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Effect of Gel Secretome Hypoxia Mesenchymal Stem Cell on Expression of TGF-β and IL-6 (In Vivo Experimental Study in Male Rats of Wistar Strains Model Hyperglycemic Wounds)

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ABSTRACT: Wounds disrupt normal skin structure, function, and shape. The healing process is hampered by hyperglycemia or high blood glucose levels resulting in wounds healing longer, resulting in an extension of the healing phase. The aim of this study was to determine the effect of administering hypoxic MSC secretome gel on the expression of the TGF-β and IL-6 genes in male white Wistar rats with a hyperglycemic injury model. True experimental in vivo research with a Post Test Only Control Group design. Consisting of 5 groups, namely the healthy mice group (K1), the base gel treated mice group (K2), the standard intervention treatment group (positive control) with gentamicin (K3), the secretome treatment and intervention group with a dose of 100 µL (K4) and a dose of 200 µL (K5). The research sample consisted of 30 male Wistar rats. Mouse skin tissue was analyzed on day 46 after termination to see the expression of the TGF-β and IL-6 genes using the quantitative RTq-PCR method. There was a significant difference in TGF-β gene expression between treatment groups using the One-way Anova test 0.024 ($p<0.05$), various doses of MSC hypoxic secretome gel had an effect on increasing TGF-β gene expression using Tamhane's Post Hoc test ($p<0.05$) showed the highest increase at a dose of 200 µL compared to the others, while IL-6 gene expression had a significant difference with the Kruskal-Wallis test 0.001 ($p<0.05$), using MSC hypoxia secretome gel had an effect on decreasing IL-6 gene expression with results lowest at a dose of 200 µL. MSC hypoxic secretome gel at a dose of 200 µL/BW effectively increased TGF-β gene expression and decreased IL-6 gene expression in male white Wistar rats with a hyperglycemic injury model.

KEYWORDS: Hyperglycemic wounds, MSC hypoxic secretome gel, TGF-β, IL-6

I. INTRODUCTION

Wounds are disorders of the structure, function, and normal shape of the skin that can be distinguished according to the healing time of acute wounds and chronic wounds. The integrity of the skin and the architecture of skin tissue are disturbed in its form and function.[1] Several conditions, including hyperglycemia hamper the healing process. Wounds heal more slowly than usual when blood glucose levels are high, in this condition there is an extension of the healing phase.[2] Disruption of the wound healing process is associated with regulatory cytokines, growth factors, and cell signaling molecules, increased oxidative stress, epithelialization delay, collagen synthesis, angiogenesis, and impaired endothelial function. Impaired production of transforming growth factor-β (TGF-β) and vascular endothelial growth factor (VEGF), impairing wound healing in hyperglycemic conditions.[3] Hyperglycemia is closely related to the pathogenesis of diabetes mellitus, increased free fatty acids, and insulin resistance that can cause endothelial damage.[4] Susceptibility to wound infection and inflammation cannot be pathologically separated from elevated levels of pro-inflammatory cytokines such as TNF-α and IL-6, often.[5] being a serious complication leads to ischemic conditions or even amputation. Advances and technological developments in the health sector also gave rise to various alternative treatments, one of which was treatment using secretomes of hypoxic mesenchymal stem cells (MSC).

The incidence of injuries every year is increasing, both acute and chronic wounds. According to American studies, 3.50 patients out of every 1000 people have injuries. Surgical or traumatic injuries account for the majority of injuries in the world's population (48.00%), followed by foot ulcers (28.0%) and pressure sores (21.0%).[6] An American Wound Association called MedMarket Diligence conducted research on the incidence of injuries in the world based on the etiology of disease. According to surgical

wound data, there are 110.30 million cases of injuries worldwide. Trauma injuries 1.60 million cases, there are 20.40 million cases of abrasions, 10 million cases of burns, 8.50 million cases of decubitus ulcers, 12.50 million cases of venous ulcers, 13.50 million cases of diabetic ulcers, 0.20 million amputations per year, 0.60 million cases of carcinoma, 0.10 million cases of melanoma, and complications of skin cancer up to 0.10 million cases.[6]

Hyperglycemia is one of the early symptoms of metabolic disorders.[2] Impaired recovery due to physiological conditions leads to the occurrence of chronic injuries.[1] Increased oxidative stress, delayed collagen synthesis, decreased angiogenesis, impaired epithelialization and glucose metabolism, fibroblasts, and endothelial cell dysfunction have been reported as vital pathophysiological factors in delaying the wound healing process.[7] Coordination between various cells, the release of local growth factors and cytokines affect the rate of wound repair whereas disruption in this cycle leads to delayed wound healing.[8] MSC secretomes produce and release cytokines that stimulate angiogenesis, extracellular matrix remodeling, cell recruitment, immunomodulation, extracellular matrix remodeling, and nerve regeneration which all aid wound healing.[9] Hypoxic preconditioning increases cytoprotective impact, maintains multipotency, increases proliferation, and the capacity of MSC hypoxic secretomes to survive in environments that are difficult to locate damaged tissues.[10]

TGF-β is the most important growth factor for the improvement of collagen synthesis, and plays an important role in wound healing by activating fibroblasts, which promotes healing and contributes to the formation of scar repair.[11] Hyperglycemic wounds are more susceptible to infection and hyper-inflammation, causing an increase in the pro-inflammatory cytokines TNF-α and IL-6. IL-6 is thought to play an important role in wound repair, including keratinocyte stimulation and fibroblast proliferation, synthesis and breakdown of extracellular matrix proteins, fibroblast chemotaxis, and regulation of immune responses.[12]

Research by Kuntardjo et all, 2019 reported that topical administration of MSC-CM gel doses of 100 µL and 200 µL was more effective compared to combination subcutaneous injection.[13] Administration of MSC secretome can improve wound condition in mice by inhibiting inflammatory response through decreasing levels of IL-6, IL-1β, TNF-α, and IL-4, increasing the anti-inflammatory cytokine TGF-β.[14] Utilization of various precondition techniques to increase the regenerating ability of cell secretion using MSC secretomes, hypoxic conditions are strategies to increase growth factor secretion, protect tissues, and accelerate cell regeneration in wound healing.[15] However, administration of MSC hypoxic secretome gel against TGF-β and IL-6 gene expression in hyperglycemic wound model mice has never been reported. Based on this background, research is needed to determine the effect of MSC hypoxic secretome gel on TGF-β and IL-6 gene expression in male Wistar rat hyperglycemic wound models.

II. MATERIAL AND METHOD

Study Design and Experimental Animals

This research is a true experimental in vivo research with Post Test Only Control Group Design. The study subjects used male Wistar white rats aged 2-3 months with a weight ranging from 200-250 grams that fit the inclusion and inclusion criteria, acclimatized for 7 days. This study used 5 treatment groups, a group of healthy rats (K1), a group of mice treated with base gel (K2), a treatment group that received standard intervention (positive control) with gentamicin (K3), a treatment group, and secretome intervention dose of 100 µL/kgBW (K4), and a treatment group and secretome intervention dose of 200 µL/kgBW (K5).

Research Materials

The study material used rat umbilical cord, Streptozotocin, citrate buffer, water-based gel, Ketamine, Xylasine, dinitrophenyl-bovine serum albumin (DNP-BSA), aluminum hydroxide gel (Al(OH)), 2,4-Dinitrochlorobenzene 1.5% (DNCB), acetone-olive oil NaCl 0.9%, PBS, DMEM, FBS, fungizone, and penstrep.

Research Equipment

This research uses equipment consisting of a Biosafety Cabinet (BSC), micropipette, CO2 incubator, Disecting kit, Flask 75T, Hypoxic chamber, oxygen meter, beaker glass, stirring rod, 1 cc injection syringe, balance sheet, Gluco test easy touch, tweezers, surgical scissors, handscoon, and sterile swab.

MSC Isolation from Umbilical Cord

Work on biosafety cabinet class 2, equipment used and work carried out in sterile conditions, the steps are as follows: **(1)** The umbilical cord is cut from the rat fetus and the blood vessels are removed. **(2)** An Umbilical cord is collected and placed in a sterile container containing 0.9% NaCl. **(3)** Using tweezers, the umbilical cord is placed on a petri dish, and the umbilical cord is washed thoroughly with PBS. **(4)** The umbilical cord is finely chopped and inserted in flask 25T then flattened and allowed to stand for 3 minutes until the tissue sticks to the surface of the flask. **(5)** The complete medium consisting of DMEM, fungizon, penstrep, and FBS is added slowly until it covers the network. **(6)** The explant is then incubated in an incubator at 37°C and 5% CO2. **(7)** Cells

appear about 14 days after the start of the culture process. **(8)** Media is replaced every 3 days by removing half of the media and replacing it completely with new media. **(9)** Cell maintenance is carried out so that the cell obtains 80% contingency.

Hypoxia and Tangential Flow Filtration Manufacturing Process

(1) MSC that has obtained 80% consensus is then added complete medium of up to 10 mL. **(2)** Then the flask containing MSC is inserted into the hypoxic chamber. **(3)** Nitrogen gas is flowed through the inlet valve and an oxygen meter is placed in the sensor hole to measure the oxygen concentration in the chamber. **(4)** Nitrogen is then added until the indicator needle shows an oxygen concentration of 5%. **(5)** After 24 hours, the culture media will be removed and then filtered using Tangential Flow Filtration to obtain SH-MSC and then it will be matched with K4 and K5.

Gel Preparatory Manufacturing Process

(1) SH-MSC gel preparation is made by mixing SH-MSCs with gel to obtain a concentration of 250 µL/g. SH-MSC 200 mg gel preparation contains SH-MSCs at doses of 100 µL/kgBW (K4) and 200 µL/kgBW secretome (K5). **(2)** The mixing process is carried out aseptically until a homogeneous mixture is formed based on the physical properties observed using a microscope.

Treatment of Hyperglycemic Mouse Model

(1) Rats are adapted for 7 days, then after 7 days are satisfied for 8-12 hours. Rats were injected once intraperitoneally with streptozotocin 65 mg/kgBW. Validation of the blood glucose test is carried out on the 36th day after initiation. Hyperglycemia was established when the rats' blood glucose was >200 mg/dl. **(2)** After 30 days of Streptozotocin injection, the rats were anesthetized with a mixture of ketamine (60 mg/kgBW) and Xylazine (20 mg/kgBW), and the back of the rats were shaved. **(3)** Using sterile and aseptic techniques, a circular incision with a diameter of 6 mm and a depth of 2 mm was made on the skin of the rat's back with a disposable incision tool. Then the wound is closed using tegaderm for 6 days.

Validation of Microscopic Examination of Hyperglycemic Wounds

Hyperglycemic lesions are viewed macroscopically using photographs of the specimen and microscopically with HE staining. Skin samples were taken to make histological preparations by paraffin wax method and hematoxylin-eosin (HE) staining. The steps are as follows: **(1)** Skin samples of mice of all groups were taken and fixed in a 10% NBF solution. **(2)** Rinse the skin sample with 70% alcohol until it is free of fixative residue. **(3)** Skin samples were soaked in 70%, 80%, 90%, 96% alcohol, and absolute alcohol for 30 minutes respectively for the dehydration process. **(4)** Soak the skin sample in toluol until clear or transparent for 1 hour. **(5)** Paraffin infiltration is carried out in an oven at 56-60°C by placing skin samples in a mixture of toluol and paraffin in a ratio of 3:1, 1:1, and 1:3 for 30 minutes each. Skin samples were placed on pure paraffin I, pure paraffin II, and pure paraffin III for 30 minutes at each treatment. (6) In Embedding, a sample of pure paraffin peel is implanted into a mold of a paraffin block that contains liquid paraffin and waits for the paraffin to harden. **(7)** Skin samples in paraffin blocks are cut into 6 µm thick pieces using microtomes. **(8)** Next, the tissue sample slices are attached to the glass by applying Mayer's albumin and then given a little aqueous droplet and then heated on a hot plate until the slices stick firmly. **(9)** The histological preparation is deparafined by placing it on xylol for 24 hours. **(10)** The staining process is carried out by hematoxylin-eosin staining. The xylol content is taken with filter paper, then added in alcohol 96%, 90%, 80%, 70%, 60%, 50%, 40%, 30%, and aqueous is done for 1-2 minutes. The preparation is placed on Hematoxylin dye for 5-10 seconds, then rinsed under running water for 10 minutes. The next process is to soak the preparation in 30%, 40%, 50%, 60%, and 70% alcohol for 3-5 minutes. The preparation is laid out in eosin for 5-10 minutes each, then soaked in 70%, 80%, 90%, and 96% alcohol for 3-5 minutes, and then dried with filter paper. The preparation is put into xylol for 15 minutes, then the histological preparation is given drops of canada balsam. **(11)** The preparation is covered with a cover glass, named, and stored in a supply box.

Hyperglycemic Wound Care

Then on the sixth day after the formation of hyperglycemic wounds, rats were treated for 10 days with topical gel administration every day, with SH-MSC gel composition dose 100 µL/kgBW in 200 mg gel (K4) and dose 200 µL/kgBW in 200 mg gel (K5). The positive control mice (K2) received standard antibiotic therapy and the negative control mice received the base gel treatment (K3). After 10 days of wound treatment, the mice were terminated using the cervical dislocation method, and skin samples were taken for parameter analysis.

RNA Extraction Process and cDNA Synthesis

(1) A total of 50-100 mg of skin samples are cut into small pieces and inserted in a tube containing 0.5 mL of Iso Plus RNA. The pieces of skin are smoothed with micro pastels and DNA RNAse free, then added RNAIso Plus as much as 0.5 ml and stored at room temperature for 5 minutes. **(2)** Then add 0.2 mL chloroform and vortex until the solution until it becomes milky white.

Incubation for 5 minutes at room temperature and centrifugation for 15 minutes at 4°C at a rate of 15,000 xg-force until solution in a three-layer tube. **(3)** The topmost layer containing mRNA is transferred into a 2 mL microtube, with the same ratio of isopropanol added. **(4)** Shake the 2 mL microtube tube until a white thread appears, then centrifuge at 4^{0C} at a speed of 12,000 xg-force for 10 minutes. The supernatant is removed until white pellets appear at the bottom of the tube. **(5)** After drying, add 100 µL of 75% ethanol to the solution (Diethyl pyrocarbonate) DEPC then turned back several times then centrifuged again at 40C, speed 15,000 xg-force for 5 minutes. **(6)** Supernatants are removed and DEPC is added as much as 50-100 µL at -80^{0}C or can be used in further research. **(7)** Then as many as 2 µL of RNA samples were quantified using NanoDrop with a wavelength of 260 nm. The quantification result will be calculated to make 3000 ng. **(8)** cDNA synthesis process by mixing the previously calculated RNA sample with 1 µL of OligoDT and PCR water to a volume of 10 µL, then the mixture is put into a thermal cycler and incubated for 5 minutes at a temperature of 700C. (9) Then added 5X buffer 4 µL, DEPC-Treated H2O 5 µL, ReverTraAce 1 µL. (10) The mixture is then incubated at a temperature of 45-500C for 30 minutes.

Diagnosis of TGF-β and IL-6 gene expression RTq-PCR method

(1) Analysis of TGF-β and IL 6 expression using RTq-PCR, a mixture of 1 µL cDNA sample, KAPPA SYBR FAST qPCR master mix as much as 10 µL, specific forward and reverse primers in each target gene as much as 1 µL and Nuclease Free Water as much as 7 µL. **(2)** PCR products are then analyzed using RTq-PCR illumine with predaturation temperature at 950C for 2 minutes, then denaturation 950C with a time of 5 seconds, then aneling 55-600C for 20 seconds. With a total number of cycles 40 times. The primary sequences of GADPH used are: F: 5'-GCG ACA GTC AAG GCT GAG AATG -3' and R: 5'-TCT CGC TCC TGG AAG ATG GTGA -3'. **(3)** The primary sequence of TGF-β used is F: 5'-TAC CAT GCC AAC TTC TGT CTG GGA-3' and R: 5'-ATG TTG GAC AAC TGC TCC ACC TTG-3'. **(4)** The primary sequences of IL-6 used are F: 5'-TCC TAC CCC AAC TTC CAA TGC TC-3' and R: 5'-TTG GAT GGT CTT GGT CCT TAG CC-3'. **(5)** Increased gene expression was analyzed in the ratio of improvement to gene housekeeping using EcoStudy software.

III. RESULT

Isolation of the Hypoxia Secretome of MSCs

The MSC hypoxic secretome used in this study was the result of filtration of MSC culture medium under 5% hypoxic conditions for 24 hours using Tangential flow filtration (TFF). MSCs were isolated from Umbilical Cord rats aged 19 days and cultured on a flask using a specific culture medium. MSC validation is performed after the 4th phase. The results of morphological analysis showed that MSCs have spindle-like morphology similar to fibroblast cells. The results of membrane marker analysis using flow cytometry showed that MSC expressed membrane proteins CD90 (97.6%), CD29 (96.4%), and slightly expressed CD45, (1.7%) and CD31 (3.9%). Analysis of the differentiation potential of MSCs into el adipocytes and osteocytes was carried out using Oil Red O and Alizarin Red Staining painting. MSC shows the ability to differentiate into osteocytes and adipocytes indicated by calcium and fat deposits in the form of red color.

MSC hypoxic secretomes were performed using morphological analysis, cell surface markers, and differentiation potential, morphological analysis using inverted microscopy, cell surface marker analysis using flow cytometry, and adipogenic and osteogenic differentiation analysis using Oil Red O and Alizarin Red Staining. The validated cells were then cultured and incubated under hypoxic conditions (5% O2). The culture medium is collected and filtered by the Tangential Flow Filtration (TFF) method.

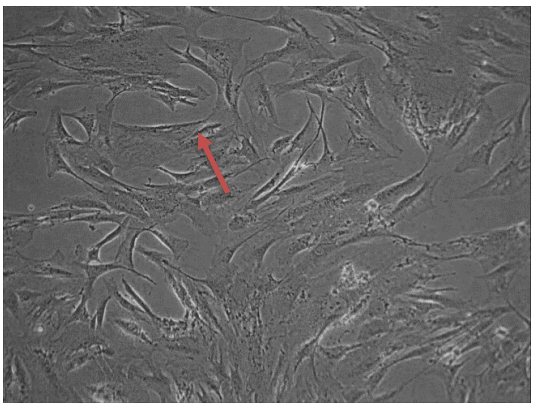

Figure 1. Isolation of MSC with spindle-like cells at 100x magnification.

Effect of Gel Secretome Hypoxia Mesenchymal Stem Cell on Expression of TGF- β and IL-6 (In Vivo Experimental Study in Male Rats of Wistar Strains Model Hyperglycemic Wounds)

The results of the isolation of MSC hypoxic secretomes were validated by flow cytrometry to demonstrate MSC's ability to express various special surface markers. Figure 2 shows that MSCs express CD90 (97.6%), CD29 (96.4%) and express CD45 (1.7%) and CD31 (3.9%)

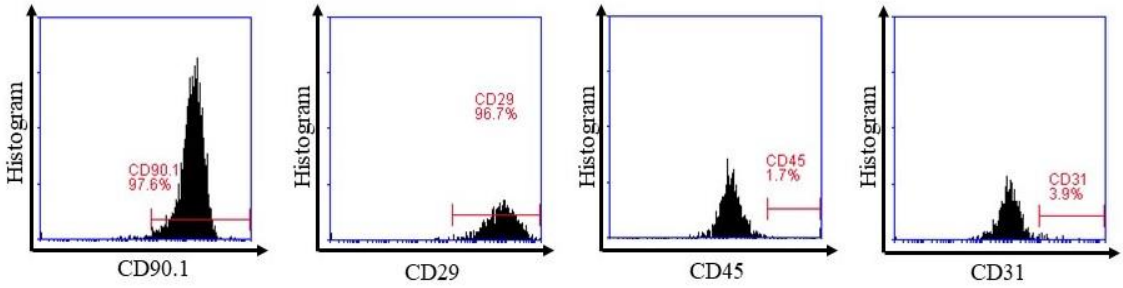

Figure 2. Flow cytometry analysis of CD90, CD29, CD45 and CD31 expression

Analysis of MSC's ability to differentiate into a wide variety of mature cells. MSCs are given special media to differentiate into osteophytes and adipocytes. red coloring using Alizarin Red staining and oil Red dye on osteogenic and adipogenic cultures at 100x magnification, respectively, seen in Figure 3 and Figure 4:

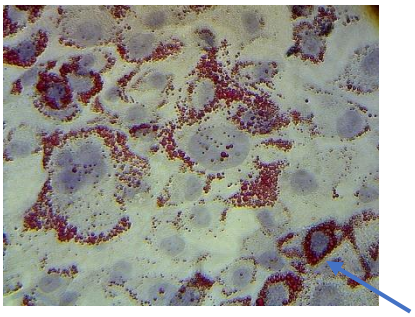

Figure 3. MSC differentiated into adipocytes

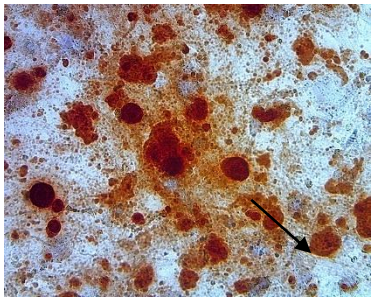

Figure 3. MSCs differentiate into osteocytes

MSC cultures containing secretomes were collected and screened using the Tangential flow filtration (TFF) method based on molecular weight measuring 10-50 kDa containing cytokines IL-10 and TGF-β.[60] TGF-β is a dimeric growth factor molecule with a molecular weight of 25 kDa that helps reduce the duration of the inflammatory response.[60]

In this study, the isolation of growth factors contained in MSC hypoxic secretomes was carried out to obtain pure MSC hypoxic secretomes. Researchers used TFF media to isolate molecules using filter tapes of 10-50 kDa 50%, 50-100 kDa 25%, and 100-300 kDa 25%. After screening, measurement of growth factor content in MSC hypoxic secretomes was carried out using the ELISA test. MSC hypoxic secretomes are known to produce TGF-β levels of 372.53 ± 9.18 pg/mL, this value is higher than the TGF-β concentration in normoxic MSC secretomes which range from 10-100 pg/mL.

IL-10 and TNF-α gene expression in mouse models of alopecia-like by administration of SH-MSCs

The results of TGF-β gene expression analysis using the RTq-PCR method using the Illumina Eco Real-Time PCR tool obtained the following results:

Table 1. Average levels, normality, and homogeneity of TGF-β gene expression between treatment groups

Group	Base gel (K2)	Gentamicin (K3)	Sec 100 µL (K4)	Sec 200 µL (K5)
Tikus 1	0,72	0,45	0,85	1,34
Tikus 2	0,78	0,89	0,93	1,85
Tikus 3	0,51	0,81	1,53	2,00
Tikus 4	0,40	0,95	0,59	1,72
Tikus 5	0,36	0,63	0,61	0,61
Tikus 6	0,97	0,62	0,66	0,54
Mean	0,62	0,72	0,86	1,34
SD	0,24	0,19	0,35	0,64
Shapiro wilk	0,64*	0,71*	0,06*	0,21*

Information:

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

** Levene's Test (p> 0,05 = homogeneous)

Based on the results shown in Table 3 and Figure 6 graph, the average expression of TGF-β genes in the base gel group (K2) was 0.62 ± 0.24, the average gentamicin group was 0.72 ± 0.19, the average secretome group 100 µL (K4) 0.86 ± 0.35 and the average secretome group 200 µL (P2) 1.34 ± 0.64. TGF-β gene expression data of the four groups were normally distributed with Shapiro-Wilk test values with p >0.05 values and had homogeneous data variants with Levene's Test test results with p values = 0.02 (p > 0.05). It was concluded that the distribution and variant of TGF-β gene expression data were normal and inhomogeneous.

Statistical analysis with the One Way Anova test obtained a value of p = 0.024 (p < 0.05) so it was concluded that there was a significant difference in the average expression of the TGF-β gene between the four groups. One-Way Anova test results followed by Post Hoc Tamhane test to determine the most influential dose group, shown in Table 2.

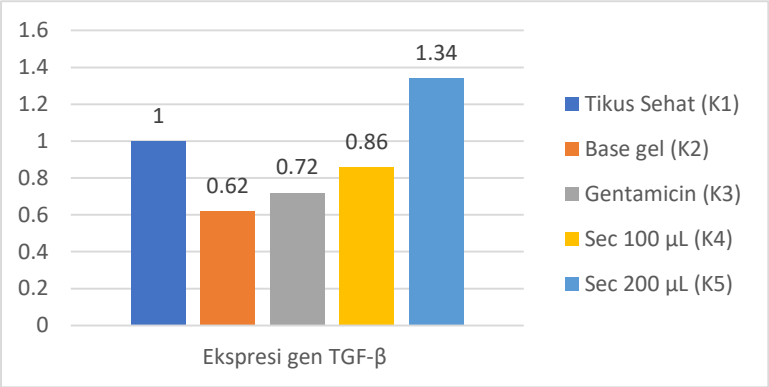

Figure 4. Average levels of TGF-β gene expression between treatment groups

Table 2. Post Hoc Tamhane test of TGF-β gene expression in each group

Group	Comparison Group	Sig.	95% confidence interval	
			Lower Limit	Upper Limit
Gel Base	Gentamicin	0.66	-0.5765	0.3732
	Secretom100	0.308	-0.7132	0.2365
	Secretom200	0.005*	-1.1948	-0.2452
Gentamicin	Gel Base	0.66	-0.3732	0.5765
	Secretom100	0.555	-0.6115	0.3382

	Secretom200	0.013*	-1.0932	-0.1435
Secretom100	Gel Base	0.308	-0.2365	0.7132
	Gentamicin	0.555	-0.3382	0.6115
	Secretom200	0.047*	-0.9565	-0.0068
Secretom200	Gel Base	0.005*	0.2452	1.1948
	Gentamicin	0.013*	0.1435	1.0932
	Secretom100	0.047*	0.0068	0.9565

The * indicates a significantly different group

Based on the data of Table 4 above, the average comparison of the base gel group (K2) with the gentamicin group (K3) 0.66 and secretome group 100 (K4) 0.308 was not significantly different, while the group (K2) with secretome group 200 (K5) was significantly different with a value of 0.005 ($p < 0.05$). Group (K3) compared with group (K2) and group (K4) were not significantly different while group (K3) compared with group (K5) was significantly different with a value of 0.013 ($p < 0.05$).

Group (K4) compared to group (K2) and group (K3) were not significantly different while group (K4) compared to group (K5) was significantly different with a value of 0.047 ($p < 0.05$). Group (K5) compared to group (K2) was significantly different with a value of 0.005, Group (K5) compared to group (K3) was significantly different with a value of 0.013, and group (K5) compared to group (K4) significantly different with a value of 0.047 ($p < 0.05$).

Tamhane's Post Hoc test results on TGF-β gene expression results showed that administration of MSC 200µl/kgBW hypoxic secretome gel can increase TGF-β gene expression in male Wistar rat hyperglycemic wound models.

Effect of MSC hypoxic secretome gel administration on IL-6 gene expression.

Table 3. Mean levels, normality, and homogeneity of IL-6 gene expression between treatment groups

Group	Base gel (K2)	Gentamicin (K3)	Sec 100 µL (K4)	Sec 200 µL (K5)
Rat 1	1,13	1,95	0,80	0,31
Rat 2	1,75	0,86	1,16	0,91
Rat 3	1,65	0,95	0,28	0,63
Mouse 4	1,96	1,95	0,91	0,63
Rat 5	1,48	0,90	1,01	0,91
Rat 6	1,13	0,95	0,28	0,63
Mean	1,52	1,26	0,74	0,67
SD	0,34	0,53	0,37	0,22
Shapiro wilk	0,49*	0,005	0,20*	0,20*
Levene test	0,036			

Information:
*Saphiro Wilk test ($p > 0.05$ = normal)
** Levene's Test ($p > 0,05$ = homogeneous)

The results of the study based on Table 5 average expression of the IL-6 gene in the base gel group (K2) of 1.52 ± 0.34, the average gentamicin group of 1.26 ± 0.53, the average secretome group of 100 µL (K3) of 0.74 ± 0.37 and the average secretome group of 200 µL (K5) of 0.67 ± 0.22. IL-6 gene expression data of the four groups were not distributed with Shapiro Wilk test values in the

gentamicin group (K3) of p = 0.005 (p < 0.05) and had inhomogeneous data variants with the results of the Levene's Test test with a value of p = 0.036 (p < 0.05). It was concluded that the distribution and variant of IL-6 gene expression data were abnormally distributed and inhomogeneous.

The Kruskal-Wallis non-parametric test obtained results p = 0.001 (p < 0.05) so it was concluded that there were significant differences between treatment groups for IL-6 gene expression

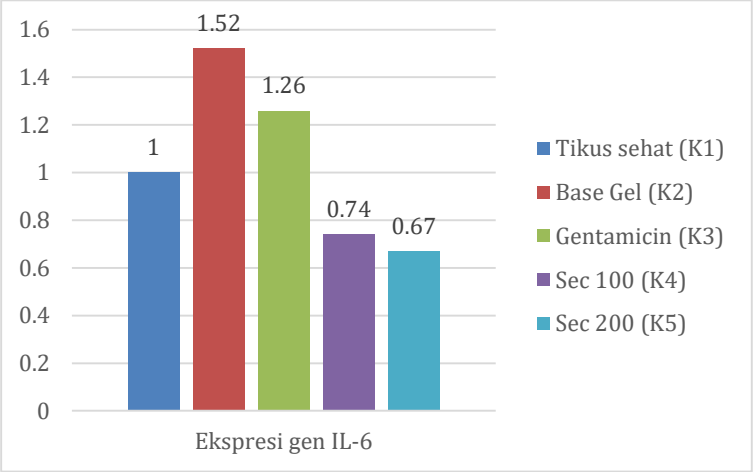

Figure 5. The average of the IL-6 gene expression between treatment groups

Comparison of TNF-α gene expression between groups in mouse models of alopecia-like with administration of SH-MSCs

Validation of Blood Sugar Levels

Blood glucose examination is carried out gradually on day 0, day 30, and day 36, following the results of the glucose level examination in Table 4.

Table 4. The results of glucose level

Group	Healthy H36	THE H36
Rat 1	102	388
Rat 2	112	314
Rat 3	96	346
Mouse 4	99	456
Rat 5	105	498
Rat 6	110	327
Mean	104	388.2
SD	6.23	74.41

Based on Table 4, the average glucose level in the day 36 hyperglycemia treatment group showed a significant increase from normal at 104 ± 6.23, and the average glucose level in the day 36 hyperglycemic treatment group showed a significant increase from normal at 388.2 ± 74.41. The results of glucose levels showed a significant increase so hyperglycemic conditions were declared valid for hyperglycemic wound mouse models.

Histopathological Validation of HE Staining

The results of observations of hematoxylin-eosin (HE) staining histopathological preparations in a group of healthy rats with hyperglycemia treatment are as follows:

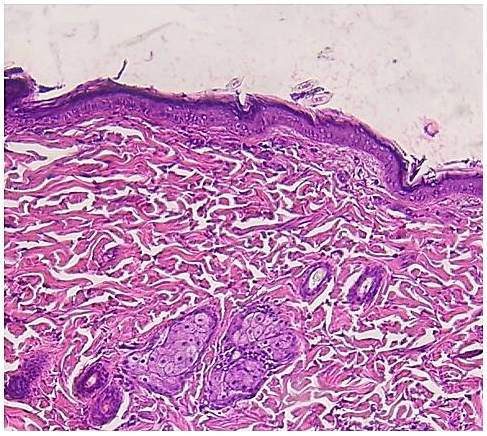

Figure 7. HE staining on healthy rat group

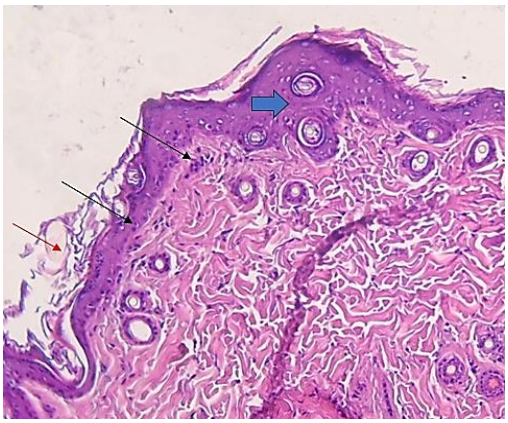

Figure 8. HE staining on hyperglycemia rat group

Based on Figure 8, HE staining in the hyperglycemic group showed thickening of the epithelial wall shown in the blue arrow, while the black arrow showed inflammation in immune cells and the red arrow necrosis in epithelial cells.

Macroscopic Picture of Hyperglycemic Wounds

Based on Figure 6, macroscopic results of hyperglycemic wounds were observed on day 36, day 43, and day 46. The results showed the acceleration of wound repair using secretomes of 200 µL on day 43 experienced the smallest macroscopic healing and day 46 wounds had healed.

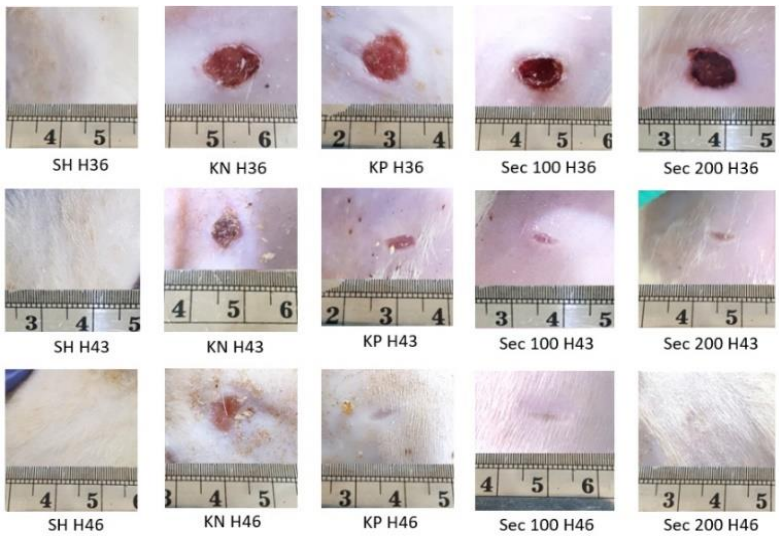

Figure 9. Macroscopic picture of hyperglycemic wounds

IV. DISCUSSION

Impaired wound healing due to hyperglycemia causes changes in endothelial cell function and vascular dysfunction in wounds resulting in infection and in many cases amputation. Delayed wound healing results from changes in several endogenous factors that contribute to wound healing, including decreased production of growth factors such as PDGF and TGF-β, mediators involved in angiogenic response, macrophage function, collagen synthesis, keratinocyte migration, and unstoppable inflammatory fibroblast proliferation.[42]

Recent developments in stem cell research, particularly involving MSCs to stop prolonged inflammatory processes as well as the recovery and repair of damaged tissues.[61] Growth factors that can trigger damage tissue repair include Basic Fibroblast Growth Factor (BFGF), Vascular Endothelial Growth Factor (VEGF), Transforming Growth Factor beta (TGF-β), Platelet-Derived Growth Factor (PDGF), Angiopoietin-1 (ANG-1), Placental Growth Factor (PIGF), and Monocyte Chemoattractant Protein 1 (MCP-1). [62]

Wound healing depends on the growth and migration of keratinocytes in the epidermis and the formation of granulation tissue in

the dermis. This tissue growth will not occur before the inflammatory process is complete. Inflammation that occurs results in tissue death which triggers the release of DAMP and causes macrophages (M1) to secrete proinflammatory cytokines, namely IL-6. With the involvement of T cells that also secrete IFN-γ M1 will continue to secrete IL-6 and cause local and systemic inflammation.[40,63]

The results showed that the highest IL-6 gene expression was found in the base gel group (K1) of 1.52 ± 0.34, and the average of the lowest IL-6 gene expression group was found in the secretome group of 200 µL (K5) of 0.67 ± 0.22. It showed that administration of MSC hypoxic secretome gel in the 200 µL (K5) secretome group had the most significant effect on decreasing IL-6 gene expression. Other studies have shown that damaged cells cause inflammation, which promotes the production of inflammatory mediators including IL-6. Decreased IL-6 levels cause MSCs to actively migrate towards damaged areas.[64] MSC cells that migrate in the wound area will secrete anti-inflammatory cytokines such as IL-10 and growth factors such as PDGF which play an important role in the wound healing process.[65]

Previous research has found that IL-10 can suppress inflammation by inhibiting nF-kB activity, which leads to the decline of pro-inflammatory cytokines such as IL-6. In addition, IL-10 is able to trigger polarization of M1 and M2, so macrophages are anti-inflammatory. Secretomes containing IL-10 can also play a similar role in suppressing inflammation in the wound area.

Another study by Pan et al. 2017, using UC-SH MSCs showed a significant reduction in serum IL-6 and TNF-α levels after UC-MSCs treatment in DM mice and improved insulin resistance.[66] In another study by Cevey et al. 2019, showed a decrease in inflammation through the IL-10 and STAT3 pathways, inducing phosphorylation of STAT3, IL-10 binds to IL-10R, activating JAK 1. The STAT3 protein enters the nucleus and activates the SOSC3 mRNA sequence which is then expressed intracellularly and inhibits proinflammatory signaling pathways, specifically NF-κB. Production of proinflammatory cytokines, including IL-6, is reduced when the NF-κB pathway is suppressed.[67]

This study aims to reveal the role of the secretome in IL-6 and TGFb expression in hyperglycemic wounds. The results showed that TGF-β gene expression increased significantly in the secretome group of 200 µL (P2) with an average of 1.34 ± 0.62. The results of the analysis using the Post Hoc Temhane test found that K5 was significantly different compared to the base gel group (K2), gentamicin group (K3), and secretome group 100 (K4). In line with the 2019 study of Beserra et al., using lupeol in wound healing in streptozotocin-induced hyperglycemic rats, found that TGF-β expression increased in mice that experienced improved and accelerated wound healing. Elevated levels of IL-10 were also known to occur in the lupeol-treated group compared to the control group.[42]

In another study by Ormazabal et al. 2022, the hMSC-EC secretome promotes wound healing in hyperglycemic mice. hMSC-EC, being a therapeutic alternative, administration of hMSC-EC generated under tightly controlled conditions could represent a new therapeutic approach for treating chronic wounds in diabetic patients.[16] In wound healing, the role of TGF-β is important in the activation of fibroblast cells, leading to the production of ECM which acts as a scaffold for MSC cells. TGF-β is mostly released by type 2 macrophages which are anti-inflammatory cells.[68] Therefore an increase in TGF-β correlates with wound healing.

V. CONCLUSION

Administration of MSC hypoxic secretome gel at a dose of 200 µL/kgBW was shown to significantly increase TGF-β gene expression in male Wistar rat hyperglycemic wound models. Administration of MSC hypoxic secretome gel at a dose of 200 µL/kgBW was shown to significantly affect the decrease in IL-6 gene expression in male Wistar rat hyperglycemic wound models.

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