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A new innovation in topical diabetic foot ulcer; hyaluronic acid platelet-rich fibrin (HAPRF) gel - a study in inflammation and angiogenesis

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ABSTRACT

Background: Indonesia ranks 6th out of ten countries with the highest number of diabetes patients. An increase followed this condition in the prevalence of diabetic foot ulcers by around 15%. This study demonstrated the ability of combination of Platelet Rich Fibrin (PRF) and Hyaluronic Acid (HA) to reduce inflammation in diabetic foot ulcer (DFU) healing.

Methods: In the baseline, all patients received the same treatment including wound debridement. There were 20 subjects divided into two groups; HAPRF and PRF. A part of sample was processed into lysate and stored in -80°C. The rest is applied as a topical therapy in DFU base on intervention. We analyzed the inflammation using two methods; swab in DFU and fibrin gel of HAPRF or PRF and ELISA on day-3 and day-7. We also measure the granulation index by digital photograph and analyze use ImageJ at the same time.

Result: There were 20 diabetic patients with DFUs, performed analysis of IL-6 lysate gel HAPRF decrease significantly compare with PRF on day 3 ($p = 0.038$) and day-7 ($p = 0.034$). Granulation index evaluation, in HAPRF increase on day-3 ($p = 0.043$), day-7 ($p = 0.049$), and day-14 ($p = 0.041$).

Conclusion: A-PRF +HA administration leads to reduce IL-6 levels, and accelerated wound healing of DFUs patients. HAPRF directly aids epithelialization and granulation index.

Keywords: diabetic foot ulcer, platelet, fibrin, hyaluronic acid, inflammation, granulation index.
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INTRODUCTION

The prevalence of diabetes in adults aged 18–99 years was estimated to be 8.4% in 2017 and predicted to rise to 9.9% in 2045. Meanwhile, diabetic foot ulcer (DFU) prevalence was 6.3% which was higher in males (4.5%) than in females (3.5%) and higher in type 2 diabetic patients (6.4%) than in type 1 diabetics (5.5%). In DFU which chronic hyperglycemia due to insulin resistance will result in deficiency of growth factors, especially Platelet Derivate Growth Factor (PDGF), Epidermal Growth Factor (EGF), Transforming Growth Factor -β (TGF-β), Vascular Endothelial Growth Factor (VEGF), and Fibroblast Growth Factor (FGF). This situation will affect the healing of DFU receiving growth factor therapy.[1]

Various modalities have been identified for chronic wound therapy. One such modality is the use of hyaluronic acid as a therapy for chronic wounds. Hyaluronic acid is one of the agents used as a topical agent to accelerate wound healing has been reported for acute injuries and chronic wounds.[1,2]

Hyaluronic acid(HA) is a polysaccharide that belongs to the glycosaminoglycan family and consists of glucuronic acid and N-acetyl-glucosamine. Since its discovery in 1934, hyaluronic acid, also known as hyaluronan, has been used as a medium for various skin medications, whose function is to improve drug transport to the epidermis and localize the drug into wounds. In previous study administration of hyaluronic acid was statistically effective in reducing wound size.[3] In diabetic foot ulcers also found no statistically significant difference in the wound size reduction between the wound dressings containing hyaluronic acid derivatives versus the wound dressings of paraffin gauze. Hyaluronic acid produces a microenvironment that stimulates growth factors, proliferation and

migration of fibroblasts, endothelial cells, keratinocytes and angiogenesis.[4]

Platelet-rich fibrin (PRF) is the second generation of platelet-rich plasma (PRP). Platelet-rich fibrin is the 2nd generation PRP and is superior to PRP because it is easier to prepare and does not use bovine thrombin, thereby reducing the likelihood of cross-infection and the risk of coagulopathy.[5] The smooth and flexible PRF structure is beneficial for cellular and cytokine enmeshment and helps hemostasis in bleeding wounds.[6]

Platelet-rich fibrin was developed in France by Choukroun et al.[7] in 2001 to improve bone healing in implant cases. The stimulation of wound healing occurs because PRF releases growth factors and cytokines stored in the fibrin matrix. Once activated, platelets release growth factors to stimulate new tissue. Platelets contain angiogenesis stimulating factors, namely bFGF, VEGF and angiostatic factors such as endostatin and thrombospondin-1.[8]

Interesting functions of PRF such as a reservoir of bioactive molecules to support wound healing and tissue regeneration. The advantage of PRF is by increasing the polarization of macrophages from the pro-inflammatory M1 to the pro-resolving M2 phenotype. Expression of the M1 marker genes interleukin 1β (IL1β) and interleukin 6 (IL6) together with the M2 arginase-1 and chitinase-like 3 markers (Chil3 or YM1). It was reported that PRF lysate could decrease the pro-inflammatory response of primary macrophages indicated by IL1β and IL6 expression. Increased polarization of M2, PRF lysate and medium conditioned PRF increased the expression of arginase-1 and YM1 in primary macrophages. PRF has anti-inflammatory activity and shifts macrophage polarization from M1 to M2 phenotype.[9]

This study aimed to analyze whether combine Hyaluronic Acid and PRF (HAPRF) might reduce inflammation and increase angiogenesis in topical DFU during 14 days.

MATERIALS AND METHODS

We conducted a randomized controlled trial from July 2019 to April 2020. The Ethics Committee approved the study of the Faculty of Medicine of Universitas Indonesia KET-668/UN2.F1/ETIK/PPM.00.02/2019 Informed consent was obtained from the patients, including for the use of photographs (ImageJ).

Figure 1. Preparation of PRF and HAPRF.

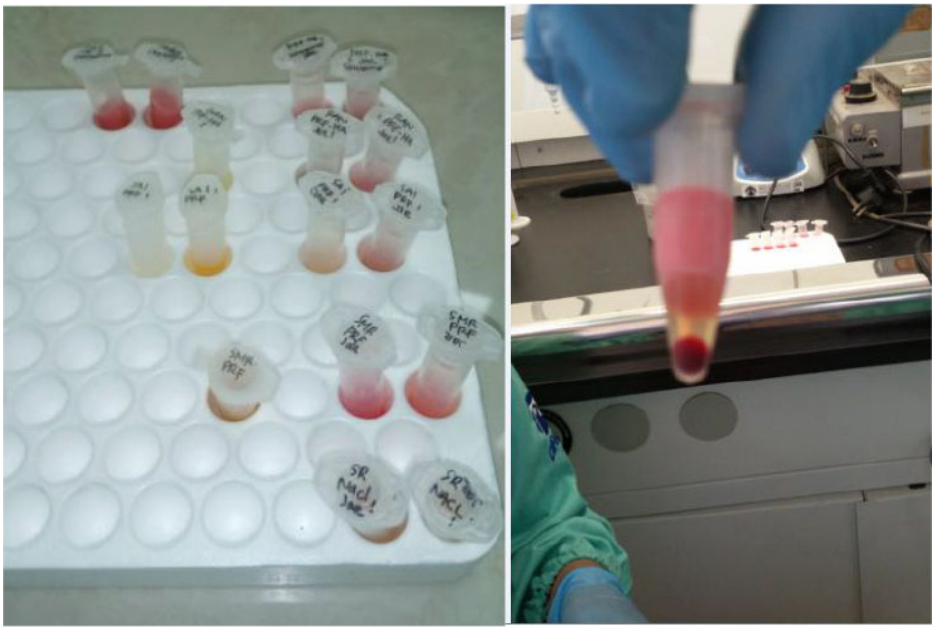

Figure 2. The Lysate of HAPRF and PRF before ELISA.

The study was conducted at Koja District Hospital and at Gatot Soebroto Hospital in Jakarta. Patients with DFU and an average wound duration of 3 months, categorized as Wagner-2, ulcer <40 cm2, were chosen as the subjects of the study.

The 20 subjects divided randomly into two groups, namely the HAPRF (n = 10) and PRF (n = 10). Each group was drawn 20 cc of blood, and processed into PRF. In HAPRF group, HA 0.2% added in fibrin use vertex machine. 1 cc of both groups' fibrin is made into lysates and stored at freezer with temperature -80° C. The rest of fibrin of both groups was applied to DFU according to their group. Evaluation of laboratory uses ELISA from fibrin gel. Inflammation was evaluated use IL-6 level of fibrin gel. Otherwise angiogenesis was assessed by VEGF level. Evaluation of clinical appearance of DFU by looking symptoms of inflammatory such as edema and pain day-0 and day-14 and the size of wound area.

Preparation of PRF HAPRF

In this study, PRF made from 20-40 mL vein blood collected in a non-anticoagulant tube, centrifuged at 200×g for 15 minutes. In HAPRF group, 0.6 mL HA 0,2% was added in fibrin 1 mL PRF (**Figure 1**). Fibrin was used in topical DFU, and a part of samples (1 mL fibrin gel) was stored in DMEM and immediately processed into lysate. The resulting lysate was examined using enzyme link immunosorbent assay (ELISA) method to obtain baseline levels of IL-6 and VEGF (Figure 1).

Biomarker ELISA Evaluation

HAPRF and PRF were evaluated IL-6 and VEGF by ELISA in day-0, day-3 and day-7. ELISA was done using ELISA reagents used are Human VEGF / ELISA Kit, Human IL-6 / ELISA Kit and Human VEGF/ELISA Kit with Insert Kit from LifesSpan BioSciences, Inch. Cat:LS-F4604. (Figure 2)

Evaluation Inflammation Sign in DFU Healing

In DFU, inflammation is obtained as a physiological response to the body through release immune response. Immune cells will respond inflammation in neutrophils and macrophages. Persistent inflammation will lead to dysregulation, especially chronic non-healing inflammatory DFUs.

Infectious Disease Society of America (IDSA) introduce clinical sign of inflammation in DFU such as increasing pain, erythema, edema, heat and purulent exudate.

Evaluation Angiogenesis and Granulation Area in DFU Healing

One of the factors that influence the formation of granulation tissue is the angiogenesis process. Angiogenesis is the process of forming new blood vessel shoots that support the construction of granulation tissue. Angiogenesis might be represented by VEGF sample got from gel fibrin and analyze use ELISA. The clinical appearance might evaluated by using digital photographs and imageJ software before and after treatment.

Statistical analysis

Data are presented and analyzed using SPSS version 20 test and analyzed using Independent t-test or Mann Whitney test and GraphPad Prism 9.0. All values considered significant if $p<0.05$.

RESULT

We recruited 20 subjects, consisting of 8 men and 12 women. The median age was 64 years in women and 61 years in men. The subjects were randomly divided into two groups (HA PRF and PRF), comprising 10 subjects per group (Table 1).

Table 1. Subjects baseline characteristics

Characteristic	HA+PRF (n = 10)	PRF (n = 10)	p-value
Age (year)[a]	59.8 (SD 12.7)	64.7 (SD 12.0)	0.626
Sex n (%)			
Male	1 0/30	12 (30)	
Female	2 2/30	1 6 (30)	
BMI[a]	28.9 (SD 2.7)	27.3 (SD 2.08)	0.337
Haemoglobin (g/dL)[b]	12.7 (27.4–39.0)	13.1 (SD 1.3)	0.224
Haematocrit e (%)[b]	36.3 (29.2–42.9)	35.6 (SD 4.6)	0.145
Leucocyte (10^3/µL) [a]	13.30 (SD 1.08)	11.08 (SD1.33)	0.985
Platelet (10^3/µL) [a]	354.9 (SD 167.5)	338.8 (SD 164.5)	0.880
Random Blood Glucose mg/dL[b]	286.0 (170–390)	243.8 (SD 47.4)	0.104
HbA1C (%)[a]	11.34 (SD 1.30)	9.0 (SD 0.68)	0.950
Cholesterol total (mg/dL) [a]	214.5 (SD16.9)	249.3 (SD_16.1)	0.096
Albumin (mg/dL) [b]	3.3 (2.8–4.2)	3.1 (2.8–4.2)	0.662

[a]mean (SD), Independent t-test
[b]median (min-max), Mann Whitney test

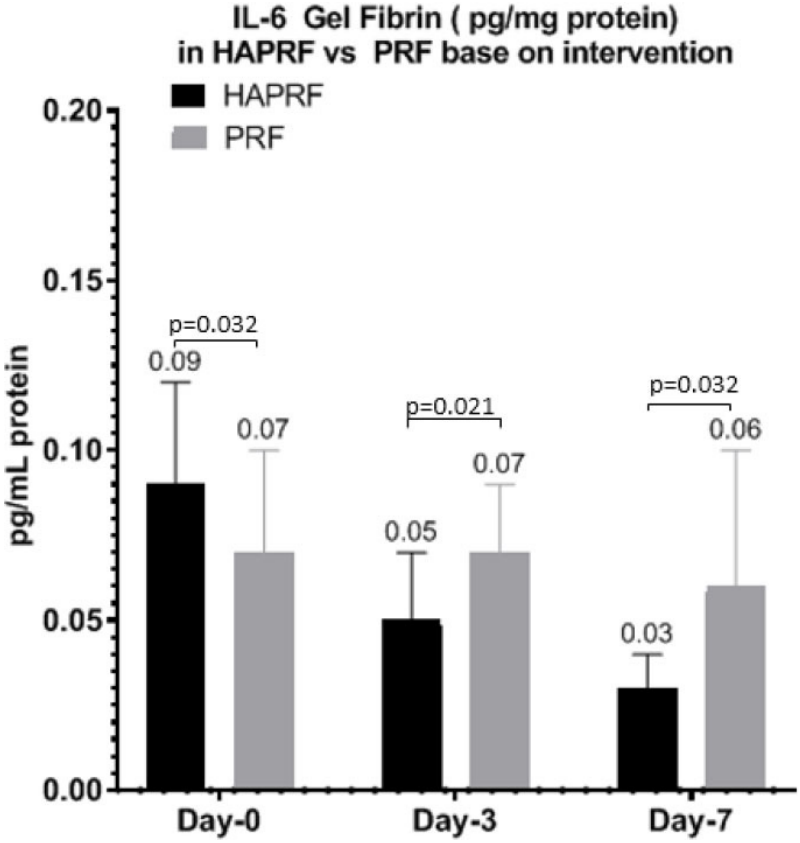

Figure 3. IL-6 Gel Fibrin (pg/mg protein) in HAPRF vs PRF base on intervention.

Evaluation IL-6 level in HAPRF versus PRF

Another way to inflammation level, the IL-6 sample got from fibrin gel and analyzed using ELISA. Figure 3 shows that in the HA+PRF group, IL-6 levels decreased from day-0 to day-7 (0.09 pg/mg protein to 0.03 pg/mg protein), while group PRF also reduced from 0.07 pg/ml protein to 0.06 pg/mg protein) (Figure 3).

In addition to analyzing the IL-6 levels between HAPRF and PRF, a delta analysis of changes in day-3 and day-7 was also carried out compared to the initial measurement. In the HAPRF group compared to PRF, there was a decrease in Δ IL-6 gel fibrin levels on day-3 (p = 0.002) and day-7 (p<0.001), independent t-test (Table 2).

Table 2. Delta IL-6 Gel Fibrin in HAPRF vs PRF base on intervention.

Intervention	HA + PRF (n=10)	PRF (n=10)	p-value
Δ Day 0–3	-0.05(SD 0.04)	0.01 (SD 0.01)	0.002
Δ Day 0–7	-0.06(SD 0.03)	0.03 (SD 0.01)	<0.001

*Mean (SD), independent t-test

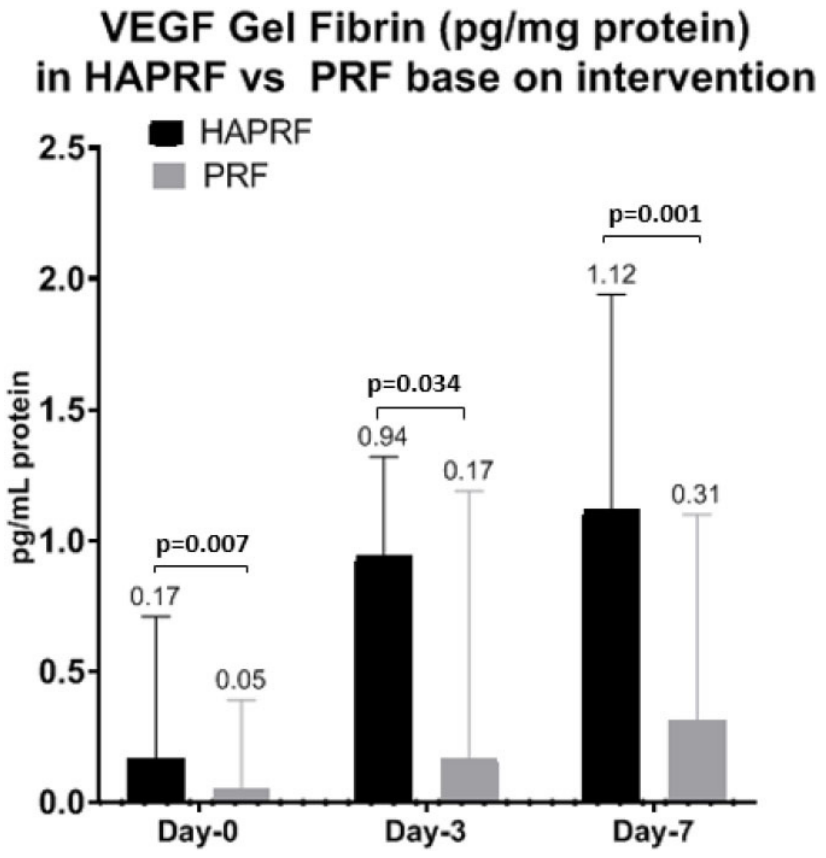

Figure 4. VEGF Gel Fibrin (pg/mg protein) in HAPRF vs PRF base on intervention.

Table 3. Delta VEGF Gel Fibrin in HAPRF vs PRF base on intervention Gel Fibrin in HAPRF vs PRF base on intervention.

Intervention	HA + PRF (n=10)	PRF (n=10)	p-value
Δ Day 0–3	0.62(-0.02–0.88)	0.12 (0.01–0.84)	0.096
Δ Day 0–7	0.92(0.45–1.87)	0.22 (0.02–1.07)	0.002*

*Significant (p<0.05)

Evaluation VEGF level in HAPRF versus PRF

Figure 4 shows that in the HAPRF group, VEGF levels increased from day-0 to day-7 (0.17 pg/mg protein to 1.12 pg/mg protein), while group PRF also decreased from 0.05 pg/ml protein to 0.17 pg/mg protein) (Figure 4).

In addition to analyzing the VEGF levels between HAPRF and PRF, a delta analysis of changes in day-3 and day-7 was also carried out compared to the initial measurement. In the HAPRF group compared to PRF, there was an increase ΔVEGF gel fibrin levels on day-7 (p=0.002) Mann Whitney test (Table 3).

Clinical appearance of granulation area in HAPRF versus PRF

An analysis of granulation tissue (percentage of granulation area) was performed as measured by imageJ software to see the correlation between inflammation and angiogenesis. The average of granulation area was shown in Figure 5.

In addition to measuring the granulation area of the DFU with topical HAPRF or PRF flow, a delta analysis was also carried out on the change in area gravity from days 3 and 7 compared to the initial measurement (Table 4).

Differences in Pain Scores in DFU Subjects

Numeric Pain Score (NPS) at baseline examination of the two groups was 7-8 (severe pain). After the intervention, pain scores decreased in all two groups but in the HAPRF group there was a decrease on day 3, 7 and 14 compared to the other two groups (Table 5).

Evaluation NPS in HAPRF compare with PRF group on day-3 (p<0.001), day-7 (p=0.007), and day-14 (p=0.002), Mann Whitney test (Figure 6).

DISCUSSION

The Role of Combination of HA with PRF decrease inflammation

Inflammation signs can be tested through serum to see systemic inflammation. In this study, the HAPRF group has decreased Δ IL-6 gel fibrin levels compared to PRF on day-3 (p=0.002) and day-7 (p=0.001). It showed that used topical HAPRF gel

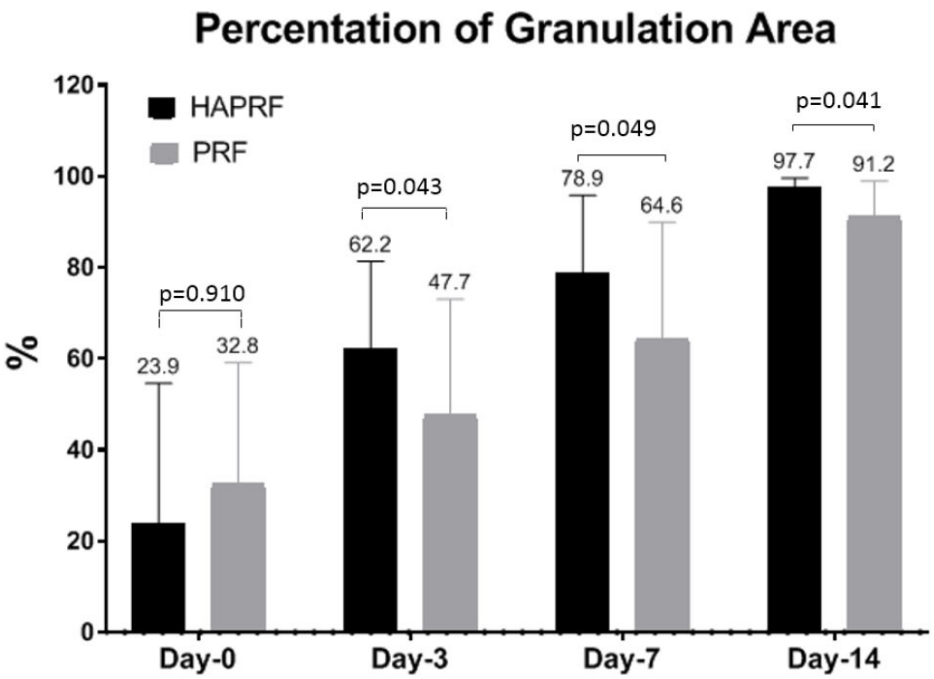

Figure 5. Percentage (%) of granulation area in topical HAPRF vs. PRF in HAPRF vs. PRF base on intervention.

Table 4. Delta VEGF Gel Fibrin in HAPRF vs PRF base on intervention Gel Fibrin in HAPRF vs PRF base on intervention.

Intervention	HAPRF (n=10)	PRF (n=10)	p
Δ Day 0–3	26.0 (SD 8.4)	12.5 (SD 6.2)	<0.001
Δ Day 0–7	41.7 (SD 13.8)	29.0 (SD 9.2)	0.042
Δ Day 0–14	57.7 (SD 14.1)	50.9 (SD 17.6)	0.999

Table 5. Different Numeric Pain Score (NPS) in DFU Base on Intervention.

Intervention	PRF+ HA (n = 10)	A-PRF (n = 10)	p-value
Baseline	8 (8–9	8 (7–8)	0.164
Day-3	4 (3–5)	5 (5–6)	<0.001*
Day-7	2,5 (1–3)	3 (3–5)	0.007*
Day-14	2 (2–3)	2 (2–3)	0.002*

*Significant (p<0.05)

might reduce inflammation di topical DFU.

Hyaluronic acid affects three main receptors in tissue regeneration modulation, namely migration, proliferation, and activation of keratinocyte cells (CD44).[10] This is done to restore the epidermis, fibroblast migration (RHAMM), control of inflammation and neoangiogenesis (ICAM-1), and promotion of ECM deposits such as collagen fibers that contribute to wound healing.[11-19]

Nazirzade et al.[11] reported PRF lysates which incubate conditioned medium has greatly decreased the pro-inflammatory response of primary macrophages with presence of a decrease of IL1β and IL6 level These PRF lysates may cause an M2 phenotype, arginase-1 (ARG1) and YM1 gene expression was measured in primary macrophages.

Afat et al.[12] reported that combine HA and PRF could be used to minimize postoperative edema after mandibular third molar surgery. It also decreases the inflammation in dental surgery. HAPRF gel fibrin reduced edema after oral surgery of the 3rd molar, although HA links with Intracellular adhesion molecule-1 (ICAM,-1) and Vascular Cell Adhesion Molecule (VCAM) receptor. This link will reduce vascular leakage of neutrophil and reduce edema

Russo et al.[13] also reported that combining HA and PRF might decrease synovial inflammation in osteoarthritis compared with PRF alone. The combination of HA+ PRP reduces pro-inflammatory cytokines and increases articular chondrocyte proliferation and chondrogenic differentiation via the HA-dependent Erk1/2 pathway and the PRP-dependent Smad 2/3 pathway. The clinical application of the combination of PRP and AH is more effective than PRP or HA alone; both are therapeutic options for osteoarthritis and chronic tendinopathy.[14]

The Role of Combination of HA with PRF to Accelerate DFU Granulation

In this study, homogenous HAPRF increases the granulation index in day-3 dan day 7 in which HAPRF has increased VEGFgel fibrin compared with PRF on day-7 (Figure 7 and figure 8).

Iio et al.[15] reported that the combination of AH with PRF stimulated growth factors such as TGF-b, significantly increasing the proliferation index and collagen deposition. AH also interacts with the TGF-ß1 transformation of PRF, thereby protecting growth factors from skeptic and collagen degradation by protease enzymes. It is thought that PRP stimulates the healing process of new tissue by producing growth factors and cytokines released by platelets. The addition of HA to PRP can increase the release of growth factor on day 5.[16-19]

Wound healing is initiated and developed by a complex integration process of cellular, physiological, and biochemical events, such as inflammation, cell migration and proliferation. Interleukin-6 is a multifunctional cytokine and can regulate the wound healing process's inflammatory response

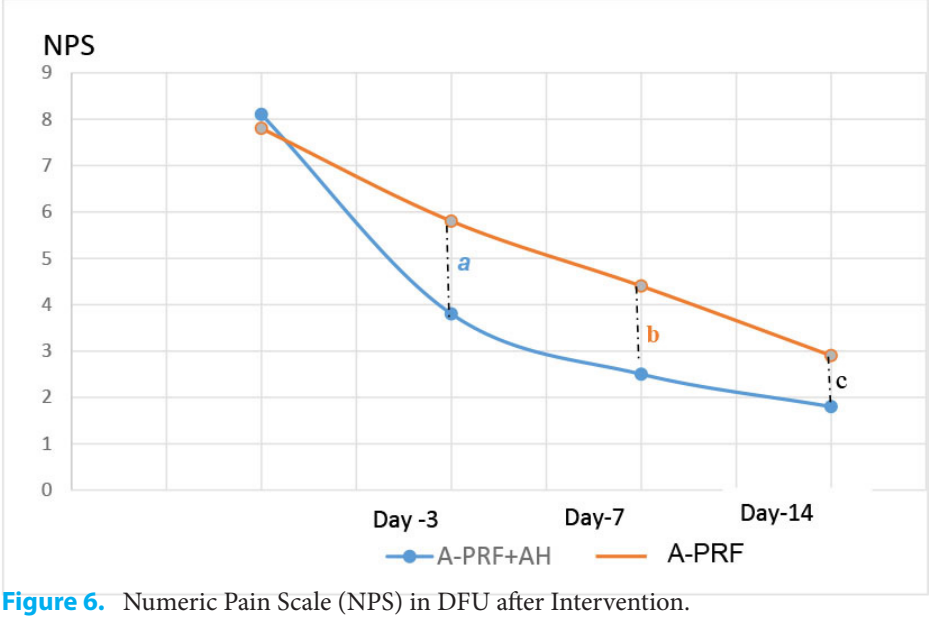

Figure 6. Numeric Pain Scale (NPS) in DFU after Intervention.

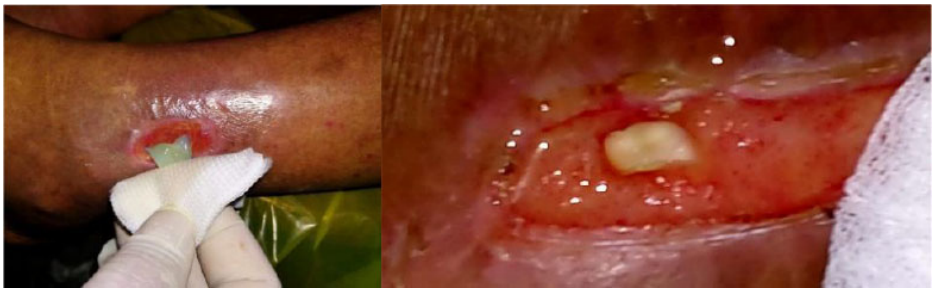

Figure 7. Combination HAPRF in DFU.

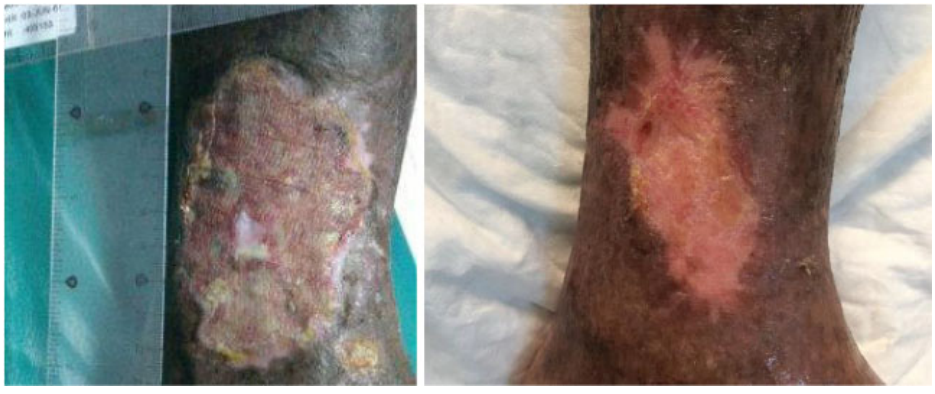

Figure 8. Combination HAPRF in Pre –tibial DFU

in a timely manner. Hyaluronic acid is an important component of ECM and makes a significant contribution to cell proliferation and migration. The combination of PRF and AH provides a synergistic effect on the migration of wound healing cells with ERK (Extracellular signal-51) activation.[17]

Although many studies have shown a significant effect of the combination of HAPRF on wound healing, the results of this study have not demonstrated complete wound closure because there are several influencing factors such as age, uncontrolled blood glucose control, and inadequate HA concentrations and a short length of observation.[18,19,20] The combination of HA+PRF will also synergize to accelerate the polarization of pro-inflammatory M1 macrophages to become anti-inflammatory M2 macrophages, anti-inflammatory properties, immunoregulation, collagen deposit and tissue regeneration.[21,22] HA has shown to increase angiogenesis. CD44 and PKCδ influence the HA-induced angiogenesis mechanism for HA receptor-mediated cell motility (RHAMM). RHAMM receptors are required for HA-promoted cellular invasion and endothelial cell tube formation.[23,24] The bond between PRP and HA will increase P-selectin expression after PRP interacts with HA.[25] In platelets that are not activated P-selectin is stored in α-granules. P-selectin's function as a Cell Adhesion Molecule (CAM) on the surface of endothelial cells lining the inner surface of blood vessels. It seems the added Hyaluronic Acid will increase growth factor release in Platelet Rich Fibrin.[26] Several pathways that a combination of HAPRF can increase angiogenesis and a granulation index tissue are decreasing ECM degradation and epithelial permeability, hinder linking ICAM, VCAM receptor also anti-inflammation in DFU.[27,28] These is proposed mechanism of how a combination of HAPRF may increase granulation index and decrease pain score (Figure 9).

CONCLUSIONS

Topical combination therapy HAPRF increased angiogenesis and granulation index significantly better on day-14 than PRFalone in the treatment of DFU. It was described by a significant increase in VEGF and PDGF in the HAPRF compared to PRF alone in day-14. The proposed mechanism is increased angiogenesis and anti-inflammation. Topical HAPRF application may be considered in DFU therapy with the same characteristics because it effectively accelerates the formation of granulation tissue. From this proposal, this mechanism can explain the role of the combination of HAPRF in accelerating granulation tissue formation and reducing pain in the healing process of DFU, but further studies are needed to determine the optimal dose of HA when

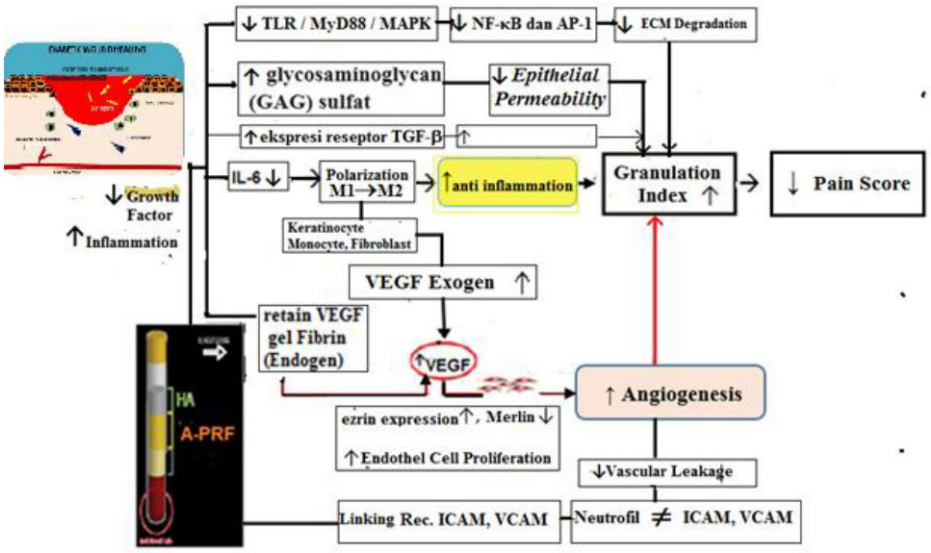

Figure 9. Proposed mechanism HAPRF increase angiogenesis and granulation tissue.[27,28]

combined with A-PRF.

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CONFLICT OF INTEREST

All author declares there is no conflict of interest regarding the publication of this study.

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AUTHOR CONTRIBUTION

Ronald W. Kartika: first author: data collecting, writing, statistics. Idrus Alwi: advisor in writing. Franciscus D. Suyatna: advisor in writing, pharmacology references. Em Yunir: advisor in writing, diabetic and metabolic endocrine references. Sarwono Waspadji: advisor in writing. Suzzana Immanuel: advisor in writing, clinical pathology references. Todung Silalahi: advisor in writing. Saleha Sungkar: advisor in writing. Jusuf Rachmat: advisor in writing, angiogenesis references. Saptawati Bardosono: advisor in writing, statistic references. Mirta Hediyati Reksodiputro: (Corresponding Author) advisor in writing.

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