 11.862	0.644
Duration of ulcer, (month)	12.00 ± 19.661	5.38 ± 4.274	5.44 ± 5.747	0.608
Location of ulcer, n (%)	7 (77.8)	7 (77.8)	10 (100)	0.268
Foot	1 (11.1)	2 (22.2)	0 (0)	
Heel	1 (11.1)	0 (0)	0 (0)	
Leg				

Data are presented as mean ± SD or number (%). FBS: fasting blood sugar. Hb: Hemoglobin. Chol: cholesterol. TG: triglyceride. BP: blood pressure. ABI: ankle brachial index. BMI: body mass index.
PG: platelet gel, PPP: platelet poor plasma, placebo: lubricant gel.

the use of amniotic membrane allograft [3,9,10], but it causes some concerns such as preparing, obtaining, storing the material as well as the risk of infectious disease transmission. Some other alternatives are skin auto grafts which have been used for wounds [10], but this type of graft requires a donor site wound. Cultured allogeneic adult keratinocytes is another approach [11], but one or more dermis-like substrates need to be incorporated because allogeneic adult keratinocytes in itself are fragile and unstable and its culture is costly and time-consuming [12]. Growth factors have been used to treat wounds since 1985 [13]. The healing process can be accelerated by using growth factors such as platelet-derived growth factor [3]. Numerous studies were conducted about the effectiveness of PRP for the treatment of chronic wound [14], but one of the major issues of this approach is that the patients with chronic wound usually have anemia and poor general health. Therefore, large volumes of blood are needed to produce autologous PRP or PG [15,16] in addition, some drugs or organ failures affected platelet and diminished function and limit the application of autologous platelets such as dialysis, uremia, anticoagulant drugs, third generation cephalosporin. Another restriction in the said-mentioned treatment process is to have access to good vein. To overcome these limitations, PG derived from UCB is used to heal the diabetic foot ulcers in this study. The aim of this trial is to show the effectiveness of platelets derived from cord blood on the healing of diabetic ulcers (wound size reduction, rates of complete healing, presentation of osteomyelitis and infection) versus the placebo group (the standard of care only).

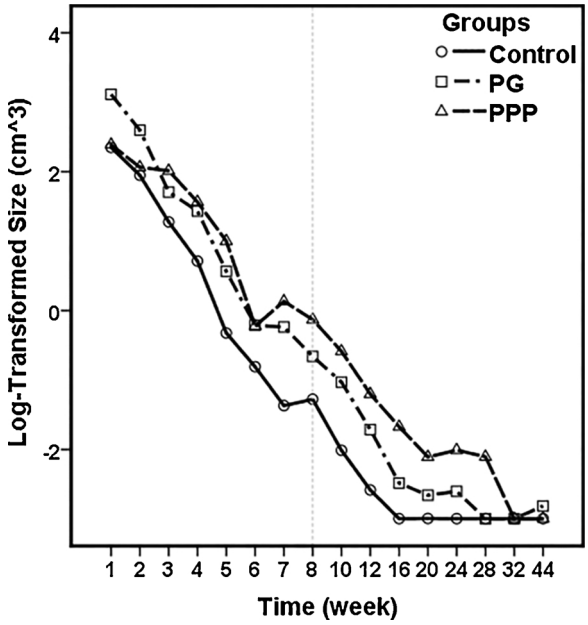

Fig. 2. Line plot of natural logarithm of size during of time in three groups. PG: Platelet Gel, PPP: Platelet Poor Plasma, placebo: lubricant gel.

2. Methods

2.1. Patients

This study is a randomized double blind placebo-controlled trial in the diabetic foot ulcer groups. This clinical study lasted from May 2013 to January 2015 in Shariati Hospital as a research collaborator of Royan Institute. For enrollment, patients who were selected had the following inclusion criteria with history of type 2 diabetes, diabetic ulcer located in any part of foot, ankle-brachial index (ABI) above 0.7 (according to standard method for peripheral artery disease [17]), glycosylated hemoglobin (HbA1c) < 12 %, serum creatinine < 3.0 mg/dL, Hb (hemoglobin) > 10 g/dL, controlled diabetes and willingness to continue evaluation processes thereunto. Patients would be excluded if they had history of cancer, received radiation or chemotherapy in the past 12 month, diagnosed or suspected to suffer from malignant disease, diagnosis of autoimmune disease, pregnant women or breastfeeding, severe cardiovascular disorder, patients with ischemic change of leg, limb ischemia or gangrene, participation in another clinical trial, bleeding disorders such as hemophilia, sickle cell disease, leukemia or a thrombocytopenia and wounds whose source are electrical trauma, chemical or radiation burns, previous treatment with growth factors or stem-cell therapy, patients with a life expectancy of less than 12 months and albumin < 3.5 mg/dL.

The protocol was approved by institution review boards and ethics committee of Royan Institute (NO: ec/90/1073) and registered in clinical trials.gov (Identifier: NCT02134132). Before launching the project, the procedure was described for all patients and written informed consent was obtained.

2.2. Randomization

Eligible Patients criteria were randomized to receive either PG, PPP, or lubricant gel (placebo) (ratio 1:1:1). The randomization code list was accessible only to the head of the laboratory to determine the type of product to patients. The investigators, clinical research personals, patients and statisticians were masked to treatment allocation in the entire process. PG, PPP and placebo were similar in their appearance and were beyond the recognition from each other.

2.3. Product preparation

UCB-derived platelet lysate (PL): Healthy UCB samples were obtained from Royan public Cord Blood Bank. To obtain PL from UCB, we used PRP method optimized in our lab. Briefly, about 3–4 UCB samples were pooled in a 450 mL transfer bag (Besat, Ind. Co, Qom, Iran), and was centrifuged in 300*g* at 20 °C for 22 min (acceleration = 5, deceleration = 0) in a centrifuge with rotor for blood bag (Rotixa 50S, Hettich, Germany). In our experiments, any centrifugal speed higher or lower than 300*g* (RCF) yields recovery less than 70 %. This step commonly known as light spins and leads to platelet PRP separation. Then, PRP was collected gently by using plasma extractor. Later on, PRP was centrifuged again in 5000*g* (hard spin) at 20 °C for 30 min (acceleration = 5, brake = 2). Platelets were precipitated firmly at the bottom of

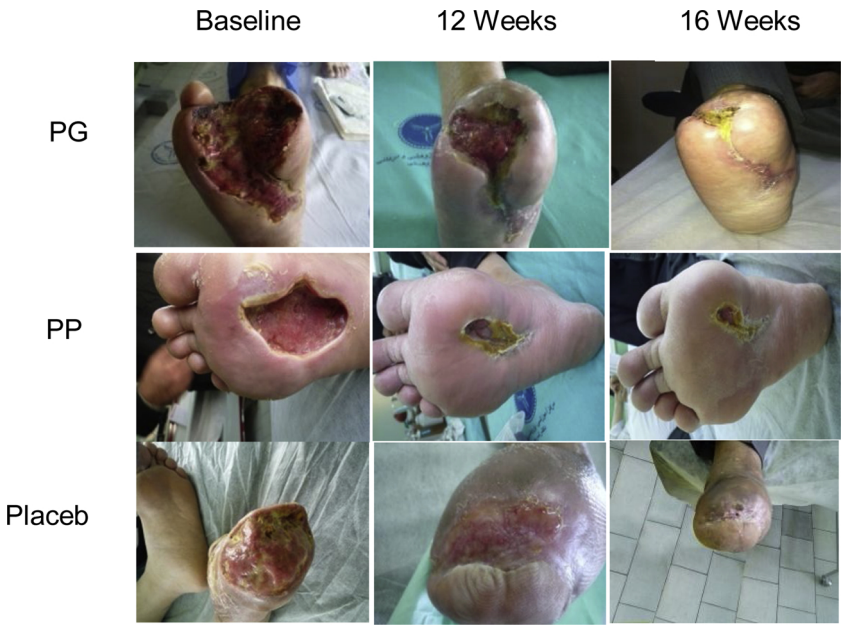

Fig. 3. Diabetic foot ulcer healing process. Images show diabetic foot ulcer healing in baseline, then 12 and 16 weeks after treatment. PG: platelet gel, PPP: platelet poor plasma, placebo: lubricant gel.

Table 2
Study outcome: healing duration in each group separately.

TIME (weak/s)	Groups, Mean ± SD [*P*-value**]		
	PG (N = 10)	PPP (N = 10)	Placebo (N = 10)
1*	49.62 ± 76.76	41.97 ± 97.94	14.30 ± 13.16
2	39.24 ± 75.83 [1.000]	12.21 ± 12.25 [1.000]	10.30 ± 11.63 [1.000]
3	28.19 ± 62.78 [.435]	37.41 ± 37.41 [1.000]	7.24 ± 11.23 [1.000]
4	22.05 ± 45.66 [1.000]	28.21 ± 64.78 [1.000]	5.42 ± 9.61 [0.641]
5	6.72 ± 11.49 [.011]	17.30 ± 40.06 [.219]	1.37 ± 1.82 [0.004]
6	2.87 ± 3.70 [<0.001]	1.59 ± 2.45 [.010]	1.15 ± 1.69 [0.002]
7	2.40 ± 4.28 [.001]	11.15 ± 26.85 [.006]	0.68 ± 1.20 [<0.001]
8	1.49 ± 2.12 [<0.001]	15.06 ± 31.84 [.006]	0.68 ± 1.16 [0.001]
10	0.86 ± 1.22 [<0.001]	9.26 ± 19.98 [<0.001]	0.29 ± 0.62 [<0.001]
12	0.32 ± 0.45 [<0.001]	6.37 ± 15.25 [<0.001]	0.06 ± 0.13 [<0.001]
16	0.12 ± 0.27 [<0.001]	7.50 ± 16.77 [<0.001]	0.00 ± 0.00 [<0.001]
20	0.07 ± 0.18 [<0.001]	3.57 ± 9.45 [<0.001]	0.00 ± 0.00 [<0.001]
24	0.08 ± 0.20 [<0.001]	3.13 ± 7.65 [<0.001]	0.00 ± 0.00 [<0.001]
28	0.00 ± 0.00 [<0.001]	3.57 ± 9.45 [<0.001]	0.00 ± 0.00 [<0.001]
32	0.00 ± 0.00 [<0.001]	0.00 ± 0.00 [<0.001]	0.00 ± 0.00 [<0.001]
44	0.06 ± 0.11 [<0.001]	0.00 ± 0.00 [.049]	0.00 ± 0.00 [<0.001]

* Reference Time: comparison of the wound size of the second week up to week 44 with the first week the initial wound.
** *P*-value was computed by natural logarithm of size.
PG: platelet gel, PPP: platelet poor plasma, placebo: lubricant gel.

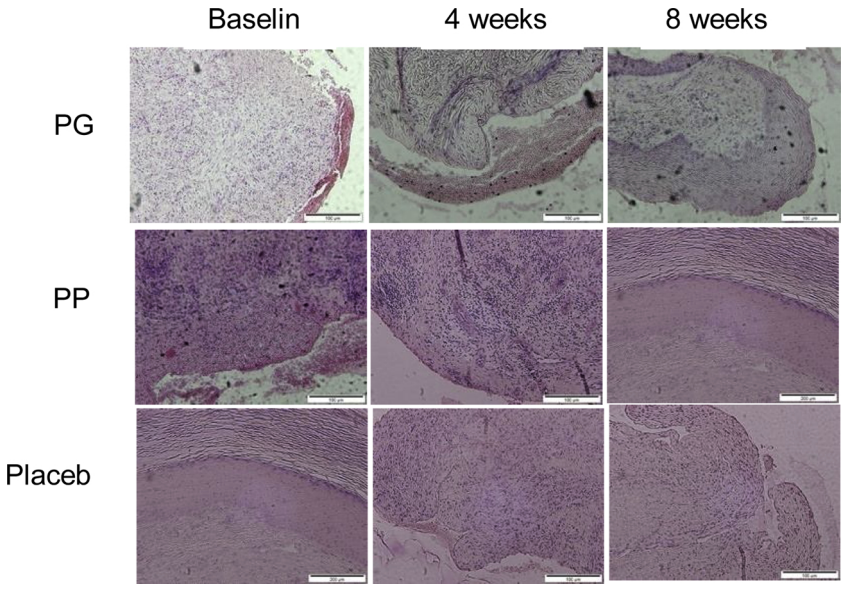

Fig. 4. Hematoxylin-and-eosin stain (H&E). Histological changes baseline (0 day) and after 4 and 8 weeks of treatment. Magnifications of the images are 20 × . The PG: platelet gel, PPP: platelet poor *plasma, placebo: lubricant gel.*

blood bag and only 76 mL (mean) remained on the platelets. Platelet concentrate (PC) was counted by an automated hematology analyzer (Sysmex Corporation, Kobe, Japan). We used PCs with a minimum platelet count 1×10^9/mL and this cut-off used for other PC product. PCs were transferred to −70 °C overnight and were quarantined to test for both infectious disease including HIV-I/II, HCV, HBV, CMV, HTLV-I/II and microbiology. Then, frozen PCs were completely thawed at 37 °C water bath and centrifuged in 3000*g* for 30 min at 4 °C in order to remove platelet bodies. PL obtained was aliquoted in 5–10 mL volumes.

Growth factor assay: the concentration of PDGF-AB, TGF-β_1, IGF-I, and bFGF in PL were assessed by ELISA (enzyme-linked immunosorbent assay) kits (all from R&D Systems, Minneapolis, MN, USA) and was performed according to manufacturer's instructions.

Measurement of protein concentration: Bradford procedure was used for total protein assay. First, Bradford reagent (Coomassie Brilliant Blue G) is prepared as 1X concentration. Then, plasma gamma globulin was used as calibrators 3, 6, and 9. All test samples were diluted with H_2O to 20 × . All tubes including blank, calibrator, and test samples reached 520 µL final volumes. Samples were added to flat-bottom 96 well microplate in duplicate. OD was read at 595 nm with ELISA reader and the best-fitted linear curve was plotted. The Concentration of sample test was calculated by using in-built software of ELISA reader (Multiskan Spectrum Microplate Spectrophotometer, Thermo Scientific, USA).

Preparation PG from PL: to obtain PG, 4 parts of PL, 2 parts of $CaCl_2$ and 1 part of thrombin (which was available from our Public Cord Blood Bank) in 50 mL conical tube are combined. After being thoroughly mixed, the liquid product was incubated at 37 °C for about 10–15 min. On the basis of our experiments, the gel is formed during this time. The gel product was placed on ice pack and transferred immediately to the hospital to prevent the proteins destruction or the gel consistency reduction.

Placebo: a commercial dye is added into the neutral lubricant gel to get a similar appearance compared with PG and PPP groups.

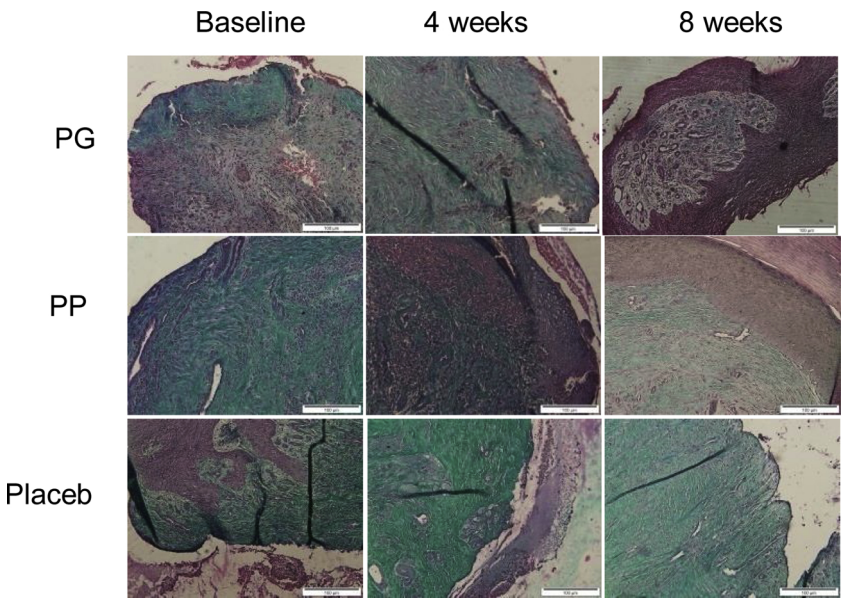

Fig. 5. Photomicrographs with Masson's trichrome (MT) stain. Histological changes baseline (0 day) and after 4and 8 weeks of treatment. Magnifications of the images are 20 × .The PG: platelet gel, PPP: platelet poor plasma, placebo: lubricant gel.

2.4. Procedures

If both legs were affected, the leg with the lowest pressure index or ABI would be chosen as the one which is to be treated and the other leg would be reported to case-report and is not analyzed in this study. After enrollment in this study, the patients must not participate in any clinical trial. Prior to the commencement of treatment procedures, a career nurse trained all target groups. The training schedules are performed such as daily dressing change, wound rinse under running water with use baby soap, daily bandage changing, daily inspection of their wound to detect any signs of infection and observance of appropriate regime. At every session, before the gel is used for patients, the medically sharp debridement is made when necessary. Necrotic and infection tissues were removed and the wound was rinsed with a sterile normal saline solution. Then, the gel products were applied to each patient like ointment on the wound and then it was covered by Molnlycke Mepitel Transparent Dressing (SKU: MKN-28972101, Model: 289,700). The gels were applied to patients of each group (PG, PPP and placebo) twice a week with 3–4 days' interval. The process continued for 8 weeks. Follow-up procedures continued initially with the 2 weeks' interval, and then it is followed once per month up to nine months. The ulcer was measured with graded centimeter ruler (length, width and depth (cm^3)). Measurements were taken after the debridement. The application was done by an expert nurse and patients of three groups underwent the same procedure (gel investment, dressing change) and followed up like wise.

2.5. Histological assays

Punch biopsy with 2 cm diameter were performed from the edge of the lesion and samples were fixed by perfusion with 10 % formal saline at 1, 4, and 8 weeks' interval during the treatment phase. Biopsies submitted for histological examination and each samples were stained with Masson's trichrome (MT) and hematoxylin-and-eosin (H&E) [18] (Fig. 4 and 5) with an Olympus BX61. Digital Images were captured by using an Olympus DP70, 12-megapixel cameras (Olympus, Tokyo, Japan).

2.6. Study outcome and data analysis

The purpose of this investigation was to determine the efficacy of PG versus standard care in the treatment of diabetic foot ulcers. Primary study outcome included reduction of wound size. Final evaluation outcome of the research continued up to week 44 for those patients with ongoing enrollment. The association between qualitative variables was evaluated by using χ^2 test with odds ratio and 95 % confidence interval (CI). Wound surface area reduction was evaluated by using a mixed effect model. Shapiro–Wilk test was used on the basis of this assumption that variable distribution has been normal. To investigate differences between wound surface areas of groups in baseline, ANOVA or Kruskal-Wallis test was used. The R software was used to analyze the data. The level of statistical significance was set at $P < 0.05$.

3. Results

210 patients were screened for eligibility and 30 were randomly selected (Fig. 1). With the exception of diastolic blood pressure being significant among groups; there were no statistically significant differences in subject baseline demographics, medical history, and disease characteristics among treatment groups (Table 1). The mean age of patients was 57.0 ± 9.9 year (mean ± SD). Six patients were female (29.3 %) and 24 patients were male (70.7 %). 25 patients were past or present smokers (25 %) and 25 patients had a history of heart disease (25 %). The average size of the wound at the beginning of treatment in PG group was 49.6 ± 76.7, PPP group 41.9 ± 97.9 and placebo group 14.3 ± 13.1 mm. No significant differences were detected between groups at baseline of wounds ($P = 0.09$).

The analysis made by using mixed effect model shows that there is no significant difference in the mean size of wound among three groups during treatment and follow-up period ($P = 0.174$).In other words, there is no statistically significant interaction between three groups during follow-up process. The main effect of groups has no significant difference (Fig. 2 and 3).

The major impact of the Follow-up time in this model has significant difference ($P < 0.001$). It is concluded that the reduction of sore volume bears significant difference during the whole treatment and follow up period. Significant statistical correlation direct was observed between the level of HbA1c, duration of ulcer prior to platelet gel use and ABI before treatment and ulcer size. In a way that with the increase of each unit of HbA1c level, the logarithm of wound volume increase 0.63. Also, patients who took longer to attend the clinic and have a lower

ABI, had larger size of the wound ($P = 0.037$).

If the size of the wound in each group is separately considered, it is concluded that the size of the wound has significant differences in PG and placebo groups after the fifth week and PPP group as of the sixth week on the size of the wounds. This means that the wounds have become significantly smaller. The first week was considered as the base of comparison. Second week up to the 44th week was compared with the first week (Table 2).

No patient was lost to follow-up in this trial. No adverse events occurred in the patients after randomization.

Histological evaluation of biopsy specimens from the margin of ulcer of placebo group after 4 and 8 weeks revealed severely ulcerated epidermis and fibrotic dermis without dermal appendices.

Comparison of histological evaluation of biopsy specimens from the margin of ulcer of PPP and PL groups after 4 weeks confirmed the following differences:

- Repairing epidermis in PPP group but ulcerated and destroyed epidermis in PL group observed.
- Repairing process with severe fibrosis in the dermal component in PPP group but ongoing edema and inflammatory process in PL group.
- Both PPP and PL groups revealed more degree of epidermal repairing and less degree of dermal inflammation than the placebo group (Figs. 4 and 5).

4. Discussion

Recently, platelet-derived GFs have been favored by many researchers in regenerative medicine not only for soft-tissue reconstruction but also for wound healing acceleration process. The reasons for this preference was mainly attributed to numerous GFs being present in alpha granules [9]. PG has many advantageous effects on the healing of cutaneous chronic wounds particularly diabetic lower extremity ulcers. This product can be obtained simply from PRP or platelet lysate-the product of degraded platelets by freezing plus thrombin and calcium chloride.PG promotes dramatically granulation tissue formation and decrease patient hospitalization period [7]. Animal studies conducted in rabbits by using heterologous PRP also resulted in acceleration of the wound healing without any side effects [12]. It has been obvious for many years that growth factor presented in rhPDGF-BB can accelerate significantly the recovery time of chronic ulcers in patients with diabetes [4]. In one study launched by Bhansali et al. in India, comparison between rhPDGF-BB gel and standard wound care showed that rhPDGF-BB had substantial effect on both healing duration and size of diabetic neuropathic ulcers [5]. In spite of many advantages for rhPDGF-BB (known under commercial name Becaplermin or Regranex) its' application may be limited in some countries due to financial bottleneck. In wealthy country such as Sweden the availability of this recombinant drug is relatively simple and has shown to save the money in combination with general wound care [6]. In addition, rhPDGF-BB failed to show the beneficial effect on wound healing in practice of some people with diabetes [17]. There are also some combinations of platelets and other biologic materials to treat the diabetic ulcer. One of them is FIBRINET® device preparing platelet-rich fibrin matrix from whole blood. The primary data has demonstrated the good effect of ulcer treatments, but more investigation still is ongoing [8]. Some instruments have been made in order to facilitate the PRP gel preparation as point-of-care. These systems can significantly decrease the time of delivery to the hospital and are best appropriate for antilogous gels [13]. It is noteworthy that some of diabetic foot ulcers are difficult to usual treatments such as debridement, dressing and wound moistening with normal saline. Under such conditions, the autologous PRP may be used as material for non-healing chronic wounds. [9,11]. Cost-effectiveness of PG was compared with other interventions for non-healing diabetic foot ulcers in a long-term study (5 years) and data confirmed that these biologic components obtained from platelets may be considered to decrease the cost care and health issues in people with diabetes [18]. It seems that combination of PG and skin graft may be a good option for treatment of large-size and intractable diabetic foot ulcers [15].

Finally, there are no significant differences between placebo, PPP, and PG group. It was found that a significantly reduced trend in the size of the wound during the follow-up period if the analysis is made in each group separately. This result may be obtained due to a good nursing care. In this research, a career and well-trained nurse visited the patients every week and provided them with necessary training pertaining care and debridement of the wound in preventing diabetic foot ulcers in high-risk patients [19,20]. The training provided by the nurse will create awareness and enhance self-care procedure.

The removal of necrotic tissue, foreign debris, bacterial growth, and callus, wound edge, and wound bed tissue from chronic wounds are called debridement. This clearance and removal induce stimulation of the wound healing process. Debridement mediates stimulation of the wound healing through which the conversion of chronic non-healing wound environment to an acute healing environment occur [21]. Steed's study showed that the debridement has important role on heeling of diabetic ulcer [22].

There are some explanations for these results since our primary hypothesis based on an output in PG group. First, we used PG derived from UCB and we did not compare whether allogenic peripheral blood or autologous PG may lead to better improvement in diabetic foot ulcers. Second, we did not categorize our diabetic patients in terms of the severity of wounds and if we have used PG in diabetic with better life style, we would acquire a significant result in PG group. Thus, it needs to further investigations to assay the efficacy of UCB-PG compared with other treatments for diabetic foot ulcers especially autologous PG and rhPDGF-BB. The point which should be evaluated is to specify the performance result of career and amateur nurse.

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Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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