Wound Healing in Diabetic Foot Ulcer Patients Using Combined Use of Platelet Rich Fibrin and Hyaluronic Acid, Platelet Rich Fibrin and Placebo: An Open Label, Randomized Controlled Trial

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ABSTRAK

Latar belakang: *Manajemen perawatan standar luka kaki diabetes (LKD) terdiri atas debridemen, kompres NaCl, kontrol infeksi dan kontrol glikemik yang ketat. Saat ini penggunaan autologous platelet-rich fibrin (PRF) dianggap sebagai metode tambahan untuk mengobati LKD. Tujuan penelitian ini untuk mengevaluasi peran kombinasi A-PRF + hyaluronic acid (HA) dalam pengobatan LKD.* ***Metode:*** *Desain penelitian adalah RCT open label yang dilakukan di RSUD Koja dan RS Gatot Soebroto pada Juli 2019 - April 2020. Pasien LKD dengan durasi luka tiga bulan, Wagner-2 dengan ukuran ulkus <40 cm^2 diikutsertakan dalam penelitian ini. Subjek dibagi secara acak menjadi tiga kelompok, yaitu kelompok A-PRF+AH, A-PRF dan NaCl 0,9% sebagai kontrol masing-masing 10 subjek. Biomarker VEGF dan IL-6 diambil dari LKD dengan usap lidi kapas dan dianalisis menggunakan ELISA. Pemeriksaan biomarker dan indeks granulasi pada hari ke-0, ke-3 dan ke-7. Data dianalisis menggunakan SPSS versi 20 dan diuji dengan Anova dan Kruskal Wallis untuk membandingkan pengaruh angiogenesis dan inflamasi pada ketiga kelompok sedangkan indeks granulasi diukur dengan ImageJ.* ***Hasil:*** *Pada kelompok A-PRF+AH terjadi peningkatan signifikan VEGF pada hari ke-0 (232,8 pg/mg protein) hingga ke-7 (544,5 pg/mg protein) dibandingkan A-PRF pada hari ke-0 (185,7 pg/mg protein) sampai ke-7 (272,8 pg/mg protein), dan kontrol pada hari ke-0 (183,7 pg/mg protein) sampai ke-7 (167,4 pg/mg protein).Didapatakan peningkatan VEGF pada kelompok A-PRF-HA dibandingkan kelompok A-PRF dan kontrol NaCl pada hari ke-3 (p= 0,022) dan ke 7 (p = 0,001) IL-6 menurun signifikan pada kelompok A-PRF+AH dari hari ke-0 (106,4 pg/*

mg protein) sampai ke-7 (88,7 pg/mg protein) dibandingkan PRF dari hari ke-0 (91,9 pg/mg protein) sampai ke-7 (48,8 pg/mg protein) dan IL-6 meningkat pada kelompok NaCl hari ke-0 (125,3 pg/mg protein) sampai ke-7 (167,9 pg/mg protein). Didapatakan penuruan IL-6 pada kelompok A-PRF-HA dibandingkan kelompok A-PRF dan kontrol NaCl pada hari ke- 7 (p = 0,041). ***Kesimpulan:*** *Kombinasi A-PRF+HA dapat meningkatkan angiogenesis dan mengurangi inflamasi pada LKD sehingga dapat menjadi metode terapi baru pada LKD.*

Kata kunci*: ulkus kaki diabetik, fibrin kaya trombosit, asam hialuronat.*

ABSTRACT

Background: *Autologous platelet-rich fibrin (A-PRF) is an adjunctive method for diabetic foot ulcer (DFU) in addition to glycaemic control and debridement. This study aimed to evaluate the role of A-PRF + hyaluronic acid (HA), A-PRF and sodium chloride 0.9% (control) in DFU wound healing. Nowadays, the use of PRF autologous consider as adjuvant therapy in DFU treatment.* ***Methods:*** *This open-label randomized controlled trial was conducted at Koja District Hospital and Gatot Soebroto Hospital from July 2019 to April 2020. DFU patients with wound duration of three months, Wagner-2, and ulcer size < 40 cm^2 were recruited and randomly assigned into A-PRF + AH, A-PRF and control group. On day-0, day-3 and day 7, samples and photographs were taken. Samples were analysed with ELISA and photographs were analysed with ImageJ to calculate granulation index (GI). Statistical analysis was performed using SPSS version 20.* ***Results:*** *Topical therapy with A-PRF + AH was associated with a significant increase in VEGF from day 0 (232.8 pg/mg) vs day 7 (544.5 pg/mg) compared to A-PRF on day 0 (185.7 pg/mg) vs day 7 (272.8 pg/mg), and the controls on day 0 (183.7 pg/mg) vs day 7 (167.4 pg/mg). On evaluation of VEGF swab, there is increasing significantly in A-PRF+HA group compare others group in day 3 (p=0.022) and day 7 (p= 0.001).In the A-PRF + AH group, there was a significant decrease in IL-6 from day 0 (106.4 pg/mg) vs day 7 (88.7 pg/mg) compared with PRF on day 0 (91.9 pg/mg) vs day 7 (48,8 pg/mg). IL-6 was increased in the control group from day 0 (125.3 pg/mg) vs day 7 (167.9 pg/mg). On evaluation of IL-6 swab, there is decreasing significantly in A-PRF+HA group compare others group in day 7 (p= 0.041).* ***Conclusion:*** *The PRF + HA combination increased angiogenesis and reduced inflammation in DFUs and may represent a new DFU therapy.*

Keywords*: diabetic foot ulcer; platelet-rich fibrin; hyaluronic acid.*

INTRODUCTION

Diabetic foot ulcers (DFUs) are challenging to health care professionals due to limited availability of effective topical therapeutic interventions. Growth factor treatment has shown to be beneficial for healing DFUs in conjunction with extensive surgical debridement.[1] Neuropathy and peripheral vascular disease are two major causal factors of foot ulcers in patients with diabetes.[2]

The major problem with DFUs is the length of time needed to heal. There is chronic inflammation that show elevated levels of matrix metalloproteinase (MMPs) and tissue inhibitor of metalloproteinase (TIMP), which significantly contribute to delayed healing.[3] Inflammatory cells release cytokines, including interleukins (e.g., IL-1 and IL-6) and tumour necrosis factor-α (TNF-α).[4] Neuro-ischemic disease in DFUs limits oxygen and nutrients supply to the wound, preventing wound healing. Due to the lack of oxygen and nutrients, epithelial cells at the wound site are unable to express essential factors for healing, such as vascular endothelial growth factor (VEGF) and platelet derived growth factor (PDGF).[5] The decreased levels of growth factor will delay the healing process.[6] As an adjunct to topical DFU therapy, a dressing is needed that can contain growth factors and reduce inflammation.

Platelet rich plasma (PRP) has been used for wound healing, but it requires a fairly complicated process and there may be an allergic effect from CaCl2 which is needed to activate plasma platelets. Thus, the second generation of PRP, namely platelet rich fibrin (PRF) have been developed. It has been proposed as an

adjunct for the treatment of DFUs and other chronic and acute wounds.[7] The alpha granules of platelets contain growth factors, including PDGF, VEGF, and transforming growth factor-β (TGF-β), which stimulates cell proliferation and differentiation that results in new tissue formation.[8] For over 20 years, PRF gel has been used to stimulate wound healing.[6] Autologous PRF gel consists of cytokines, growth factors, chemokines, and a fibrin scaffold derived from the patient's blood.[7,8] Advanced-PRF (A-PRF) contains additional growth factors that results in faster and better wound healing.[9] Since patients with DFUs have low growth factors and prolonged inflammation, hyaluronic acid (HA) is added to A-PRF to optimize growth factor release and control inflammation.[10] HA is a glycosaminoglycan that can enhance angiogenesis, wound healing, as well as reduce chronic inflammation in DFUs[11]

The combination of A-PRF + HA has not been used for the treatment of DFUs. Therefore, this study is required to identify the effect of combined A-PRF and HA for the treatment of DFUs.

METHODS

We conducted an open-label randomized controlled trial from July 2019 to April 2020. Informed consent was obtained from patients, including the use of photographs (ImageJ). This study was approved by The Ethics Committee of the Faculty of Medicine Universitas, Indonesia (ID 0855/UN2.F1/ETIK/2018).

The study was conducted at Koja District Hospital and Gatot Soebroto Hospital, Jakarta. Diabetic patients with chronic DFUs with an average wound duration of three months, categorized as Wagner 2, ulcer < 40 cm^2 were included in the study. All patients had their blood sugar under control with an oral antidiabetic in the range of 150 – 200 mg/dl. The HbA1c range was 6 – 7.5 mg/dl. Patients exhibiting the following criteria were excluded: platelet dysfunction syndrome, critical thrombocytopenia, unstable hemodynamics, and pregnancy. Patients who failed to complete follow up visits were dropped out.

A-PRF + HA Preparation

A-PRF was used in the form of a gel. To make the A-PRF gel, 20 – 40 mL venous blood was centrifuged at 200 × G for 8 minutes to obtain the fibrin, buffy coat, and erythrocyte layers.

The erythrocyte layer was carefully removed from the buffy coat with sterile scissors and transferred to a new sterile tube. A-PRF was formed by the fibrin and buffy coat, in which platelets (rich growth factor and cytokine) were entrapped in the fibrin.

To make homogenous A-PRF + HA gel, 1 mL A-PRF was mixed with 0.6 mL 0.2% HA using a vortex machine for approximately 20 seconds.

Wound Treatment and Monitoring

In this study, the subjects were divided into three groups: 1) A-PRF + HA; 2) A-PRF; and 3) 0.9% sodium chloride (NaCl) as a control.

A-PRF + HA was used as a single application to the surface of the wound and covered with a protective bandage. Similar procedures were performed in other groups. The wounds were photographed before and after treatment using a digital camera. Images were uploaded and analysed using ImageJ. Granulation Index (GI) was calculated using red-yellow-black analysis.

Biomarkers of Wound Healing

To identify the process of angiogenesis and inflammatory conditions during DFU healing, samples were collected using a cotton swab from the DFU surface before topical therapy. The level of VEGF and IL-6 was measured using ELISA.

The data were analysed using SPSS version 20. The association between the variables was analysed using an ANOVA and Kruskal Wallis test.

RESULTS

We recruited 36 DFU patients in Metabolic Endocrine Internal Medicine Out Patient Discharge (OPD). From the 36 subjects, only 30 subjects satisfied the inclusion criteria and has completed follow-up. The subjects, consisted of 12 men and 18 women. The median age was 64 years in women and 61 years in men. The subjects were randomly divided into three groups (A-PRF + HA, A-PRF, and control) comprised of 10 subjects per group.

Table 1. Subjects baseline characteristic.

Characteristic	A-PRF + HA (n = 10)	A-PRF (n = 10)	Control (n = 10)
Age (year)[a]	59.8 (SD 12.7)	64.7 (SD 12.0)	66 (36–71)
Sex n (%)			
Male	5/10	4 (40)	3 (30)
Female	5/10	6 (60)	7 (70)
BMI[a]	28.9 (SD 2.7)	27.3 (SD 2.08)	28.4 (SD 2.5)
Haemoglobin (g/dL)	12.7 (27.4−39.0)	13.1 (SD 1.3)	12.05 (10.1−16.5)
Haematocrit e (%)	36.3 (29.2−42.9)	35.6 (SD 4.6)	33.8 (24.4−40.8)
Leucocyte (10^3/µL)[a]	13.30 (SD 1.08)*	11.08 (SD1.33)	9.23 (SD 1.66)
Platelet (10^3/µL)[a]			
Random Blood	354.9 (SD 167.5)	338.8 (SD 164.5)	319.9 (SD 128.4)
Glucose mg/dL[b]	286.0 (170−390)	243.8 (SD 47.4)	254.7 (SD 58.6)
HbA1C (%)[a]	11.34 (SD 1.30)	9.0 (SD 0.68)	8.5 (SD 0.72)
Cholesterol total (mg/dL)	214.5 (SD16.9)	249.3 (SD_16.1)	202.3 (SD 38.6)
Albumin (mg/dL)	3.3 (2.8–4.2)	3.1 (2.8–4.2)	3.2 (SD 0.39)

[a]mean (SD), ANOVA test
[b]median (min-max), Kruskal Wallis test

Biomarker Output

Table 2 shows the level of VEGF collected from the DFU swabs. At baseline, the VEGF levels were not significantly different among the three groups, meaning that the data was homogenous. The evaluation on day three and day 7 showed a significant increase in the VEGF levels in the A-PRF + HA group compared with the A-PRF and control groups.

The VEGF levels were increased in the A-PRF + HA group from day-0 to day 3 (232.8 pg/mg to 320.6 pg/mg) and to day 7 (232.8 pg/mg to 544.5 pg/mg). In the A-PRF group, there was a decrease in the VEGF levels on day 3 (185.7 pg/mg to 180.4 pg/mg) but an increase on day 7 (185.7 pg/mg to 272.8 pg/mg). In the control group, there was also a decrease in the VEGF levels on day 3 (183.7 pg/mg to 144.8 pg/mg) and day 7 (183.7 pg/mg to 167.4 pg/mg). Table 2 shows an evaluation of VEGF swab. There is a significant increase in the A-PRF+HA group compared to others groups in day 3 (p=0.022) and day 7 (p= 0.001). In the subgroup analysis using Post-hoc ANOVA test, in day 3, A-PRF+HA increase significantly compare with A-PRF (p=0.014) and control (p=0.003). In day 7, A-PRF+HA increase significantly compare with A-PRF (p=0.002) and control (p<0.001). Meanwhile increases in the A-PRF group was no significant compared with controls on day 3 (p=0.612) and day 7 (p=0.186)

Another method of evaluating inflammation is through detecting base VEGF increase before therapy. **Figure 1** shows the delta of the VEGF based on each intervention. The delta of the VEGF levels in the A-PRF + HA group increased: Δ 0−3 (43.1 pg/mg) and Δ 0−7 (275.8 pg/mg). In the A-PRF group, there was an increase in Δ0−3 (1.8 pg/mg) and 0−7 (104.7 pg/mg). In the control group, there was a decrease in Δ 0−3 (4.9 pg/mg) and an increase Δ 0−7 (28.3 pg/mg). In the A-PRF + HA group, there was an increase in ΔVEGF compared to the A-PRF and control groups on day 3 (p = 0.003) and day 7 (p < 0.001).

Following the intervention (**Table 3**), there was a decrease in IL-6 in the A-PRF + HA group

Table 2. Mean VEGF in different treatment groups

Intervention	A-PRF + HA Mean (SD)	A-PRF Mean (SD)	Control Mean (SD)	P
Day 0	232.8 (125.7)	185.7 (100.8)	183.7 (127.2)	0.568
Day 3	320.6 (165.8)	180.4 (87.4)	144.8 (87.7)	**0.022**
Day 7	544.5 (266.8)	272.8 (97.7)	167.4 (98.8)	**0.001**

mean (SD) , anova test

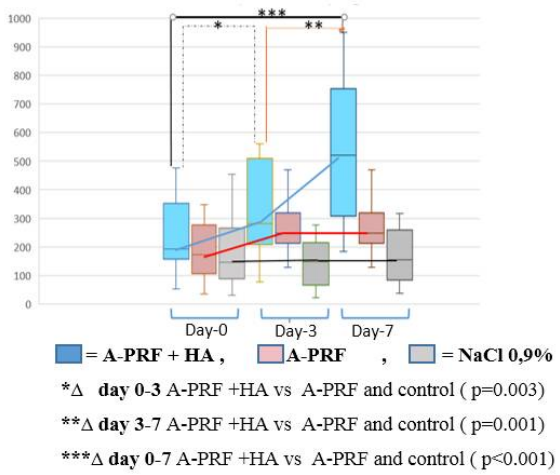

Figure 1. ΔVEGF based on intervention group.

on day 3 (106.4 pg/mg to 99.5 pg/mg) and day 7 (106.4 pg/mg to 88.7 pg/mg). In the PRF group, the IL-6 levels decreased on day 3 (91.9 pg/mg to 72.8 pg/mg) and day 7 (91.9 pg/mg to 4 pg/mg). In the control group, the IL-6 levels increased on day 3 (125.3 pg/mg to 131.1 pg/mg) and day 7 (125.3 pg/mg to 167.9 pg/mg). IL-6 was significantly decreased in the A-PRF + HA group compared with the A-PRF and control groups on day 7 (p = 0.041). In the subgroup analysis using Post-hoc ANOVA test, in day 3, there is a significant decrease in the A-PRF+HA group compared to controls (p=0.049). In day 7, A-PRF+HA group also had a significant decrease compared to A-PRF group (p=0.023) and controls (p=0.041).

Another method of evaluating inflammation is through detecting base VEGF increase before therapy. **Figure 2** shows the ΔIL-6 each intervention based on a baseline. In the A-PRF + HA group, there was a decrease in Δ 0−3 (10.9 pg/mg), and Δ 0−7 (18.3 pg/mg). In the A-PRF group, there was a decrease in Δ 0−3 (3.7 pg/mg), and Δ 0−7 (7.8 pg/mg). In the control group, there was a decrease in Δ 0−3 (4.3 pg/mg) and Δ 0−7 (35.5 pg/mg).

In the A-PRF + HA group, there was a decrease in ΔIL-6 compared with the PRF and control groups on day 3 (p = 0.018) and day 7 (p = 0.015).

Clinical Outcomes

Table 4 shows that the granulation index increased significantly in the A-PRF + HA group compared with the A-PRF and control groups on day 3 and day 7.

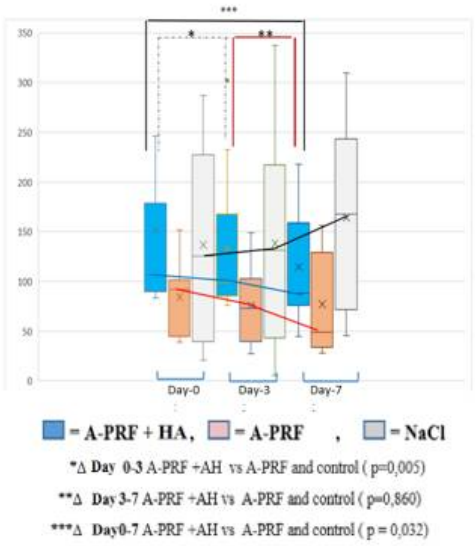

Figure 2. Δ IL-6 based on intervention group.

Table 3. Mean level of IL-6 from DFU swab following intervention.

Intervention	A-PRF + HA median (min-max)	A-PRF median (min-max)	Control median (min-max)	P
Day 0	106.4 (83.1−407.6)	91.9 (38.6 −151.6)	125.3 (20.3−287.0)	P=0.287
Day 3	99.5 (76.3−302.2)	72.8 (27.1−148.9)	131.1 (5.3−337.5)	p=0.141
Day 7	88.7 (44.3−217.9)	48.8 (27.7−156.2)	167.9 (27.7−156.2)	p=0.041*

(min-max), Kruskal Wallis test

Table 4. Mean of Granulation Index Based on Intervention

Intervention	A-PRF + AH	A-PRF	Control	p
Day 0	42.1 (18.4–57.8)	33.6 (14.1–58.9)	42.6 (19.6−52.6)	0.921
Day 3	83.1 (58.9–101.9)	68.3 (28.083.19)	59.7 (37.2–75.7)	0.004
Day 7	97.4 (94.7−99.8)	81.9 (48.4–96.8)	66.0 (47.7–86.4)	< 0.001

*Data median (min-max), Kruskal Wallis

DISCUSSION

The use of topical A-PRF + HA induced the formation of healthy granulation tissue and allowed successful granulation of the wound area. Improvement of the granulation tissue was observed because A-PRF works synergistically with HA to increase the release of growth factors and to trigger angiogenesis. In addition, the combination of A-PRF + HA decreases inflammation as shown by the decrease in IL-6.[12]

Growth Factor Release from A-PRF +HA

Type 2 Diabetes Mellitus is associated with chronic hyperglycaemia and can often cause tissue injury. Dysregulation of growth factors in DM occurs through biochemical and hemodynamic pathways. In some tissues affected by DM, excessive growth factors are induced, whereas a relative deficit of growth factors occurs in other sites. The changes in growth factors contribute to tissue pathology, including fibrosis, persistent inflammation, or a combination of the two.[13]

In this study, there is significant increase of VEGF in the swab sample in A-PRF+HA group compare with A-PRF and control NaCl on day-3 ($p=0.022$) and day 7 ($p=0.001$).

The increase of VEGF level in A-PRF+HA cause granula -α of platelets trapped in HA and slow release growth factors. In an *in vitro* study, some growth factors (PDGF, VEGF and TGF-β) contained in PRF incubated in Dulbecco's modified Eagle's Medium (DMEM) were able to survive over 14 days of observation, with a peak on day 7.[17] The addition of HA to PRF increases the release rate of VEGF because VEGF released from PRF will adhere to the HA and be slowly distributed onto the DFU surface. Adding VEGF to the DFU will stimulate endothelial cells to undergo angiogenesis and stimulate the formation of granulation tissue.[18] Furthermore, it was observed that the new formulation of A-PRF released significantly higher total quantities of growth factors when compared to traditional PRF. In A-PRF, using low time (only 10 minutes) and low speed (200G) settings on the centrifuge prevents platelet breakdown. The addition of HA will induce the release of growth factors from A- so that it can work more effectively.[19]

In the present study, adding HA to the PRF increased the concentration of the released growth factors.[19,20] This study showed that VEGF levels would increase on day 3 and day 7, evidently filling the literature gap of the effect of combined use of PRF and HA.

The addition of HA to PRF can increase the selective permeability of activated platelet α granules. This causes changes in inflammation, angiogenesis, and immunity. Therefore, HA administration can also inhibit platelet aggregation and affect the release of growth factors from platelet concentrate.[20]

The Combination of A-PRF + HA Reduces Inflammation

In this study, effect combination HA to A-PRF also decreases the inflammatory progression in DFU, as shown by decreased levels of IL-6 on day 7 ($p=0.041$) compare with A-PRF and NaCl.. These findings conclude that stimulatory effect of HA on growth factor also decreases the inflammatory progression in DFU. Both PRF and HA injections are therapeutic options for osteoarthritis and chronic tendinopathy. Although several studies on PRF and HA have been published, the effects of mixing PRP and HA are not fully understood. PRF can stimulate the healing process of different tissues by delivering various growth factors and cytokines that are released by platelets. Ilio et al.[19] reported that the expression of P selectin was dramatically increased after PRF interacted with a bio macromolecule complex film (HA collagen (I)/chitosan). HA engagement of CD44 leads to increased MAP kinase-dependent trafficking of TGF-β receptors to lipid raft-associated pools, which facilitates increased receptor turnover and attenuation of TGF-β1-dependent alteration in proximal tubular cell function. Further investigation is needed to elucidate the associated mechanism on growth factor delivery.[21]

A-PRF + HA also increases M1 to M2 polarization as an anti-inflammatory effect. The topical application of A-PRF + HA exerts a protective effect by decreasing the release of cytokines (IL-1β, IL-6 and TNF-α) during initial inflammatory process. This effect allows for the acceleration of the inflammatory phase, in the otherwise prolonged inflammation due to hyperglycaemia in DFU.[21]

The topical application of A-PRF + HA will bind to the ICAM-1 and VCAM-1 receptors, which will inhibit the prolonged response of leukocytes (lymphocytes and monocytes) to prevent chronic inflammation.[22] An in vitro study by Chen et al.[23] showed a synergistic effect of anabolic HA and PRP on cartilage regeneration in osteoarthritis. It was also reported that the combination of HA and PRP reduced pro-inflammatory cytokines and increased articular chondrocyte proliferation and chondrogenic differentiation via the Erk1/2 pathway in HA and the Smad2/3 pathway in PRP.[24] **Figure 3** shows the propose mechanism by which A-PRF + HA increases granulation in diabetic foot healing.

Propose Mechanism Combine of A-PRF+HA in DFU Healing

The pathomechanism of A-PRF + HA in increasing the granulation in diabetic foot healing is shown in **Figure 3**.

CONCLUSION

The combination of A-PRF + HA increased the release of VEGF and decreased IL-6 levels on day 3 and day 7. A-PRF + HA elevated granulation index through angiogenesis and anti-inflammatory pathways. Thus, the combination of A-PRF and HA provides beneficial effect in DFU healing process.

ACKNOWLEDGMENTS

This study was a dissertation of the Doctoral Program of Medical Science Universitas Indonesia.

CONFLICT OF INTEREST

This study was funded by Universitas Indonesia, Jakarta.

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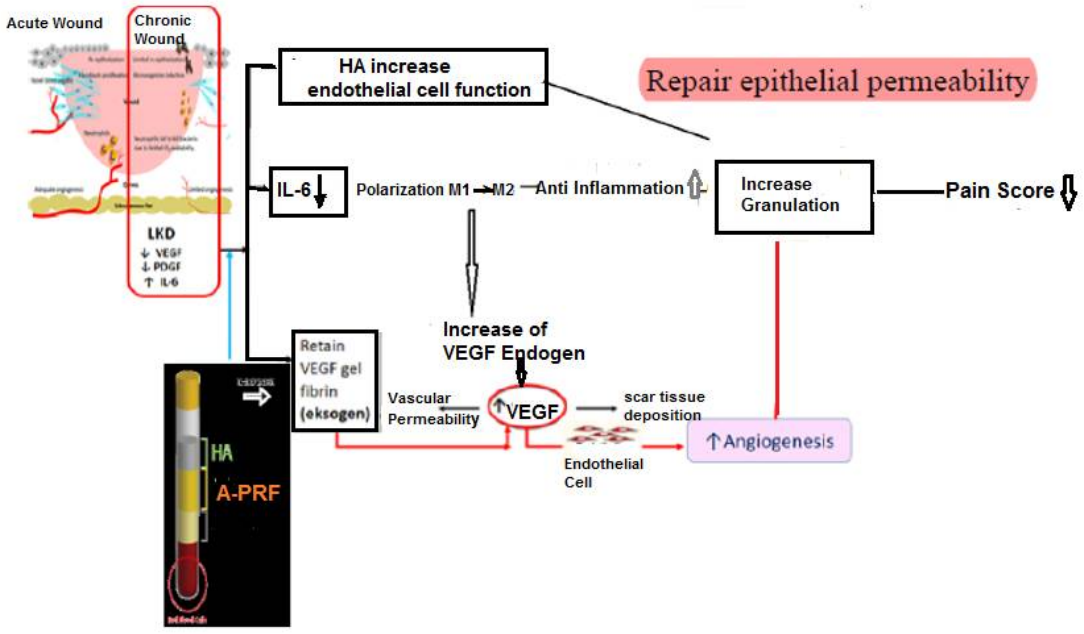

Figure 3. Proposed pathomechanism that allows A-PRF and HA to increase granulation in DFU healing.

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