

RESEARCH ARTICLE

Hyaluronic Acid Accelerates VEGF and PDGF Release from Advance Platelet Rich Fibrin in Diabetic Foot Ulcer

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Received date: Feb 19, 2021; Revised date: Aug 25, 2021; Accepted date: Aug 25, 2021

Abstract

BACKGROUND: Hyaluronic acid (HA) is an essential component of extracellular matrix and mediates signaling in wound healing. HA could induce growth factor release from Advanced Platelet Rich Fibrin (A-PRF), including Vascular Endothelial Growth Factor (VEGF) and Platelet-derived Growth Factor (PDGF). However, concentrations of the released-VEGF and PDGF have not been clearly disclosed. Therefore, current study was conducted to measure the release of these growth factors in HA + A-PRF gel of diabetic foot ulcer (DFU) subjects.

METHODS: Twenty DFU subjects were included in the study and treated with A-PRF or HA+A-PRF. A-PRF was derived from autologous peripheral blood and processed with low-speed centrifugation. HA was added with a ratio of 1:0.6. A-PRF or HA + A-PRF was applied topically on DFU. Upper tips of A-PRF or HA + A-PRF gels were

collected on day 0, 3 and 7 for measurements of VEGF and PDGF concentrations with Enzyme-linked Immune-sorbent Assay (ELISA) methods.

RESULTS: On day-3, both VEGF and PDGF concentrations of HA + A-PRF group were significantly higher than the VEGF ($p=0.000$) and PDGF ($p=0.019$) concentrations of A-PRF group. The VEGF and PDGF concentrations were continuously and significantly increased on day-7 of HA + A-PRF group, compared to the VEGF ($p=0.000$) and PDGF ($p=0.004$) concentrations of A-PRF group.

CONCLUSION: Combination HA+A-PRF induces VEGF and PDGF release from A-PRF. A mixture of A-PRF and HA could be more effective than A-PRF alone for treatment of DFU.

KEYWORDS: hyaluronic acid, advanced platelet rich fibrin, PRF, growth factor, VEGF, PDGF, diabetic foot ulcer

Indones Biomed J. 2021; 13(3): 332-6

Introduction

In tissue regeneration, the damaged tissues could be recovered by using a stable fibrin (1), mesenchymal cells, fibroblasts, and epithelial cells.(2,3) Autologous platelet with high concentrations of fibrin is widely used in tissue regeneration (4-6) as adjuvant in oral and maxillofacial surgery, sports medicine, orthopedic surgery, and aesthetic plastic surgery in the last decade (7-9). To promote tissue healing and regeneration, platelet concentrates are also used to obtain local release of growth factors.(10) In addition, platelet concentrates have been shown to enhance and stimulate the wound healing process and accelerate angiogenesis.(11)

Platelet concentrates are classified as platelet rich plasma (PRP) or platelet rich fibrin (PRF). Several studies have reported the suboptimal benefit of autologous platelet gel in the healing of diabetic foot ulcer (DFU). This is due to the low level of growth factor contained in the platelet gel, hyperglycemia, chronic inflammation, and high protease enzymes on the surface of DFU.(12) Therefore, better PRF quality should be produced. With low-speed centrifugation, the red blood cell, and its components such as fibrin, platelets, growth factors, leukocytes, and other circulating cytokines and proteins, can be separated and concentrated, so that advanced PRF (A-PRF) could be obtained.(13,14)

Hyaluronic acid (HA) is an essential component of extracellular matrix (ECM), therefore the addition of HA in A-PRF could improve wound healing-related ECM signaling.(15) Hyaluronic acid was reported to induce growth factors release for angiogenesis such as Vascular Endothelial Growth Factor (VEGF) and Platelet-derived Growth Factor (PDGF) (16), which will be crucial in DFU healing. However, concentrations of the released-VEGF and PDGF have not been clearly disclosed. Therefore, current study was conducted to measure the release of these growth factors in HA and A-PRF gel of DFU subjects.

Methods

Study Design

An open label randomized controlled study had been conducted from July 2019 until April 2020 in Koja District Hospital, Jakarta and Gatot Soebroto Army Hospital, Jakarta. Informed consent was obtained from all subjects. DFU subjects with ulcer area <40 cm², categorized as

Wagner 2, were included. Other inclusion criteria were blood sugar under control within the range of 150-200 mg/dL, taking oral antidiabetic medication, and hemoglobin A1c (HbA1c) within 6-7.5 mg/dL. Meanwhile, subjects with platelet dysfunction syndrome, thrombocytopenia, thrombotic thrombocytopenic purpura (TTP), unstable hemodynamic or pregnancy, were excluded.

Twenty mL of peripheral blood was withdrawn on selected subjects, followed by A-PRF production. A-PRF with/without HA were used to treat DFU topically. The study protocol was approved by The Ethics Committee of the Faculty of Medicine Universitas Indonesia (No. 0855/UN2.F1/ETIK/2018).

The A-PRF and HA+ A-PRF Fibrin Gels Production

Blood was centrifuged at 200xg for 8 minutes. Resulted erythrocytes layer was carefully removed. A-PRF was gradually formed by fibrin and buffy coat. Fibrin clot formed in center of the tube was collected and used for the treatment. For making the mixture of A-PRF and HA, 1 mL of A-PRF was added with 0.6 mL of 0.2% HA in vaseline and vortexed. The mixture was placed in a clean cup and ready to be used for the treatment.

Measurement of VEGF and PDGF Concentrations

To measure the concentration of VEGF and PDGF, on each day 0, 3 and 7, small samples were collected in transfer medium containing 0.9% NaCl by cutting the upper tips of A-PRF and HA+A-PRF fibrin gels. The samples were centrifuged, lysed, and kept in -70°C storage. For VEGF measurement, Human VEGF Enzyme-linked Immunosorbent Assay (ELISA) Kit (Catalog No.: MBS355343, MyBiosource, San Diego, CA, USA) was used. Meanwhile for PDGF measurement, Human PDGF-AA ELISA Kit (Catalog No.: MBS2506128, MyBiosource) was used. Briefly, both kits were based on sandwich ELISA technology with detection range of 31.2-2000 pg/mL (for VEGF) and 15.63-1000 pg/mL (for PDGF), sensitivity: <1 pg/mL (for VEGF) and 9.38 pg/mL (for PDGF). Anti-VEGF or anti-PDGF-AA polyclonal antibody was pre-coated onto 96-well plates and the biotin conjugated anti-VEGF or anti-PDGF-AA polyclonal antibody was used as detection antibodies. Avidin-Biotin-Peroxidase Complex was added. The 3,3',5,5'-Tetramethylbenzidine (TMB) substrate was catalyzed by HRP to produce a blue color product that changed into yellow after adding acidic stop solution. The optical density (OD) of yellow was measured at 450 nm in a microplate reader, and then the concentration of VEGF and PDGF were calculated.

Statistical Analysis

All calculated datas of VEGF and PDGF were presented as mean±SD. Statistical analyses were performed using SPSS for Windows software version 20 (IBM, Armonk, NY, USA). The data were analysed using SPSS version 20. The *p*-value<0.05 was considered as significant.

Results

Twenty subjects with DFU were selected and randomly divided into two groups for A-PRF or HA + A-PRF fibrin gel application. A-PRF fibrin gel-treated subjects were 6 women and 4 men with average age of 64, while HA + A-PRF-treated subjects were 5 women and 5 men with average age of 59.

VEGF Concentration in Fibrin Gel of A-PRF and HA + A-PRF Groups

As shown in Figure 1, on day-0, the VEGF concentration of A-PRF group (137.11±119.45 pg/mL) was lower than the VEGF concentration of HA + A-PRF group (181.75±160.87 pg/mL), but not significant (*p*=0.226, Mann-Whitney test). On day-3, VEGF concentration of HA + A-PRF group (910.62±307.35 pg/mL) was significantly higher (*p*=0.000, Mann-Whitney test) than the one of A-PRF group (279.99±141.49 pg/mL). The VEGF concentration was continuously and significantly increased on day-7 (*p*=0.000, Mann-Whitney test), the VEGF concentration of HA + A-PRF group (1,105.60±344.39 pg/mL) compared to the one of A-PRF group (436.16±269.93 pg/mL). The VEGF concentrations were further analyzed by measuring the increment (Δ) of VEGF concentration on day-3 and day-7 with subtraction of baseline (day-0) (Table 1). The Δ VEGF

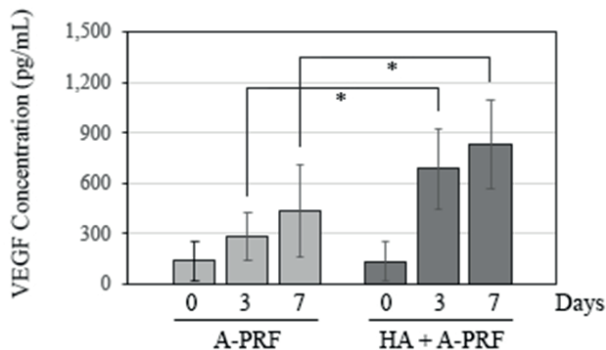


Figure 1. VEGF concentrations of fibrin gels. After the treatment of A-PRF or HA + A-PRF for 0, 3 or 7 days, upper tips of fibrin gel were collected and processed for ELISA to detect VEGF according to Methods. **p*<0.05, Mann-Whitney test.

concentration of HA + A-PRF group was significantly higher than the one of A-PRF group for both Δ 0-3 and Δ 0-7.

PDGF Concentration in Fibrin Gel of A-PRF and HA + A-PRF Groups

PDGF concentration baseline (day-0) of A-PRF group (135.18±127.34 pg/mL) was lower than the PDGF concentration of HA + A-PRF group (164.48±153.66 pg/mL), but not significant (*p*=0.880, Mann-Whitney test) (Figure 2). On day-3, PDGF concentration of HA + A-PRF group (640.56±173.31 pg/mL) was significantly higher (*p*=0.019, Mann-Whitney test) than the one of A-PRF group (405.36±228.72 pg/mL). The PDGF concentration was also continuously and significantly increased on day-7 (*p*=0.004, Mann-Whitney test), the PDGF concentration of HA + A-PRF group (876.27±257.53 pg/mL) compared to the one of A-PRF group (479.57±236.58 pg/mL). Meanwhile, Δ PDGF concentration of HA + A-PRF group was also significantly higher than the one of A-PRF group for both Δ 0-3 and Δ 0-7 (Table 2).

Discussion

Type 2 Diabetes Mellitus (T2DM) is often associated with chronic hyperglycemia which can lead to inhibition of wound healing. For DFU treatment, topical growth factors are important, since the chronic diabetes patients has a decrease growth factors and prolonged inflammation in which will inhibit healing.(17,18) In current study, the topical growth factor was provided from PRF obtained from the blood of the subjects (autologous).

Although several studies on the HA and PRF have been published, the effects of mixing PRF and HA have not been fully understood. PRF can stimulate the healing process of different tissues by delivering various growth factors and cytokines that are released by platelets.(19) In current study, adding HA to the PRF increased the concentration of VEGF

Table 1. Δ VEGF concentrations of fibrin gels. VEGF concentrations of A-PRF or HA+A-PRF for 3 and 7 days were subtracted with the concentrations of A-PRF or HA+A-PRF for 0 day. The *p*-values were the results of Mann-Whitney test.

Δ Day	A-PRF (pg/mL)	HA + A-PRF (pg/mL)	<i>p</i> -value
0-3	142.87±115.37	728.87±311.98	0.000
0-7	299.04±281.77	923.85±419.71	0.002

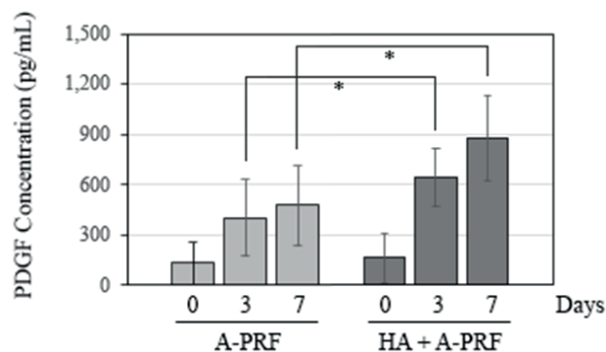


Figure 2. PDGF concentrations of fibrin gels. After the treatment of A-PRF or HA+A-PRF for 0, 3 or 7 days, upper tips of fibrin gel were collected and processed for ELISA to detect PDGF according to Methods. * $p < 0.05$, Mann-Whitney test.

and PDGF release on day-3 and day-7, which possibly could induce the effect of A-PRF on DFU.

Signal transduction plays important roles in inducing cell proliferation both in tissue regeneration and malignancy. (20-22) HA which is used in this study, could induce Cluster of Differentiation (CD)44 and Receptor for Hyaluronan Mediated Motility (RHAMM) for angiogenesis transduction in vascular endothelial cells.(23) RHAMM-ligand interaction of endothelial cells will increase endothelial cell motility, and CD44-ligand interaction increases endothelial cell proliferation.(24) Both CD44 and RHAMM work in tandem to facilitate formation of new blood vessels. HA also activates several CD44-dependent isoforms such as Protein Kinase C (PKC), Raf-1 kinase, Mitogen-activated Protein/ Extracellular Signal-regulated Kinase Kinase (MEK)-1, and Extracellular Signal-regulated Kinase (ERK)1/2, so that it will increase endothelial cell proliferation.(25) In this study, compare to A-PRF merely, HA + A-PRF significantly increased PDGF release from fibrin gel on day-3 and day-7.

Diabetes in chronic hyperglycemia has reduced capacity in the proliferation and synthesis of collagen because it is unresponsive to transformations of growth factor- β 1 (TGF- β 1) stimulation. Either platelet rich fibrin (PRF)-lysate or hyaluronic acid (HA) can restore the TGF- β 1 signaling pathway. Improving TGF- β 1 signaling was measured by the cellular proliferation index and collagen deposition. The addition of HA to PRF-lysate resulted in a significant increase in the proliferation index and collagen deposition index rather than PRF-lysate alone.(26)

In an *in vitro* study, a mixture of PRF and 3% HA was reported to increase the release of TGF- β , PDGF-BB and FGF. In the combination HA + A-PRF, can cause the platelets to be more permeable, which triggers the release of growth factors by α -granules.(27)

Table 2. Δ PDGF concentrations of fibrin gels. PDGF concentrations of A-PRF or HA+A-PRF for 3 and 7 days were subtracted with the concentrations of A-PRF or HA+A-PRF for 0 day. The p -values were the results of Independent Sample T test.

Δ Day	A-PRF (pg/mL)	HA + A-PRF (pg/mL)	p -value
0-3	270.19 \pm 174.57	476.08 \pm 181.94	0.019
0-7	344.39 \pm 292.66	711.79 \pm 328.50	0.017

Conclusion

The concentration of growth factors released by A-PRF on day-3 day-7, were increased by the addition of HA. Taken together, HA accelerates growth factor release from A-PRF. A mixture of A-PRF and HA could be more effective than A-PRF alone for treatment of DFU.

Acknowledgements

This study was funded by Medical Science Doctoral Programme, Universitas Indonesia.

Authors Contribution

RWK, IA, FDS, EY, and SB designed the study. RWK collected the study data. RWK, IA, FDS, FS, EY, and SB did the statistical analysis. RWK, IA, FDS, FS, EY, SI, TS, JR, SB, and MHR interpreted the data. All authors contributed in preparing the manuscript. FDS and FS gave writing advice. FDS collected the study fund.

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