INTERNATIONAL JOURNAL OF MULTIDISCIPLINARY RESEARCH AND ANALYSIS

ISSN(print): 2643-9840, ISSN(online): 2643-9875

Volume 06 Issue 10 October 2023

DOI: 10.47191/ijmra/v6-i10-28, Impact Factor: 7.022

Page No. 4756-4761

The Effect of Secretome Hypoxia Mesenchymal Stem Cell (Sh-MSC) Therapy on PDGF and IFN-Gama Gene Expression in Male Wistar Rat Excision Models

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ABSTRACT: Wounds are damage to normal anatomical structures and functions due to pathological processes that originate internally or externally and affect certain organs. The wound healing process consists of four highly integrated and overlapping hemostasis phases, inflammation, proliferation, and remodeling or network resolution. Therapy using Mesenchymal Stem Cells (MSC) is considered effective in improving skin wound healing because it has the potential for differentiation and immunoregulation and there is no potential for post-treatment rejection. This research aims to prove the influence of secretome hypoxia mesenchymal stem cells (SH-MSC) on PDGF and IF-Gamma gene expression in Wistar rats using the excision wound model. This research is in-vivo experimental research with Post-test Only Control Group Design. The research subjects were 18 male Wistar rats with excision wound models divided into three groups there are placebo gel (negative control), Clobetasol at a dose of 0.25g/kg (positive control), and the P1 group SH-MSC gel at a dose of 400 µl/kg BW, were given treatment for 5 days. On the 6th day, the skin tissue was examined using the RT-PCR method to see the expression of the PDGF and IF-gamma genes. Statistical analysis using One-way ANOVA and Post Hoc LSD test. The average PDGF gene expression in the P1 group was the highest, followed by the positive control. The lowest average expression of the PDGF gene was found in the negative control. The average IFN-γ gene expression in the negative control was the highest, followed by the positive control group. The lowest average expression of the IFN-γ gene was found in the P1 group. The administration of SH-MSCs topical gel 400µl/kg BW increased PDGF expression in male Wistar rats with excision wound models, whereas the administration of gel secretome SH-MSC at a dose of 400µl/kg BW can reduce IFN-γ levels in male Wistar rats with the excision wound model.

KEYWORDS: SH-MSC, hypoxia, PDGF gene, IFN-γ gene, excision wound

I. INTRODUCTION

Wounds are damage to normal anatomical structures and functions due to pathological processes that originate internally or externally and affect certain organs.[1] It is estimated that 1.5-2 million people in Europe suffer from acute or chronic injuries. These injuries receive treatment in hospitals and clinics. Patients who suffer injuries report physical, mental, and social consequences, resulting in increased demands on nursing time at significant costs, an aging population, and increased rates of morbidity associated with injuries.[2] The wound healing process consists of four highly integrated and overlapping phases: hemostasis, inflammation, proliferation, and remodeling or network resolution.[3] The phases and their biophysiological functions must occur in a precise sequence, at a certain time, and continue for a certain duration at optimal intensity. Skin wound repair is a series of highly coordinated cellular responses to injury that function to restore epidermal integrity.[4] Therapy using Mesenchymal Stem Cells (MSC) is considered effective in improving skin wound healing because it has the potential for differentiation and immunoregulation and there is no potential for post-injection rejection.[5]

Stem cells are precursor cells that are capable of differentiating into other cells and their ability to form colonies, This ability causes stem cells to be able to repair the function of tissues that have been damaged by cells.[6] Stem cells secrete a number of proteins (secretomes), Stem cell secretomes are rich and complex molecules secreted by living cells or taken up from the cell surface. Stem

cell secretome has a function to prevent acute tissue damage.[7] The secretome is important in various biological functions such as cell growth, replication, differentiation, signal transduction, apoptosis, adhesion, and angiogenesis. The secretome contains a lot of proteins, growth factors, angiogenesis factors, hormones, cytokines, extracellular matrix proteins, extracellular matrix proteases, hormones, lipid mediators, and genetic material.[8]

Culture techniques in hypoxic environmental conditions can improve the quality of MSCs in terms of proliferation, and survival ability, and secrete more cytokines and growth factors such as platelet-derived growth factor (PDGF).[9] PDGF is regulated by external stimuli, such as hypoxia, hyperglycemia thrombin, and other cytokines.[10] PDGF accelerates infiltration of inflammatory cells and fibroblasts, deposition of extracellular matrix, and formation of collagen resulting in a rapid healing process.[11] PDGF has several isoforms, namely PDGF-A, PDGF-B, PDGF-C, and PDGF-D. However, PDGF-B is the main isoform found in platelets.[12] Re-epithelialization and formation growth factor PDGF plays an important role in wound healing.[13] PDGF increases collagen synthesis by stimulating transforming growth factor-β (TGF- b).14,15 TGF-β is a powerful anti-inflammatory cytokine in regulating uncontrolled inflammatory responses.[16]Wounds at low oxygen levels activate adenosine monophosphate (AMP) that are known to activate hypoxia-inducible factor 1 alpha (HIF1a) then via IL-17 and IL-22 produced by resident T cells at the site of injury.[17] IL-1 activates toll-like receptors (TLR), and interferon (IFN), as well as TNF-α which promote M1 macrophage differentiation. The switch from M1 to M2 macrophages as well as the differential function of AMP is an important regulator of the reparative phase. Wound re-epithelialization requires regulated keratinocyte proliferation, migration, and differentiation, which is guided in part through the production of growth factors and AMPs.[17]

Therapy of Hypoxic Mesenchymal Stem Cells (H-MSC) can accelerate the wound healing process through a paracrine mechanism by increasing the expression of transforming growth factor-β (TGF-β).[24] Hypoxia is one of the factors that can trigger the release of inflammatory and vasculogenic cytokines during tissue regeneration.[25] Research conducted in June, there in 2014, showed that MSC derived from human amniotic fluid under hypoxic conditions can accelerate wound healing by increasing paracrine factors through activation Mothers against decapentaplegic homolog 2 (SMAD2) and lanefosfatidil-inositol-3-kinase/protein kinase B (PI3K)/AKT10.[26] However, research secretome hypoxia mesenchymal stem cell on the expression of PDGF and IF-Gamma has not been done. Based on this, researchers conducted influence research secretome hypoxia mesenchymal stem cell on PDGF and IF-Gamma expression of rats Wistar with an excision wound model.

II. MATERIAL AND METHOD

This research is an in vitro experimental research method using Post-Test Only Control Group Design. This study used male Wistar rats as research subjects and divided them into three groups. negative control (K-) is rats with excision wounds treated with basic gel placebo), and treatment positive control 9K+) is rats with excision wounds treated with clobetasol dose of 0.25g/kg).[58] and Group 3 (P1) is rats with excision wounds were smeared with topical SH-MSCs gel at a dose of 400µL/kg BW. Mesenchymal stem cells are capable of expressing and producing a variety of growth factors and active cytokines. As for some secretome, I mean fibroblast growth factors, such as IL-1, IL-6, TGF-β, and VEGF. Re-epithelialization and formation of growth factor PDGF play an important role in wound healing.

III. RESULT

In this study, researchers got the result that SH-MSCs were able to express CD90 (97.3%), CD29 (97.0%), and slightly expressed CD45 (1.2%) and CD31 (4.8%) in male Wistar rats depending on the dose given (Table 1).

Table 1. PDGF gene expression

Variable	Group			P value
	K- N=6	K+ N=6	P1 N=6	
PDGF expression (pg/mL)				
Mean ± SD	0,63 ± 0,23	0,84 ± 0,15	2,32 ± 0,22	
Shapiro wilk	0,49*	0,76*	0,18*	
Levene test				0,35**
One-way ANOVA				0,000***

Information:
**Saphiro Wilk test* (p > 0,05 = normal)
***Levene's Test* (p > 0.05 = homogeneous)
****One Way Anova* (p < 0.05 = there is a difference in meaning)

The Effect of Secretome Hypoxia Mesenchymal Stem Cell (Sh-MSC) Therapy on PDGF and IFN-Gama Gene Expression in Male Wistar Rat Excision Models

The effect of SH-MSC at a dose of 400µL on PDGF and IFN-γ Gene Expression

Table 1 shows that the average PDGF gene expression in the P1 group was the highest, followed by the average PDGF gene expression in the positive control group. Furthermore, the average expression of the PDGF gene in the negative control group had the lowest value. The PDGF gene expression data of the three groups were all normally distributed, as shown by the Shapiro Wilk test result obtained a value of p> 0.05, and also has a homogeneous variant of the data shown by the Levene's Test result with a value of p=0.35 (p>0.05). The distribution and variance of the PDGF gene expression data were normal and homogeneous, so a statistical analysis of One Way ANOVA was carried out and resulted in a value of p = 0.000 (p <0.05) so that it was stated that there was a significant difference in the average expression of the PDGF gene between the three groups.

Table 2. IFN-γ gene expression

Variable	Group			*P value*
	K- N=6	K+ N=6	P1 N=6	
IFN-γ (pg/mL)				
Mean ± SD	2,55 ± 0,10	0,21 ± 0,08	0,15 ± 0,12	
Shapiro wilk	0,78*	0,49*	0,26*	
Levene test				0,70**
One-way anova				0,000***

Information:

**Saphiro Wilk test* (p > 0,05 = normal)

** *Levene's Test* (p > 0.05 = homogeneous)

*** *One Way Anova* (p < 0.05 = there is a difference in meaning)

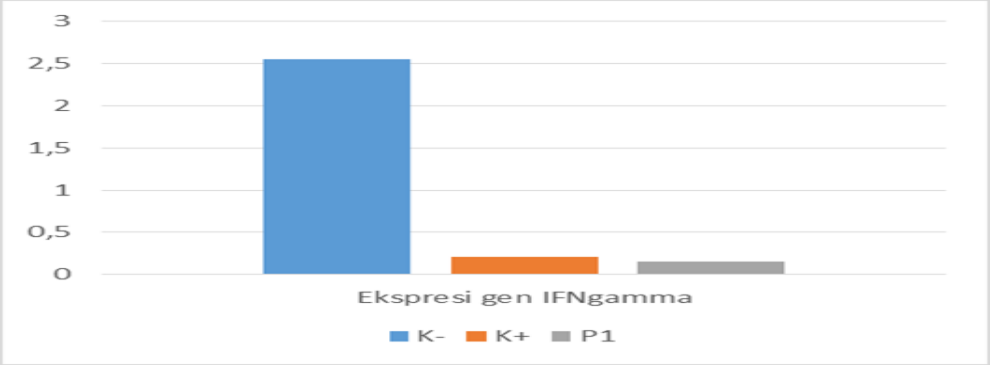

Figure 1. The Average of IFN-γ gene expression in each group

Tamhane test result (Table 2) shows that the average IFN-γ gene expression in the negative control group is the highest, followed by the average IFN-γ gene expression in the positive control group (Figure 1). Furthermore, the average expression of the IFN-γ gene in the P1 group had the lowest value. IFN-γ gene expression data for all three groups were normally distributed, as shown by the Shapiro *Wilk* test obtained a value of p> 0.05, and also has a homogeneous variant of the data shown by the Levene's Test resulted in a value of p=0.70 (p>0.05). Then a statistical analysis was carried out with a One Way Anova test resulting in a value of p = 0.000 (p <0.05) so that it was stated that there was a significant difference in the average expression of the IFN-γ gene between the three groups. One Way Anova test was significant followed by a Post Hoc test to see which groups have the most influence.

The Differences of PDGF and IFN-γ Gene Expression

Table 1 shows that there is no significant difference between K- and K+ (0.102), K- with P1, K+ with P1, P1 with K-, and P1 with K+ there is a significant difference (p <0.005). Post Hoc LSD test results by administration of SH-MSCs topical gel 400µl/kg BW can increase PDGF gene expression in male Wistar rat excision wound model.

Table 3. Post Hoc LSD test on PDGF levels in each group

Group	Comparison Group	Sig.	95% Confidence Interval	
			Lower limit	Upper limit
K-	K+	0,102	-0.4620	0.0467
	P1	0,000*	-1.9394	-1.4306
K+	K-	0,102	-0.0467	0.4620
	P1	0,000*	-1.7318	-1.2230
P1	K-	0,000*	1.4306	1.9394
	K+	0,000*	1.2230	1.7318

Based on the data in Table 3, it was found that the average K- with K+ (0.000) had a significant difference, the same thing with K- with P1, K+ with K- and P1 with K- there was a significant difference, while K+ with P1 and P1 and K+ were not significantly different between the two groups. Post Hoc LSD test results showed that administration of the secretome hypoxia mesenchymal stem cell (SH-MSC) dose of 400µl/kg BW can reduce IFN-γ levels in male Wistar rat excision wound models.

V. DISCUSSION

Excision wound is a type of wound that occurs on the surface of the skin, the bottom layer is cut with varying depths with regular wound edges.[59] Skin wound healing is an important physiological process involving the collaboration of various cell strains and their products. Recovery from lesions caused by local aggression begins early in the inflammatory stage. Repair consisting of the replacement of specific structures caused by deposition and regeneration of collagen, corresponds to the processes of cell proliferation and differentiation posteriorly through the stem cell network.[60]

The transition from the inflammatory phase to the proliferative phase is a key step during wound healing. The inflammatory phase is critical for leading to hemostasis and recruitment of the innate immune system, which protects the body from invading pathogens and helps remove dead tissue. Prolonged inflammation can hinder the normal stages of wound healing and is also associated with excessive scarring.[61] The transition from the inflammatory phase to the proliferative phase is the most crucial step during the wound healing process.[62]

In this research Mesenchymal stem cells were conditioned in hypoxic conditions and then injected with hypoxic MSC around the wound area to accelerate wound healing by shortening the transition period from the inflammatory phase to the proliferative phase. Mesenchymal stem cells injected will migrate to the wound area.[63] According to previous research by Chen et al., 2014, Widowati, et al. 2017, Peruzzaro et al., 2019 and Putra et al., 2019. Preconditioning of MSC with a hypoxic environment has survival chance which was higher in the wound area compared to MSCs cultured under normoxic conditions. During hypoxic conditions, where the oxygen concentration is at niche stem cells 2-9% can induce Hypoxia-Inducible Factor 1-α (HIF-1α) increases thereby decreasing the level of Reactive Oxygen Species (ROS) in MSC mitochondria, activating Nuclear Factor Kappa B (NFkB). HIF-1α also stimulates the synthesis of Normal Cellular Prion Protein (PrPC), and NFκB promotes the expression of anti-apoptotic proteins, repairing growth factors, and antioxidant enzymes.[64,65]

Analysis of MSC-conditioned medium showed that MSC secreted many known tissue repair mediators including growth factors, cytokines, and chemokines, particularly VEGF, PDGF, bFGF, EGF, keratinocyte growth factor (KGF), and TGF-β.[66] The increased ability of MSCs to trigger polarization was caused by more IL10 secretion by MSCs due to incubation under hypoxic conditions. Hypoxic conditions are known to encourage MSCs to secrete more cytokines.[67]

MSCs can enhance wound healing through two main mechanisms, namely by providing signals necessary for wound healing through the release of inflammatory mediators, together with cytokines and growth factors (growth factor), then through the MSC itself participates in the wound healing process.[68] MSCs communicate with other cells in the human body and undergo processes homing to the injured area in an attempt to respond to signals from cell damage, known as signal homing. The situation happens when there is TNF-α which is a pro-inflammatory mediator that comes out when the tissue is damaged so that the MSC will migrate to the damaged tissue.[69]

MSC also plays a role in every phase of wound healing. In the inflammatory phase, MSCs play a role in the regulation of inflammatory processes, suppression of TNF-α, and blockade of T cell proliferation. In the proliferative phase, MSCs play a role in the production of growth factors (VEGF, HGF, PDGF, and FGF), recruitment from keratinocytes, dermal fibroblasts, and niche stem

cells. In the remodeling phase MSCs play a role in the production of TGF-β, and KGF, regulation of MMPs/TIMPS, and regulation of collagen deposition.[70]

This study is in line with other studies using MSC secretome gel at a dose of 400µl where there was a decrease in inflammation of the peritoneal adhesions.[71] In this study, the results of giving the gel were obtained secretome hypoxia mesenchymal stem cell (SH-MSC) dose of 400µl/kg BW reduced IFN-γ levels in male Wistar rats excision wound model, in line with research by Anggarwan, et al. with the addition of MSCs into an active immune response decreased the secretion of proinflammatory cytokines TNF-α and interferon- (IFN-γ) while simultaneously increasing the production of anti-inflammatory cytokines interleukin-10 (IL-10) and IL-4.[66]

V. CONCLUSION

The Administration of gel Secretome Hypoxia Mesenchymal Stem Cells (SH-MSCs) at a dose of 400 µL/kg BW was shown to have a significant effect on increasing PDGF gene expression and decreasing IFN-γ gene expression in male Wistar rat excision wound models.

ACKNOWLEDGEMENT

The authors declare no conflict of interest and no involvement of sponsors or funding for this research.

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