

The Potential of Human Wharton's Jelly Mesenchymal Stem Cells Secretome Based Topical Gel for Therapeutic Application

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Abstract

Background: Diabetic Foot Ulcer (DFU) might be worsened by neuropathy and vascular issues. This condition can cause 14.3% fatality, stressing the need for effective wound healing therapy. Wound healing is a complex biological process, and human Wharton's Jelly Mesenchymal Stem Cells (hWJMSCs) may help manage DFU treatment issues. This research focuses on utilizing a gel carrier to deliver bioactive substances from Wharton's Jelly Mesenchymal Stem Cells secretome (hWJ-MSCs-Sec) as a possible treatment for DFU.

Methods: To maintain quality, hWJMSCs-Sec is thoroughly mixed with carbomer gel and freeze-dried. ELISA test is performed to determine the characterization of the gel of hWJMSCs-Sec such as Keratinocyte Growth Factor (KGF), Platelet-Derived Growth Factor (PDGF), Hepatocyte Growth Factor (HGF), Epidermal Growth Factor (EGF), and Heparin-Binding EGF-Like Growth Factor (HB-EGF). The antioxidant activity was also measured with Hydrogen peroxide (H₂O₂), Nitric oxide (NO), and Ferric Reducing Antioxidant Power (FRAP) assay. Proliferation assay was utilized using WST-8 and the wound healing potential was assessed *via* the migration cell ability of scratched-human skin fibroblast (BJ cells).

Results: The freeze-dried hWJ-MSCs-Sec showed higher levels of KGF, HGF, PDGF, EGF, HB-EGF, and the antioxidant activities compared to fresh hWJ-MSCs-Sec. Additionally, the gel of freeze-dried hWJ-MSCs-Sec exhibited higher levels compared to the gel of fresh hWJMSCs-Sec. This was evidenced by faster closure of scratched wounds on BJ cells treated with hWJMSCs-Sec and freeze-dried hWJ-MSCs-Sec gel.

Conclusion: The freeze-dried hWJ-MSCs-Sec gel exhibits superior quality compared to the non-freeze-dried hWJ-MSCs-Sec gel. This demonstrates that the freeze-drying procedure can maintain the bioactive chemicals found in hWJMSCs-Sec, potentially enhancing the efficacy of this gel in promoting cell regeneration for wound healing.

Keywords: Antioxidants, Carbomer gel, Freeze dried secretome gel, hWJ-MSCs, Wound healing

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Introduction

Diabetic Foot Ulcer (DFU) is an open sore or wound that is caused by complications of diabetes

mellitus. DFU affects the feet of diabetes patients and is characterized by sensory, motor, and autonomic neu-

ropathy, as well as macrovascular and microvascular disturbances. DFU can lead to ulcers, infections, gangrene, amputations, and even death. Amputation, a serious outcome of DFU, results in 14.3% of individuals passing away within a year after the procedure¹. Effective strategies for healing DFU wounds must be established to avoid the amputation². Efficient wound healing necessitates the coordination of several cellular processes facilitated by multiple growth factors, cytokines, and chemokines. There are four distinct phases involved in the process of wound healing, namely hemostasis, inflammation, proliferation, and remodeling³. The inflammatory response is triggered to help the body and initiate the healing process by widening the blood vessels and releasing certain signaling molecules such as nitric oxide, bradykinin, histamine, and prostaglandins⁴. But in cases where subsequent damage occurs, the inflammation doesn't resolve as expected, instead, it persists and intensifies, characterized by an abundance of neutrophils releasing Reactive Oxygen Species (ROS)⁵. The excessive generation of ROS has been linked to the onset of numerous persistent and degenerative disorders⁶. The antioxidant agents are needed to mitigate the harmful effect of ROS and enhance the body's ability to withstand diverse environmental stress conditions⁷.

The process of wound repair involves various growth factors, including Epidermal Growth Factor (EGF), which promotes cell proliferation and cytoprotection⁸. Other growth factors such Platelet-Derived Growth Factor (PDGF), Heparin Binding EGF Like Growth Factor (HB-EGF), and Keratinocyte Growth Factor/Fibroblast Growth Factor-7 (KGF/FGF-7) play a significant role in angiogenesis, inflammation regulation, collagen deposition⁹, keratinocyte migration, keratinocyte proliferation¹⁰ and reepithelization in wound healing¹¹. Currently, ongoing research is being conducted in the field of wound therapy with the aim of identifying appropriate treatment agents that can facilitate rapid wound healing while minimizing adverse effects. This pertains specifically to agents that possess elevated concentrations of antioxidants and growth factor proteins. One potential solution that could effectively tackle this matter is the implementation of stem cell treatment.

In the study by Park *et al*¹², it was discovered that various growth factors, including EGF, PDGF, bFGF, HB-EGF, FGF7/KGF, and HGF, are actively produced within stem cells and released through the secretome. The significant presence of these growth factors suggests their potential in wound healing, particularly in the case of DFU. Wharton's Jelly-derived MSCs (WJMSCs) are a type of stem cell derived from the umbilical cord. WJMSCs have been found to enhance the proliferation and migration of fibroblasts, speed up re-epithelialization, and facilitate wound repair through paracrine signaling¹³. The MSCs secretome, which consists of various bioactive substances released into

the surrounding cellular environment, exerts a strong impact on nearby tissues¹⁴. The Wharton's Jelly Mesenchymal Stem Cells Secretome (hWJ-MSCs-Sec) has been commonly used to enhance wound healing after severe injuries¹⁵. Therefore, the hWJ-MSCs-Sec can be developed into a gel product for wound healing or DFU treatment. Gel products create a moist environment at the wound site, fostering tissue renewal through granulation and re-epithelialization¹⁶. The excellent adhesive and spreading properties of the gel enhance its application and therapeutic effectiveness, ensuring robust adherence to the target area, uniform spreading, and ultimately optimizing the therapeutic or cosmetic effects¹⁷. To safeguard the active compounds within the secretome, the freeze-drying process is employed. This technique ensures the preservation of proteins within the secretome, enhancing the reliability and consistency of molecular assessments¹⁸.

This study aims to explore the efficacy of topical gel containing hWJMSCs-Sec for the treatment of DFU. This will be achieved through an examination of the gel's antioxidant properties by its scavenging activity towards Hydrogen peroxide (H₂O₂), Nitric Oxide (NO), and Ferric Reducing Antioxidant Power (FRAP). The analysis of the proteins content of freeze dried hWJ-MSCs-Sec gel involved the implementation of the Enzyme-Linked Immunosorbent Assay (ELISA) method, focusing on the evaluation of Keratinocyte Growth Factor (KGF), Platelet-Derived Growth Factor (PDGF), Hepatocyte Growth Factor (HGF), EGF, and hHB-EGF. Finally, the *in vitro* potential of hWJMSCs-Sec gel both freeze dried and fresh for wound healing was assessed by evaluating the cells proliferation and the cells migration of scratched-human skin fibroblast cells (BJ cells).

Materials and Methods

Formulation of carbomer gel

The carbomer gel formulation was conducted based on the study of Zhang *et al*¹⁹, with certain alterations. Disperse 7.5 g carbomer 940 slowly into 250 ml aquadest with a continuous stirring using a Stirring Hotplates (Fisher Scientific, 1110217SH). After the carbomer swells in distilled water, add 7.5 ml triethanolamine, 5 ml propylene glycol, and 5 ml glycerin drop by drop while stirring gently until the mixture forms a homogeneous gel. Lastly, add distilled water gently until it reaches 500 ml.

Production of hWJ-MSCs Freeze-Dried hWJ-MSCs-Sec Gel (FDSG)

Preparation of hWJMSCs Secretome: The hWJMSCs passage 5 were obtained from Aretha Medika Utama Bandung, Indonesia. The cultivation protocol for Human Wharton's Jelly Stem Cells (hWJ-MSCs) was elucidated by Widowati, *et al*²⁰ and has been characterized using multipotent differentiation experiments and surface phenotypic analysis in prior studies²¹. Cells were cultured in Dulbecco's Modified Eagle Me-

dium (DMEM) High Glucose (Biowest, L0475-500) with 10% FBS, 1% Antibiotic and Antimycotic (ABAM) (Biowest, L0010-100), 0.1% Gentamicin (Gibco, 15750060), 1% Amphotericin B (Biowest, L0009-100), and 1% Nanomycopulitin (Biowest, L-X16-100) and incubated with CO₂ Incubator (Thermo IH3543) at 37°C with 5% CO₂. After the hWJMSCs reached 80-90% confluence, the culture medium was collected and centrifuged with Refrigerated Centrifuge (MWP 260r) at 3000×g for 4 min at 37°C. The resulting supernatant, containing secreted factors, was purified using a Durapore (Millipore Corporation, SLGV 033 RS) filter unit²².

Production of Freeze-Dried hWJ-MSCs-Sec gel

The preparation of the gel involves incorporating fresh and fresh hWJMSCs-Sec into the carbomer gel in three sequential stages, following a specific formula (P1: 6 g carbomer gel+3 ml hWJ-MSCs-Sec, P2: 6 g carbomer gel+4.5 ml hWJ-MSCs-Sec, P3: 6 g carbomer gel+6 ml hWJ-MSCs-Sec), each mixture is stirred gently until it reaches a homogeneous consistency²³. Subsequently, the hWJ-MSCs-Sec gel is placed in a small tray and placed inside the vacuum freeze-drying machine used for the lyophilization method with a freeze dryer (FD-F-CE, China) tool²⁴. The lyophilization process using a freeze dryer was carried out for 42 hr at a temperature of 35-50°C.

Proteins assay

The Human KGF (E-EL-H0092), HGF (E-EL-H0084), PDGF (E-EL-H2211), EGF (E-EL-H0059), and HB-EGF (E-EL-H2667) level were assayed using the Elabscience kit, based on manufacturing protocols. The ELISA Assay quantification was done to measure the hWJ-MSCs-Sec gel characteristics and the protein level of hWJ-MSCs-Sec gel²⁵.

Antioxidant assay

H₂O₂ Scavenging Activity Assay: hWJ-MSCs-Sec gel was added 60 µl to the 96-well microplate. In the control and blank wells, 12 µl of 1 mM ferrous ammonium sulfate (Sigma Aldrich, 215406) were introduced. Following this, dimethyl sulfoxide (DMSO) (Supelco, 1.02952.1000) was added to the control (63 µl) and blank (90 µl) wells. Then, 3 µl of 5 mM H₂O₂ (Merck, 1.08597.1000) was added to each well of the microplate. After incubating time in a dark room for 5 min, 1,10-phenanthroline (Sigma Aldrich, 131377) was added (75 µl) into each well. Following another 10-minutes incubation, absorbance at 510 nm was measured using a spectrophotometer²⁶. H₂O₂ scavenging activity was determined using the formula:

$$\% \text{ scavenging activity} = \frac{\text{Control absorbance} - \text{sample absorbance}}{\text{control absorbance}} \times 100$$

NO scavenging activity assay

Sodium nitroprusside 10 mM (Merck, 106541) in PBS (Gibco, 1740576) as much as 40 µl was mixed

with 10 µl of the hWJ-MSCs-Sec gel sample. After incubating the mixture at room temperature for 2 hr, 100 µl of Griess reagent containing 1% sulfanilamide (Merck, 111799), 2% H₃PO₄ (Merck, 100573), and 0.1% N-(1-naphthyl) ethylenediamine dihydrochloride (Sigma Aldrich, 222488) was added to each well of 96-well plate and the absorbance was measured at 546 nm²⁷. NO scavenging activity was determined using the formula:

$$\% \text{ scavenging activity} = \frac{\text{Control absorbance} - \text{sample absorbance}}{\text{control absorbance}} \times 100$$

FRAP assay

To start preparing the reagent, 10 ml of 300 mM acetate buffer (pH=3.6) was combined with 1 ml of 20 mM ferric chloride hexahydrate (Merck 1.03943.0250) and 1 ml of 10 mM 2,4,6-Tris(2-pyridyl)-s-triazine (TPTZ) (Sigma- Aldrich, T1253). Following that, 7.5 µL of hWJ-MSCs-Sec gel sample and 142.5 µl of the FRAP reagent were introduced into the wells and incubated at 37°C for 6 min before it got measured at 593 nm²⁸. FRAP scavenging activity was determined using the formula:

$$\% \text{ scavenging activity} = \frac{\text{Control absorbance} - \text{sample absorbance}}{\text{control absorbance}} \times 100$$

Proliferation assay

The human skin fibroblast cell line (BJ cells) (ATCC, CRL-2522) was cultured based on previous study²⁹. As much as 5×10³ cells were grown in a 96 well plate. After reaching confluence, the cells were treated with various samples, while untreated cells act as a negative control (NC). WST-8 were introduced after 24 hr of treatment and the absorbance was read at 595 nm³⁰.

Cells migration

To determine the ability of hWJ-MSCs-Sec gel in wound healing, cells migration assays were conducted on scratched-human skin fibroblast cells as wounded cells model. The fibroblast cells were plated on a 24 well plate at a density of 7×10³. After reaching 90% confluence, the cells were scratched across the well to create a wound model by using a blue tip. Cells were then treated with the hWJ-MSCs-Sec gel samples, and cell without treatment act as a NC. After 24 hr, the scratched area was measured under the microscope using Image J software³¹.

Statistical analysis

The resulting data represents the mean ± standard deviation from three replicate measurements of each sample. Comparisons of treatments were undertaken using ANOVA, with a significance level of p<0.05. The post hoc test, namely Tukey Honestly Significant Difference, along with the Dunnett T3 test with a 95% confidence interval, was utilized. The statistical analy-

sis was conducted using SPSS software (version 20.0), and the findings were visualized using GraphPad Prism program (version 9.0).

Results

Protein content of Freeze-Dried hWJMSCs-Sec gel

The influence of hWJMSCs-Sec treatments on the levels of Growth Factor Proteins are elucidated in figure 1. As shown in figure 1, the hWJMSCs-Sec treatment in the FDSG regimen demonstrated an increasing trend in protein levels alongside the addition of hWJMSCs-Sec volume to the carbomer gel. On the other hand, the Freeze-Dried hWJ-MSCs-Sec (FDS) treatment significantly increased the levels of KGF, HGF, PDGF, EGF, and HB-EGF compared to other treatments, especially fresh hWJ-MSCs-Sec (S). Additionally, Freeze-Dried Gel Based (FDGB) and Freeze-Dried Medium Basal (FDMB) were used as negative

control groups ($p < 0.05$).

Antioxidant activity of Freeze-Dried hWJMSCs-Sec gel

To determine its potential as a wound healing treatment, antioxidant testing was conducted by evaluating its ability to scavenge H_2O_2 , NO, and FRAP (Figures 2-4). The antioxidant activity of FDSG exhibits a significant superiority in all antioxidant tests compared to the Secretome (S) and hWJMSCs-Sec Gel (SG). The results of antioxidant activity tend to change with different hWJMSCs-Sec Gel formulation. FDSG itself exhibits the highest antioxidant activity in the formulation of most concentrated hWJMSCs-Sec Gel, with the highest concentration being $400 \mu\text{g/ml}$.

Proliferation effect of Freeze-Dried hWJMSCs-Sec gel

Proliferation assays were conducted to assess the cells viability of freeze-dried hWJMSCs-Sec and freeze-dried hWJMSCs-Sec gel on human skin fibro-

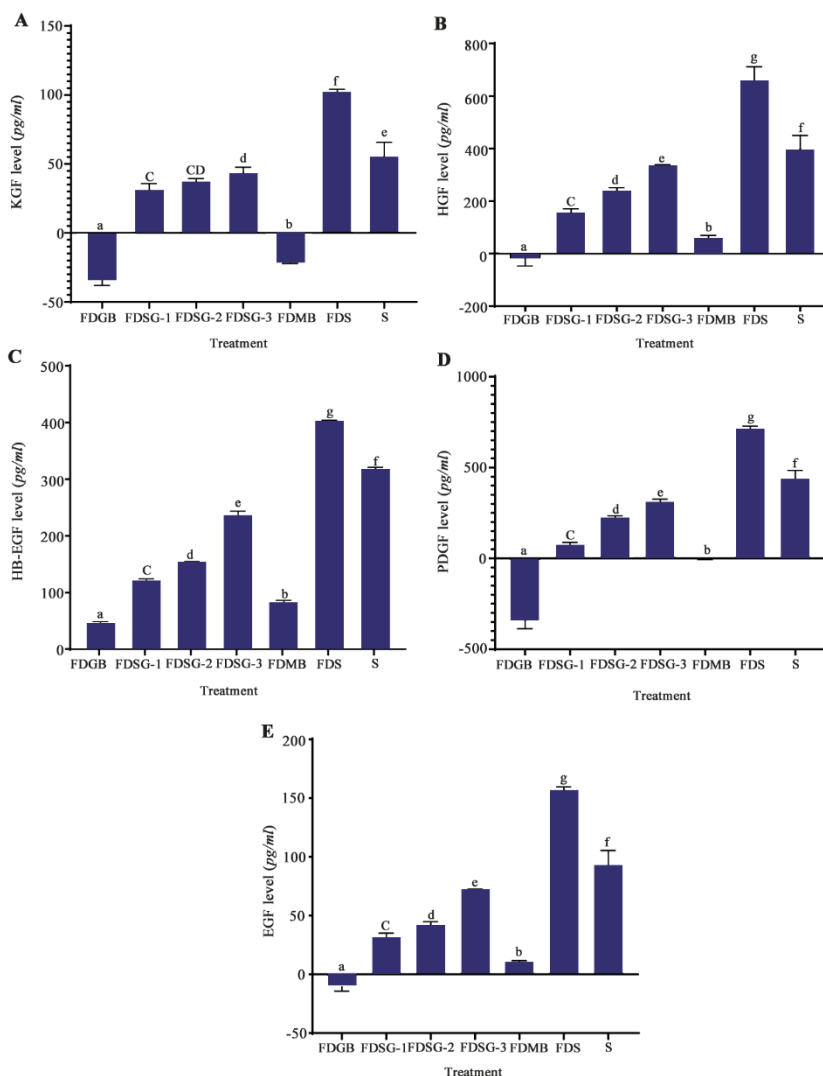


Figure 1. Effect of various treatments on hWJMSCs-Sec toward growth factor proteins level. A) KGF, B) HGF, C) HB-EGF, D) PDGF, E) EGF. The data were presented as mean \pm standard deviation. Different superscript letters on Figure A (a, b, c, cd, d, e, f), and B, C, D, E (a, b, c, d, e, f, g) showed significant differences among treatments at $p < 0.05$ (Data was analyzed using ANOVA followed by Tukey HSD post hoc test). Samples included hWJMSCs-Sec (S), Freeze-dried hWJMSCs-Sec (FDS), Freeze-dried Medium Basal (FDMB), Freeze-Dried hWJMSCs-Sec Gel (FDSG), and Freeze-dried Gel Based (FDGB). Number on samples indicate the ratio of gel to hWJMSCs-Sec, 1) 6 g: 3 ml, 2) 6 g: 4.5 ml, and 3) 6 g: 6 ml.

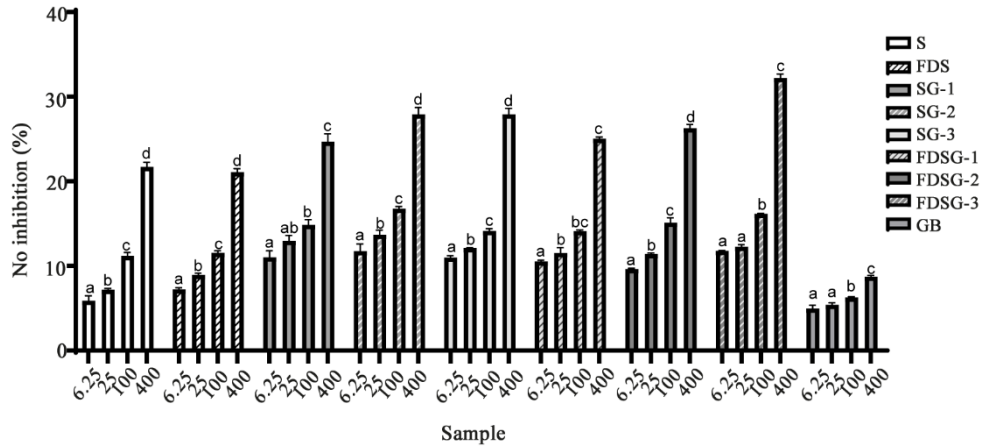


Figure 2. Antioxidant level of hWJ-MSCs-Sec gels in various formulations by its ability to scavenge H₂O₂.

*The data were presented as mean ± standard deviation. Different letters (a, ab, b, bc, c, d) showed significant variation among concentration at p<0.05. Data was analyzed using ANOVA and followed by Tukey HSD & Dunnett T3 *post hoc* test. Samples included hWJ-MSCs-Sec (S), Freeze-dried hWJ-MSCs-Sec (FDS), hWJ-MSCs-Sec Gel (SG), Freeze dried hWJ-MSCs-Sec gel (FDSG), and Carbomer gel base (GB). Number on samples indicate the ratio of gel to hWJMScs-Sec, 1) 6 g: 3 ml, 2) 6 g: 4.5 ml, and 3) 6 g: 6 ml.

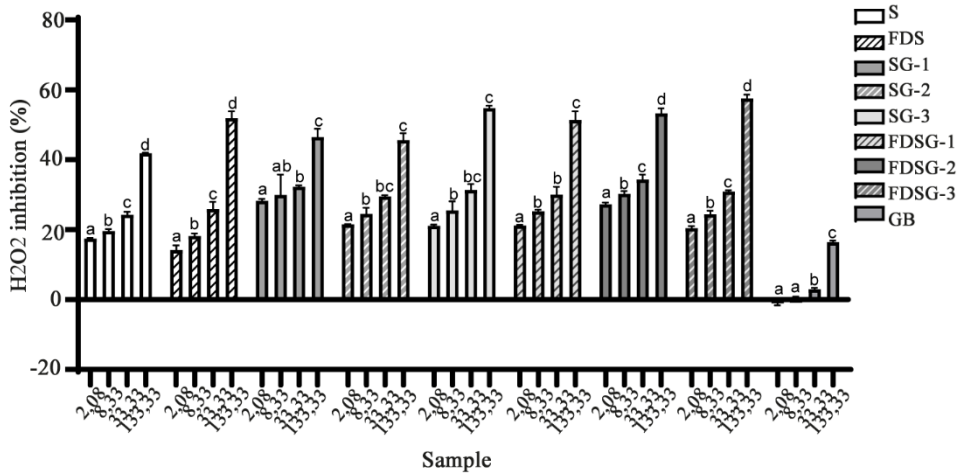


Figure 3. Antioxidant level of hWJMScs-Sec gels in various formulations by its ability to scavenge NO.

*The data were presented as mean ± standard deviation. Different letters (a, ab, b, bc, c, d) showed significant variation among concentration at p<0.05. Data was analyzed using ANOVA and followed by Tukey HSD & Dunnett T3 *post hoc* test. Samples included hWJ-MSCs-Sec (S), Freeze-dried hWJ-MSCs-Sec (FDS), hWJ-MSCs-Sec Gel (SG), Freeze dried hWJ-MSCs-Sec gel (FDSG), and Carbomer gel base (GB). Number on samples indicate the ratio of gel to hWJMScs-Sec, 1) 6 g: 3 ml, 2) 6 g: 4.5 ml, and 3) 6 g: 6 ml.

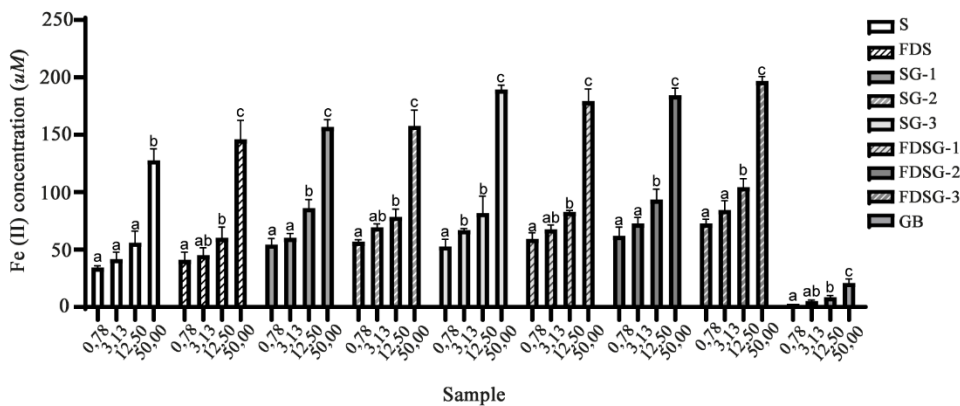


Figure 4. Antioxidant level of hWJ-MSCs-Sec gels in various formulations by its ability to scavenge FRAP.

*The data were presented as mean ± standard deviation. Different letters (a, ab, b, c) showed significant variation among concentration at p<0.05. Data was analyzed using ANOVA and followed by Tukey HSD & Dunnett T3 *post hoc* test. Samples included hWJ-MSCs-Sec (S), Freeze-dried hWJ-MSCs-Sec (FDS), hWJ-MSCs-Sec Gel (SG), Freeze dried hWJ-MSCs-Sec gel (FDSG), and Carbomer gel base (GB). Number on samples indicate the ratio of gel to hWJMScs-Sec, 1) 6 g: 3 ml, 2) 6 g: 4.5 ml, and 3) 6 g: 6 ml.

Topical Gel for Diabetic Foot Ulcers

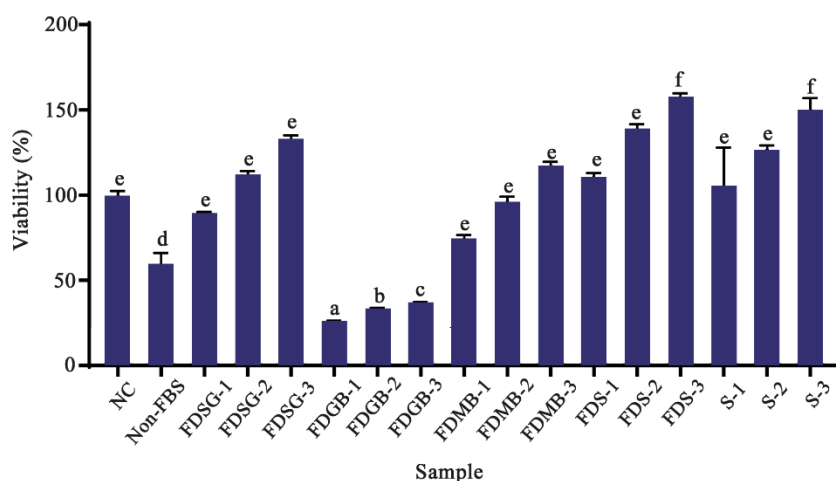


Figure 5. Proliferation effect of hWJMSCs-Sec gel towards human fibroblast cells.

*The data were presented as mean \pm standard deviation. Different letters (a, b, c, d, e, f) showed significant variation among concentration at $P < 0.05$. Data was analyzed using T-Test. Samples included: Negative Control: untreated-cells (NC), Cells without FBS (Non-FBS), Cells+hWJMSCs-Sec (S), Cells+Freeze-dried hWJMSCs-Sec (FDS), Cells+Freeze-dried Medium Basal (FDMB), Cells+ Freeze-Dried hWJMSCs-Sec Gel (FDSG), and Cells + Freeze-dried Gel Based (FDGB). Number on samples indicates the concentration used for treatment, 1) 1.3 $\mu\text{g/ml}$, 2) 12.5 $\mu\text{g/ml}$, and 3) 100 $\mu\text{g/ml}$. While for Secretome (S), the number indicates the concentration of 1) 3.25%, 2) 12.5%, and 3) 50%.

blast cells. The results of these assays are presented in figure 5. The addition of hWJMSCs-Sec at various concentrations did not exhibit any toxic activity towards fibroblast cells. Conversely, cells treated with hWJMSCs-Sec demonstrated enhanced proliferation compared to the Negative Control (NC). The freeze-dried secretome showed superior cell viability compared to the S samples. These findings indicate that the freeze-drying process preserves important bioactive components in hWJMSCs-Sec and is non-toxic to fibroblast cells.

Migration of fibroblast cells after the treatment with Freeze-Dried hWJ-MSCs-Sec gel

The proliferation and migration of fibroblast are crucial for the process of skin repair/regeneration. Therefore, migration assays were conducted on fibroblast cells that were subjected to scratching to assess the wound healing process by evaluating the effects of hWJMSCs-Sec gel on cell proliferation and migration abilities. The migration data are shown in figure 6. The results demonstrate increased cell migration values with higher addition of hWJMSCs-Sec. FDS exhibits the highest efficacy in promoting cell migration, surpassing even that of S itself. Similarly, FDSG demonstrates superior efficacy compared to S, closely resembling the outcome of FDS. This substantiates that the growth factors within the secretome are preserved through the freeze-drying process.

Discussion

Wharton's Jelly is widely regarded as a highly reliable and abundant source of mesenchymal stem cells. In the preceding investigation, a comprehensive analysis of hWJMSCs was undertaken. It has been demonstrat-

ed that hWJMSCs exhibit a high expression of CD90, CD105, CD44, and CD73, while displaying a negative expression of CD11b, CD19, CD34, CD45, and HLA-DR²¹. In this experiment, passage 5 of the hWJMSCs cells was selected due to its notable and consistent proliferation³². Carbomer is utilized as the primary component to formulate the secretome from WJMSCs into a gel form. Freeze-drying, also known as lyophilization, is a crucial technique utilized in this study to safeguard the integrity of hWJMSCs-Sec, particularly due to their susceptibility to heat-induced damage, as emphasized by previous research findings³³. This method involves the removal of moisture under low temperatures and reduced pressure, mitigating the potential harm caused by heat to the sensitive cellular structure of hWJMSCs-Sec. Moreover, freeze-drying serves as a promising approach for the preservation of biomaterial and natural cells in a desiccated state, a process that enhances their stability during storage and transportation. The significance of this technique lies in its ability to prolong the shelf life of hWJMSCs-Sec by maintaining their structural and functional characteristics, even at room temperature. Recent studies, such as the work by Merivaara *et al*³⁴, underscore the efficacy of freeze-drying in providing optimal storage conditions, making it a valuable method for the long-term preservation of biological materials.

ELISA results have illuminated a significant disparity between freeze-dried hWJ-MSCs-Sec and its conventional counterpart, particularly concerning the protein content of critical growth factors. This disparity is marked by notably higher concentrations of several growth factors, specifically KGF, HGF, PDGF, EGF, and HB-EGF, within the freeze-dried hWJ-MSCs-Sec.

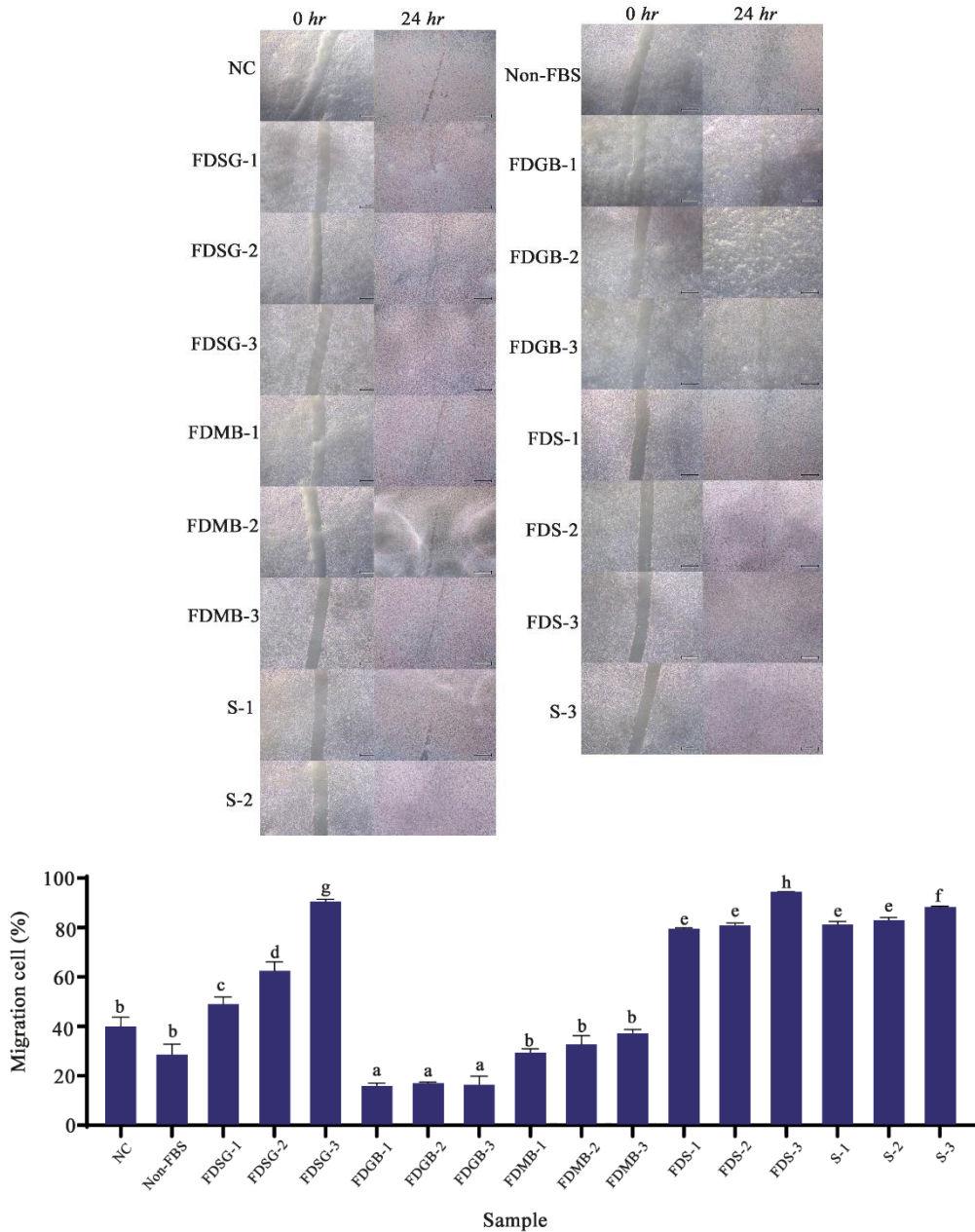


Figure 6. Effect of various treatments on hWJMSCs-Sec toward the migration of human skin fibroblast cells. *The data were presented as mean±standard deviation. Different letters (a, b, c, d, e, f, g, h) showed significant variation among samples at p<0.05. Data was analyzed using T-Test. Samples included: Scratched-cells (NC), Scratched-cells without FBS (Non-FBS), Scratched-cells+hWJMSCs-Sec (S), Scratched-cells+Freeze-dried hWJMSCs-Sec (FDS), Scratched-cells + Freeze-dried Medium Basal (FDMB), Scratched-cells+Freeze-Dried hWJMSCs-Sec Gel (FDSG), and Scratched-cells + Freeze-dried Gel Based (FDGB). Number on samples indicate the concentration used for treatment, 1) 1.3 µg/ml, 2) 12.5 µg/ml, and 3) 100 µg/ml. While for Secretome (S), the number indicates the concentration of 1) 3.25%, 2) 12.5%, and 3) 50%.

These findings bear profound implications for the applications of hWJ-MSCs-Sec in regenerative medicine and tissue engineering. The elevated content in KGF, HGF, PDGF, EGF, and HB-EGF in freeze-dried hWJ-MSCs-Sec underscores its potential in accelerating wound healing and tissue repair, demonstrating a promising avenue for therapeutic interventions ³⁵.

Growth factors are signaling molecules that play a critical role in cell growth, differentiation, and survival

³⁶. The five growth factors discussed in this article are all involved in different aspects of wound healing, from inflammation and angiogenesis to cell proliferation and extracellular matrix deposition. KGF is a growth factor that is primarily produced by keratinocytes, the cells that make up the epidermis ³⁷. KGF is a potent stimulant of keratinocyte proliferation and differentiation. While HGF is a potent stimulant of cell proliferation, migration, and differentiation. Together,

they play a role in angiogenesis and inflammation^{38,39}. The present investigation observed a positive correlation between the formulation of hWJ-MSC-Sec and the levels of KGF and HGF in FDSG. Previous research has elucidated that the levels of HGF exhibit an elevation in rats afflicted with DFU following treatment with secretome derived from umbilical cord mesenchymal stem cells (UCMSCs)⁴⁰.

PDGF is an additional growth factor that can be specifically targeted for the therapy of DFU. So far, the Food and Drug Administration (FDA) has granted permission to PDGF for its capacity to expedite the process of wound healing⁴¹ because of its capacity for attracting fibroblast⁴². PDGF was detected in FDSG samples as well in this analysis. This finding suggests that FDSG exhibits promise in the treatment of DFU. EGF and HB-EGF was also found in FDSG, as a growth factor that is produced by a variety of cells, including keratinocytes, fibroblasts, and macrophages⁴³. Kuo *et al*⁴⁴ observed that the incorporation of stem cells resulted in a significant increase in the concentration of EGF on the accelerated healing of wounds in individuals with diabetes. Another study stated that the addition of HB-EGF resulted in an increase in the formation of granulation tissue in mice with diabetic wounds⁴⁵. In all protein tests performed, FDS exhibited the highest efficacy among the samples, including FDSG. This could be influenced by the addition of carbomer gel, resulting in a lower concentration of active ingredients in the secretome gel compared to the secretome itself. As seen in figure 1, freeze-dried carbomer gel (FDGB) did not show any presence of growth factor proteins.

In addition to growth factors, the presence of oxidative stress within the wound will also exert an influence on the process of wound healing. High levels of H₂O₂ at a wound site serve as a signal for initiating chemotactic and inflammatory response⁴⁶. The inflammatory response can also be exacerbated by the presence of NO. Nitric oxide plays a pivotal role in regulating three crucial aspects of wound healing: vascular homeostasis, inflammation, and antimicrobial activity⁴⁷. These radical compounds can be identified as potential targets for wound healing interventions. The gel hWJ-MSCs-Sec exhibits a high antioxidant level (Figures 2-4). After the freeze-drying process, FDSG still exhibited remarkably high antioxidant results. This data provides evidence that the freeze-drying process effectively preserves the active compounds within the hWJ-MSCs-Sec gel. Based on the results, it was proven that FDSG comprises growth factor protein and high antioxidant levels, rendering it a promising DFU agent.

To further explore the potential of gel secretome as a wound healer, cell proliferation and migration assays on human skin fibroblast cells were conducted. FDSG shows no toxic effect towards the fibroblast cells. Instead, the cell's viability increased after the treatment

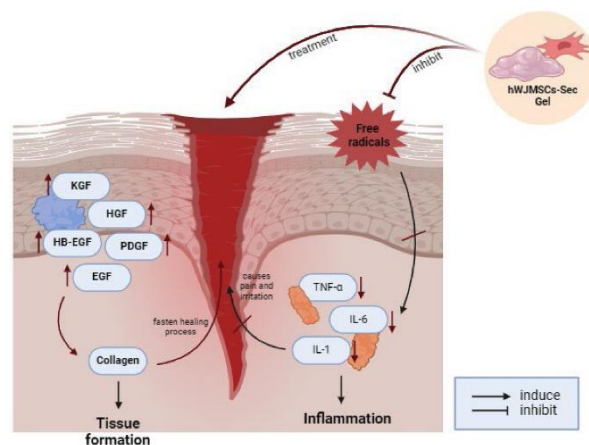


Figure 7. Proposed mechanism of hWJMSCs-Sec gel as a wound healing agent.

* The treatment of hWJMSCs-Sec gel into the wounded skin can regulate some growth factors such as KGF, HGF, HB-EGF, PDGF, and EGF. The upregulation of these growth factors can induce the production of collagen, which plays a crucial role to regenerate tissue formation. The high antioxidant hWJMSCs-Sec gel also has the ability to scavenge free radicals, and inhibit the excessive inflammation that can cause irritation, redness, and pain in the wound.

with FDSG, FDS, and S itself (Figure 5). Cell migration is pivotal in developmental processes within cells. MSCs exhibits multipotent characteristic and reparative abilities, rendering them promising sources of stem cells for degenerative therapy. MSCs possess various chemical factors such as chemokines, cytokines, and other growth factors, which support MSC migration to wound sites⁴⁸. Growth factors like EGF, FGF, TGFs, VEGF, and PDGF play a crucial role in regulating cell division, differentiation, and remodeling,⁴⁹ which facilitate cell proliferation and cell migration⁵⁰. The proposed mechanism of hWJMSCs-Sec as a wound healing agent was summarized in figure 7. The study results indicate that hWJMSCs-Sec contains several growth factors, thus supporting fibroblast cells to close faster after treatment with hWJMSCs-Sec. FDS-100 $\mu\text{g/ml}$ and FDSG-100 $\mu\text{g/ml}$ exhibited superior abilities with wound closure of 95.62% and 90.40%, when compared to NC. This result also further proves that the freeze-drying process on hWJMSCs-Sec gel can maintain quality by dehydrating the product into powder form. The dehydrated product also supports longer storages with good quality⁵¹. However, this study was hindered by a lack of verification assays. Further in vitro as well as in vivo approaches are needed to support this study.

Conclusion

In this study, hWJ-MSCs-Sec is thoroughly mixed with the carbomer gel and freeze-dried to maintain quality stability. This is evidenced by the fact that after undergoing the freeze-drying process, the protein levels of growth factors and antioxidant activity from hWJ-MSCs-Sec still yielded excellent results, even

surpassing those that did not undergo the freeze-drying process. The application of freeze-dried hWJ-MSCs-Sec *via* carbomer gel has demonstrated the preservation of growth factors and antioxidant properties. This endows it with exceptional migratory capabilities towards the wound site, indicating potential for wound healing therapy.

Ethical Issue

This research obtained ethical approval from the Research Ethics Committee of the Faculty of Medicine at Maranatha Christian University (Approval No: 097/KEP/VII/2020, dated July 25th, 2020).

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Conflict of Interest

The authors declare no conflict of interest.

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