HAYATI
Journal of Biosciences

Vol. 31 No. 5, September 2024 988-995
DOI:10.4308/hjb.31.5.988-995
ISSN: 1978-3019
EISSN: 2086-4094

The Effect of Gel Secretome Hypoxia Mesenchymal Stem Cells to Increase P38 and VEGF Expression in Rats' Diabetic Wounds

Tarrayuana Rhamadia Hasannuri[1*], Mas Rizky A.A Syamsunarno[2], Agung Putra[3]

[1]Postgraduate Biomedical Sciences Program, Faculty of Medicine, Universitas Islam Sultan Agung, Semarang 50112, Indonesia
[2]Department of Biomedical Sciences, Faculty of Medicine, Universitas Padjadjaran, Bandung, West Java, Indonesia
[3]Department of Postgraduate Biomedical Sciences Program, Faculty of Medicine, Universitas Islam Sultan Agung, Semarang 50112, Indonesia

ARTICLE INFO

Article history:
Received November 6, 2023
Received in revised form February 6, 2024
Accepted April 30, 2024

KEYWORDS:
diabetic wound,
hypoxia MSC,
secretome,
P38,
VEGF

ABSTRACT

Mesenchymal stem cells (MSCs) under hypoxic conditions can produce secretomes containing growth factors such as vascular endothelial growth factor (VEGF), accelerating angiogenesis in wound healing disorders in diabetic ulcers. This study aimed to prove the influence of gel secretome MSC hypoxia administration on increasing VEGF and P38 gene expression in rats' diabetic wounds. An *in vivo* study was conducted on 25 male Rattus norvegicus, randomly divided into four groups: base gel as a negative control, Gentamycin as a positive control, and gel secretome at a dose of 100 µL, and 200 µL/kg body weight. The differences in P38 and VEGF gene expression were tested using quantitative real-time polymerase chain reaction (qRT-PCR). Wound closure appeared to be fastest in treatment groups at a dose of 100 µL/kg body weight, followed by a dose of 200 µL/kg body weight, followed by Gentamycin and base gel group. The wound closure rate percentage was significantly different in the intervention group compared to the control group (p = 0.000). The results showed a significant difference in P38 and VEGF gene expression between the treatment and control groups (p = 0.000). This study demonstrates the administration of gel secretome hypoxia mesenchymal stem cells increases P38 and VEGF expression in rats' diabetic wounds.

1. Introduction

Diabetes is a multifaceted metabolic disease affecting over 340 million people worldwide, with approximately 20% suffering from diabetic wounds. Ulcers on the limbs or feet, commonly referred to as diabetic ulcers, are the most common microvascular complication in individuals with diabetes mellitus (DM) (Patel *et al.* 2019). The global prevalence of diabetes has led to an increase in cases of limb amputations due to its complications. Increased inflammation, reduced angiogenesis processes, and the production of growth factors, primarily vascular endothelial growth factor (VEGF), contribute to the impediment in wound healing (Chawla *et al.* 2016). The inhibition of VEGF production results in the dysregulation of P38 MAPK activation in response to stress, triggering various inflammatory pathways and dysregulating apoptosis and cell migration in angiogenesis (Jiao *et al.* 2022; Yang *et al.* 2022).

Current management of diabetic ulcers remains conventional, involving the administration of antibiotics, debridement, and surgery, often resulting in recurrence and an increased risk of amputation. Therefore, a safe and effective therapeutic approach is required for diabetic ulcer treatment, one of which is the use of secretome hypoxia mesenchymal stem cells (SH-MSC). These cells possess the anti-inflammatory potential and can detect injury signaling molecules, migrate to the injury site (homing phenomenon), remodel the extracellular matrix, promote angiogenesis, prevent apoptosis, and provide immunosuppression, all of which facilitate wound healing (Khan *et al.* 2020). Preclinical studies have shown that secretome MSC release mediators and growth factors that support skin regeneration, including anti-inflammatory cytokines (IL-10), VEGF, platelet-derived growth factor (PDGF), hepatocyte growth factor (HGF), tumor

* Corresponding Author
E-mail Address: tarrayuana@gmail.com

necrosis factor-alpha (TNF-α), fibroblast growth factor (FGF), transforming growth factor (TGF-β1), angiopoietin, IL-1, IL-6 and interferon-gamma (IFN-γ) (Zarei *et al.* 2018; Regmi *et al.* 2019). Hypoxic preconditioning enhances the cytoprotective effects, maintains multipotency, increases proliferation, and enhances the ability of MSC secretome to survive in harsh environments while targeting the injury site during transplantation (Fui *et al.* 2019).

Vascular endothelial growth factor is a signaling protein involved in vasculogenesis and angiogenesis. In endothelial cells, VEGF activates P38 MAPK through the MAPKK2K-AP receptor, which phosphorylates LIMK1. LIMK1 stimulates actin remodeling by phosphorylating and deactivating cofilin, an actin depolymerization factor. P38 regulates angiogenesis and cell motility by controlling matrix metalloproteinase (MMP). P38α MAPK-ASH produces MMP 9, which sends chemotactic signals for neutrophil migration and angiogenesis (Apte *et al.* 2019; Kim *et al.* 2022). Previous studies have shown that SH-MSC therapy is safe and effective in preclinical and clinical trials. The secretome synthesizes and releases cytokines that promote cell recruitment, immunomodulation, extracellular matrix remodeling, angiogenesis, and nerve regeneration, all of which contribute to wound healing. Several scientific publications have discussed the usage of secretome MSC for diabetic ulcer treatment (Park *et al.* 2018; Ahmadi *et al.* 2020). However, the impact of gel SH-MSC on diabetic ulcers remains unknown. Based on this background, this study aims to investigate the effects of gel SH-MSC on the expression of P38 and VEGF genes in a diabetic wound model in male Wistar rats.

2. Materials and Methods

2.1. Experimental Animals

This experimental study has a randomized post-test-only control group design. The study was carried out at Stem Cell and Cancer Research (SCCR), Faculty of Medicine, Sultan Agung University Semarang, Indonesia, and has been registered with the Ethical Clearance Number 178/V/2023/Komisi Bioetik. This study was conducted from May to August 2023. The Wistar rats used in the study were deemed suitable by a veterinarian from the Animal House, SCCR, Faculty of Medicine, Sultan Agung University Semarang. The required subjects for this experiment were 25 male Wistar rats with diabetic wounds, which were divided into four treatment groups. The experimental animals used were male Wistar rats (Rattus norvegicus) aged 2-3 months with a weight range of 200-250 grams. The experimental room had a temperature of 22-26°C, humidity of 50-60%, and artificial fluorescent lights (12:12 hours light and dark cycle). The rats were kept isolated from other laboratory animals, housed in air-filtered cages, and provided with standard laboratory while food and drinking water were served ad libitum.

2.2. Preparation of Secretome MSC

The MSCs were isolated from the umbilical cord of 21-day pregnant rats. The isolated cells were then cultured in plastic flasks in Dulbecco's Modified Eagle's Medium (DMEM) (Sigma-Aldrich, St. Louis, MO) supplemented with fetal bovine serum (FBS), fungizone and penstrep. After the fifth passage, the cultured cells exhibited an adherent appearance on the flask's bottom with spindle-like cell morphology under microscopic observation.

The isolation of MSCs was validated using flow cytometry to demonstrate the MSCs' ability to express various specific surface markers (Figure 1E). MSCs could express CD90 (99.80%) and CD29 (94.20%) and showed slight expression of CD45 (1.60%) and CD31 (6.60%). (Wright *et al.* 2021) The research also analyzed the ability of MSCs to differentiate into various mature cell types, indicated by calcium deposits and red-stained fat using Alizarin Red, Oil Red, and Crystal violet staining in the respective osteogenic (Figure 1B), adipogenic (Figure 1C), and chondrogenic (Figure 1D) (Ibrahim *et al.* 2022).

2.3. Secretome Analysis

MSCs were then incubated under hypoxic conditions with 5% O_2 concentration for 24 hours using a hypoxia chamber (Miltenyi; ThermoForma, USA). MSCs culture medium containing secretome was subsequently collected and filtered using the tangential flow filtration (TFF) (uPulse TFF, USA) method based on specific molecular weight cut-offs, resulting in molecules sized between 10-50 kDa containing cytokines such as VEGF, IL-10, and TGF-β. Secretome was obtained from MSCs preconditioning medium under hypoxia and filtered using TFF. The content analysis of secretome using ELISA (Table 1).

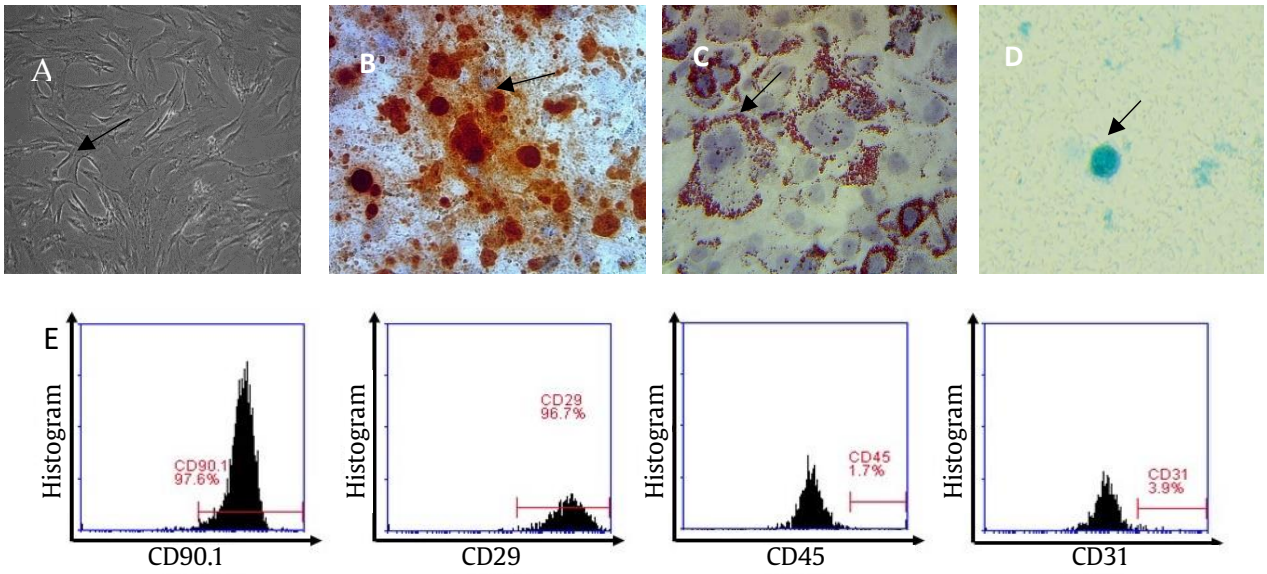

Figure 1. MSCs characterization. (A) Spindle-like cell morphology. MSCs can differentiate into various mature cell, (B) osteocytes (black arrow; calcium mineralization), (C) adipocytes (black arrow; formation of lipid droplet, (D) chondrocytes (black arrow; 3D chondrocytes), (E) surface markers

Table 1. Secretome content analysis using ELISA

Molecules	SH-MSCs value ± SE (pg/ml)
VEGF	1137.56±37.51
PDGF	947.26±34.93
bFGF	1175.54±38.82
IL-10	525.12±10.24
TGF-β	372.53±9.18

2.4. Sample Preparation of Gel Secretome

We used (acrylic acid) hydrogel (Himosap gas 100, Deripol, USA) as a base gel. The hydrogels were considered potential candidates in various biomedical applications; these polymer hydrophilic groups played a significant role in epithelial tissues and mucous membranes in wound healing. (Suhail *et al.* 2020) Secretome MSCs were mixed with base gel, and the formulation of gel secretome in the base gel was varied into 100 µL/kg body weight and 200 µL/kg body weight of secretome in 100 mg base gel.

2.5. Diabetic Rats Wound Healing Model

Male Wistar rats meeting inclusion criteria were randomly assigned to four groups: base gel administration group as a negative control, gentamycin administration group as a positive control, gel secretome administration at a dose of 100 µL/kg body weight, and gel administration at a dose of 200 µL/kg body weight in 100 mg base gel. The rats were acclimatized for one week, and the diabetic rats model induced by streptozotocin prepared in 0.1 M citrate buffer was injected intraperitoneally at a single dose of 65 mg/kg. All rats were maintained for 21 days to confirm the DM1 model, validated by blood sugar levels exceeding 250 mg/dl with glucose check at days 0, 7, and 21, followed by measurement of c-peptide insulin by ELISA. The animals were anesthetized with ketamine in the dosage of 100 mg/kg body weight and xylazine in the dosage of 10 mg/kg body weight intraperitoneally to create skin wounds under sterile conditions. Wounds were created on the rats by making a 6 mm punch excision on their back with a depth of 2 mm. Gel secretome was applied topically at the wound site six days after wounds were created. The intervention was conducted for ten days. Pictures were taken on days 0, 7, and 10 during the intervention using Image J. Wound closure rate percentage was calculated using the formula: wound closure percentage (%) = wound area $(A_0-A_t) / A_0 \times 100\%$, A_0 wound area at day 0 and At is the wound area at 7 or 10-day post excision (Hendrawan *et al.* 2021). After completion of the treatment, 50-100 mg skin samples were taken to analyze P38 and VEGF gene expression.

2.6. Measurement of P38 and VEGF

Measurement of P38 and VEGF gene expression in diabetic wound skin tissue was carried out by RNA extracted with TRIzol (Invitrogen, Shanghai, China)

according to the manufacturing's protocol and analyzed using quantitative real-time polymerase chain reaction (qRT-PCR) instrument (PCR max Eco 48) with a predenaturation temperature profile of 95°C for 3 minutes, denaturation of 95°C for 30 seconds then annealing for 20 seconds at 64°C in 40 cycles using Eco Software v5.0 (Illumina Inc, San Diego, CA, USA). The VEGF and P38 gene expression were recorded as the cycle threshold (Ct). The Ct value of the p38 and VEGF gene (target gene) normalized to the Ct value of the GADPH gene (gene reference/housekeeping) (Table 2) and calculated the expression ratio for treatment and control using the Livak Formula: $2^{-\Delta\Delta CT}$ (Hendrawan *et al.* 2021).

2.7. Statistical Analysis

Statistical analysis was conducted using IBM SPSS Statistics for Windows version 26 (IBM Corp, NY). All data variables were presented as mean ± SD. Comparability tests were assessed using the Kruskall-Wallis test to determine differences between groups, followed by post hoc Mann-Whitney analysis, with a 95% confidence interval considered statistically significant at $p<0.05$.

3. Results

3.1. Effect of Gel Secretome Administration on Macroscopic Wound Closure

The results of administering gel secretome on diabetic wounds in Wistar rats are presented in Figure 2. Progressive wound closure percentage in each group was observed on days 0, 7, and 10, calculated using the formula followed by statistical analysis using the Kruskall-Wallis were presented as mean ± SD. As mentioned previously, the rats were randomly assigned to four groups: a negative control group that received base gel only (K1), a positive control group that received gentamycin (K2), and two groups that were administered gel secretome by the dose of 100 µL/kg body weight in 100 mg gel (K3) and 200 µL/kg body weight in 00 mg gel (K4). At Day 0, the wound closure percentage in all groups was still 100±0.00. The measurement of wound closure percentage on day 7 found that K1 showed the result of 52±5.23, K2 23.20±5.36, K3 10.80±3.49, and K4 9.40±2.61. On day 10, the wound closure percentage showed improvements, with K1 yielding the result of 37.80±8.82, K2 14.80±4.55, K3 7.60±1.67, and K4 5.80±2.49. From the results, it can be concluded that wound closure appeared to be fastest in treatment groups K3 and K4, followed by K2. Meanwhile, in the K1 group, complete wound closure didn't occur by day 10. The wound closure rate percentage was significantly different in the intervention group compared to the control group ($p = 0.000$).

Table 2. Primary sequence of P38 and VEGF (genes reference) and GAPDH (housekeeping genes)

Gene symbol	Primary sequence 5'-3'
P38	Forward: 5' - GAGCGTTACCAGAACCTGTCTC
	Reverse: 5' – AGTAACCGCAGTTCTCTGTAGGT
VEGF	Forward: 5' - CTGCTGTAACGATGAAGCCCTG
	Reverse: 5' – GCTGTAGGAAGCTCATCTCTCC
GAPDH	Forward: 5' - ACTCCACTCACGGCAAATTC
	Reverse: 5' – TCTCCATGGTGGTGAAGACA

3.2. Effect of Gel Secretome Administration on P38 and VEGF Expressions

Based on the results of this study, the mean expression of P38 and VEGF showed an increasing trend in groups K2, K3, and K4 compared to K1 (Figure 3). The comparative test using Kruskall-Wallis indicated significant differences between the intervention groups ($p = 0.000$).

Post-hoc Mann-Whitney test results in Figure 3 show that the mean expression of p38 significantly increased in intervention groups K2 ($p = 0.55$), K3 ($p = 0.000$), and K4 ($p = 0.000$) compared to K1, with the most significant increase observed in group K4. A similar result was found for VEGF expression, in which the data showed significant differences in intervention groups K2 ($p = 0.000$), K3 ($p = 0.000$), and K4 ($p = 0.000$) compared to K1. These results indicate that administering gel secretome at 100 µL and 200 µL can increase p38 and VEGF expression in male Wistar rats with diabetic wounds compared to the control group.

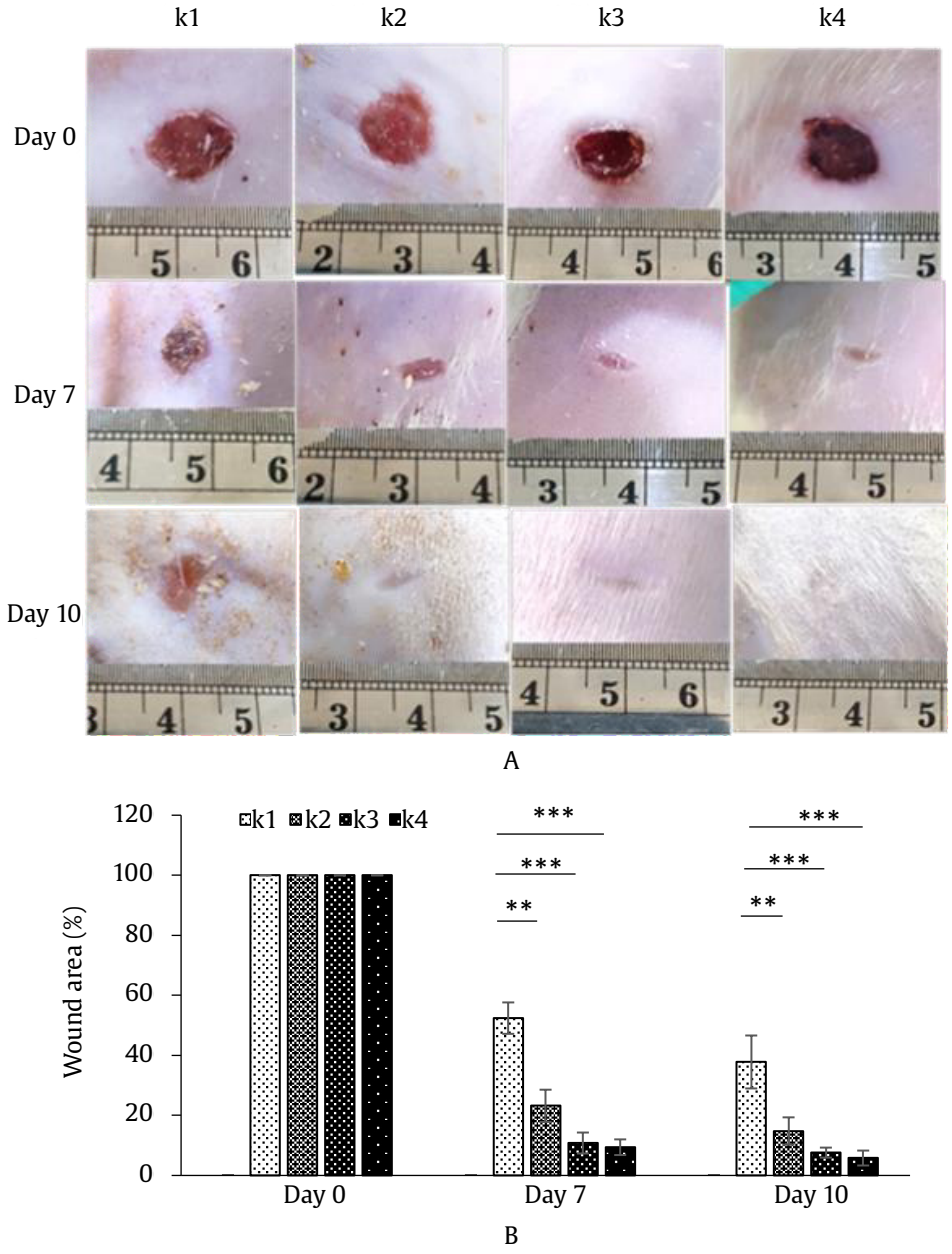

Figure 2. Wound closure process in each group. (A) Wound closure measurement in control and intervention groups on days 0, 7, and 10 after intervention. Day 0 after intervention showed that the wounds were still unhealed in each group. On day seven after the intervention, K3 and K4 showed better wound closure compared to the control group. On day 10, near-complete wound closure occurred in the intervention group, (B) graph of comparison for the wound closure area percentage after intervention on days 0, 7, and 10 in the intervention and control group. The wound closure rate on days 7 and 10 was significantly different ($p<0.05$) in the intervention group compared to the control group (*$p<0.05$, **$p<0.01$, ***$p<0.001$)

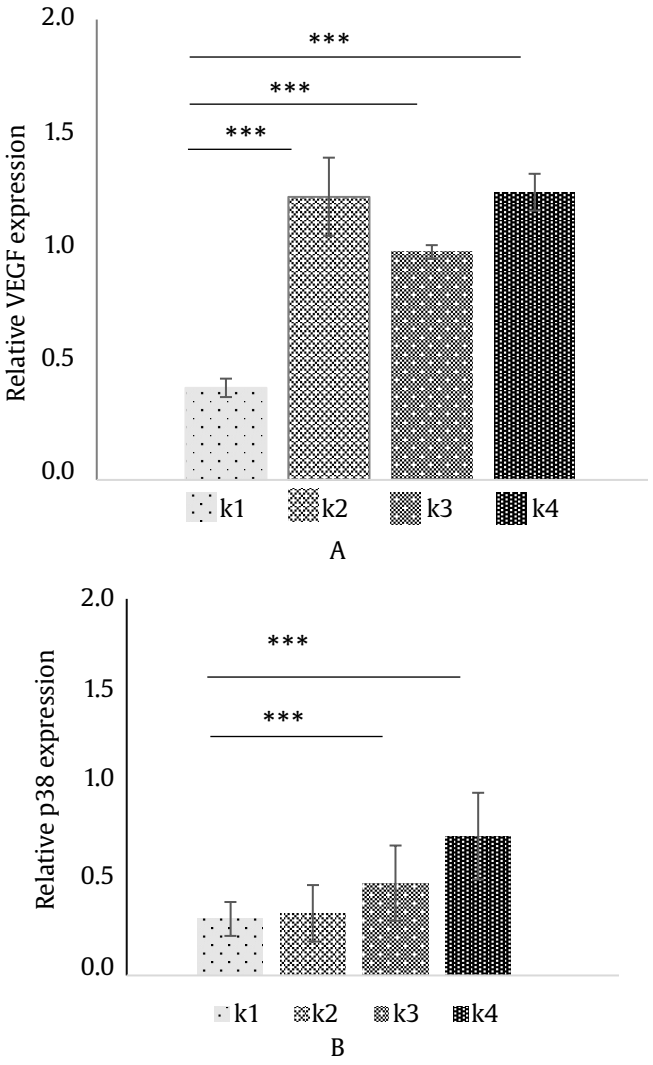

Figure 3. Comparative analysis of VEGF and P38 expression between groups. (A) Relative VEGF expression (mean±SD) between groups (K1 0.4±0.04, K2 1.23±0.17, K3 0.99±0.03 and K4 1.25±0.08), (B) Relative p38 expression (mean±SD) between groups (K1 0.3±0.09, K2 0.33±0.15, K3 0.49±0.2, K4 0.74±0.23). Comparative analysis of VEGF and P38 expression shows that there's a significant difference in the intervention group compared to the negative control (p = 0.000)

4. Discussion

This study aimed to investigate the effects of gel secretome administration with varying doses of 100 µL and 200 µL on diabetic wounds using male Wistar rats induced with Streptozotocin on the upregulation of P38 and VEGF gene expression. The study also compared the administration of gel secretome with antibiotics as the positive control group. The choice of antibiotics as the control was due to their ability to inhibit and kill microbes in open wounds, which correlated with wound healing in diabetic wounds. The research results indicate that the administration of gel secretome in the intervention groups at doses of 100 µL and 200 µL significantly enhanced the expression of P38 and VEGF compared to the control, hence the increase in cell proliferation and angiogenesis processes. The increase in P38 and VEGF expression is suspected to be due to the influence of cytokines such as IL-10 and several growth factors in the gel secretome, which function as anti-inflammatory agents and initiators of proliferation and re-epithelialization (Li *et al.* 2022)

Various bioactive molecules, including growth factors and cytokines, have been found in MSC secretomes (Ibrahim *et al.* 2022). Some of these components can activate intracellular signaling pathways in recipient cells. In the context of P38 expression, it has been observed that some components of the MSC secretome can stimulate the P38-MAPK signaling pathway in target cells (Whitmarsh 2010). Activation of P38-MAPK plays a crucial role in regulating various cellular processes, including angiogenesis. By promoting the activation of this pathway, the MSC secretome can play a central role in increasing P38 expression in target cells, which is relevant in tissue regeneration, where increased P38 expression can contribute to angiogenesis and tissue repair (Zhao *et al.* 2019). Studies have reported that components present in the MSC secretome, such as TGF-β or PDGF, can bind to receptors on target cells and initiate signaling cascades that lead to P38 activation. (Hassanzadeh *et al.* 2021; Weng *et al.* 2022). The MSC secretome also contains cytokines such as interleukin-6 (IL-6) or interleukin-8 (IL-8) that can activate MAPK pathways, including P38, in target cells. This activation likely involves target cell surface receptors and intracellular signaling pathways. In the context of angiogenesis, P38 activation by the MSC secretome can stimulate the production of factors like VEGF, which is necessary to form new blood vessels (You *et al.* 2016).

P38 activation can also modulate the expression of genes related to angiogenesis, such as VEGF and FGF, which are essential in triggering the formation of new blood vessels. Evidence suggests that the P38 MAPK pathway can play a crucial role in regulating VEGF expression. Several growth factors, such as cytokines or oxidative stress, can activate

this pathway. Activation of the P38 pathway can stimulate the transcription of the VEGF gene, leading to increased VEGF production by target cells. Previous reports indicate that VEGF is a significant growth factor that stimulates the formation of new blood vessels (Leelahavanichkul *et al.* 2014; Dai *et al.* 2021). The P38 pathway can also interact with transcription factors like hypoxia-inducible factor 1-alpha (HIF-1α), which regulates VEGF. P38 activation can influence the stability and activity of HIF-1α, which, in turn, can affect VEGF expression (Ryu *et al.* 2010).

This study showed a significant difference in the administration of gel secretome compared to the control group. The administration of gentamycin in the positive control group can help control diabetic wound infections, a crucial factor in promoting healing. By reducing the number of pathogenic bacteria in the wound, gentamycin can reduce inflammation and allow wound-healing cells to work more effectively (Hendrawan *et al.* 2021). This is also evidenced by the macroscopic wound closure rate and wound closure rate graph seen in photos taken on days 0, 7, and 10 (Figure 2).

Gentamycin administration has effects similar to those of gel secretome administration. Previous studies have stated that MSC secretome, in addition to being anti-inflammatory and regenerative, also has antimicrobial potential. Components in the MSC secretome can modulate the immune response to help direct the immune system to combat infections more effectively (Ibrahim *et al.* 2022). Other studies have revealed that the MSC secretome contains antimicrobial peptides, such as defensins, LL-37, and cathelicidin, which can naturally disrupt the cell membranes of bacteria and fungi. This results in the death of microbes and inhibits their development (Silva *et al.* 2021).

The limitations of this study include the lack of histopathological examination of collagen density and keratinocyte expression after the administration of gel secretome doses since keratinocyte migration is an essential stage in wound re-epithelialization through P38 MAPK upregulation. In potential diabetic ulcers, delayed wound healing is associated with decreased VEGF, leading to dysregulation of P38 MAPK signaling, inhibiting the migration of endothelial cells, keratinocytes, and fibroblasts, as well as the decomposition of collagen and the extracellular matrix involved in wound healing.

The administration of gel secretome at 100 µL and 200 µL doses significantly increased P38 and VEGF expression in male Wistar rats with Streptozotocin-induced diabetic wounds. Further research with measurements of anti-inflammatory parameters such as IL-10, TGF-β, and IL-4 is needed to explain the mechanism of gel secretome in increasing P38 and VEGF. Additionally, histopathological examinations of collagen density and keratinocyte expression, as well as cytokines and chemokines SDF-1/CXCR4, which play a role in endothelial cell migration in angiogenesis, are also necessary.

In conclusion, the MSC secretome may increase P38 expression by activating MAPK pathways and cellular signals that affect increasing VEGF expression in angiogenesis and tissue regeneration. A better understanding of these mechanisms will help guide the development of therapies that harness the regenerative potential of MSC secretome in clinical applications.

Acknowledgements

We would like to thank the Heads and Staff of Stem Cell and Cancer Research (SCCR), Faculty of Medicine, Sultan Agung University Semarang.

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