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Effect of Topical Gel Administration of Secretome Hypoxia Mesenchymal Stem Cells (SH-MSCs) on IL-10 and TNF- α Gene Expression (In Vivo Experimental Study in Male Rats of Wistar Strains Model of Fluconazole-Induced Alopecia)

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ABSTRACT: Alopecia is a dermatological disorder characterized by disturbances in the shorter anagen phase and longer telogen phase in the hair cycle. Therapy with irritant side effects and contact dermatitis, scalp allergies. causes increased hair loss. Alternative therapy using secretome hypoxia mesenchymal stem cells (SH-MSCs) which is safe and effective is an option. Objective to determine the effect of topical administration of SH-MSCs gel on IL-10 and TNF-α gene expression in Wistar rats with a fluconazole-induced alopecia-like model. In vivo experimental research using a Post Test Only Control Group Design research design. This study used 4 groups, namely 2 treatment groups and intervention with a topical SH-MSCs gel dose of 20 µL and a dose of 40 µL, 1 treatment group that did not receive intervention (base gel control) and 1 group of healthy mice. Skin tissue analysis was carried out on day 22 to assess IL-10 and TNF-α gene expression using the RT-PCR method. IL-10 gene expression using the One way Anova test obtained a value of 0.00 ($p<0.05$) so that there was a significant difference in IL-10 gene expression between groups in mice with the alopecia like model. The results of the TNF-α gene expression data with a value of 0.00 ($p<0.05$) showed significant differences between treatment groups. Administration of various doses of SH-MSCs topical gel increased IL-10 gene expression and decreased TNF-α gene expression in Wistar rats with a fluconazole-induced alopecia-like model, with the use of SH-MSCs topical gel at a dose of 40 µL having the greatest effect. most significant compared to other groups.

KEYWORDS: Alopecia like, SH-MHSCs topical gel, TNF-α, IL-10

I. INTRODUCTION

Alopecia is a dermatological disorder caused by bacterial or fungal infections, inflammation, or immune dysregulation. [1] The disorder is characterized by a shorter anagen phase and a longer telogen phase in the hair cycle.[1] Several proinflammatory cytokines such as tumor necrosis factor-α (TNF-α) coordinate cyclical hair growth in the pathogenesis of alopecia.[2,3] pathological T cells express IFN-C and IL-17 early in the disease, with a significant increase in the production of cytokines IL-4 and IL-10.[3] Topical therapy using minoxydil to maintain hair gives side effects such as irritants and contact dermatitis, allergies to the scalp. Cases of allergic reactions to the inactive ingredient propylene glycol found in some topical solutions containing galenic cause increased hair loss during minoxydil use.[4] There is a need for other therapies using secretomes. Safe and effective therapeutic approaches, one of which is with secretome hypoxia mesenchymal stem cells (SH-MSCs).[5] Application of secretome therapy to alopecia is still little data reported, so further research needs to be done.

In cases of alopecia, 1-2% baldness spreads throughout the scalp (alopecia totalis) or throughout the epidermis (alopecia universalis). The incidence of alopecia areata is reported to be 0.1-0.2% with a lifetime risk probability of 1.7% in both men and women. [6] For a decade of prospective studies looking at an allecian incidence of 0.7% of outpatients with new dermatology.[7]

Research shows SH-MSCs contain various anti-inflammatory cytokines, such as *transforming growth factor* (TGF-β), *interleukin-10* (IL-10).[8] IL-10 can inhibit the inflammatory process through containment of NFκB activation. On the other hand, TGF-β also plays a role in the differentiation of Th cells into T-reg which plays an important role in the immunomodulation process.[9] MSC secretome

therapy has proven to be a new option for treating alopecia, the outer root sheath cells play a role in maintaining hair follicle structure and supporting bulge areas.[10] Promotes hair follicle proliferation and reduces inflammation, and resists rat hair loss in alopecia.[11] The MSC *secretome* serves as an inflammation sensor that has anti-inflammatory and pro-inflammatory effects.[12] In another study, *Secretome* MSCs in inflammatory conditions activated anti-inflammatory cytokines such as IL-10 and TGF-β, as well as molecular proliferation, especially platelet-derived growth factor (PDGF) and vascular endothelial growth factor (VEGF) with conditioned culture media.[12,13] Using topical gel SH-MSCs proved more effective than combination subcutaneous injections in day 6 animals, the topical group experienced significant increases in PDGF and fibroblast density when compared to the combination group using subcutaneous injection.[14]

Hair transplantation can replace areas of baldness with healthy hair, but the amount of hair is not always sufficient for replacement in patients with severe loss. The use of genes, cells, and tissues as sources of new therapies is one of the hallmarks of modern medicine. Advances in regenerative medicine have increased interest in applying stem cells to engineered tissue to reconstitute damaged tissue and developing regenerative therapies for the skin. [16] By secreting TGFb1, IL10, and PGE2, MSC secretomes have the capacity to make iTreg cells and have immunomodulatory abilities that modulate the inflammatory environment.[17] Growth factor through platelets is one of several factors that can regulate cell growth, differentiation, and activation by activation of chemotaxis, angiogenesis, and fibroblast proliferation.[18] These findings suggest that dissolved molecules secreted by MSC secretomes as CM could be one of the most effective healing treatments.[14]

Previous research reported that MSC secretomes have been widely used to accelerate wound healing through topical applications, which have proven effective.[19] The immunosuppressive ability of MSC secretomes is based on the production of cytokines such as TGF-β, IDO, NO, PGE2, IL-10, and TSG-6.[20] IL-10 and TGF-β1 are powerful anti-inflammatory cytokines that help regulate excessive inflammatory responses. IL-10, in particular, dampens pro-inflammatory signals by inhibiting the release of pro-inflammatory cytokines such as IFN-, IL-2, and TNF-α.[21] TNF-α is an active stimulator molecule that increases the secretion of various inflammatory cytokines. TNF-α-activated MSC secretomes suppress inflammation by inducing IL10 production in macrophage cells.[22] A study found that IL-10 levels decreased after 24 hours with intravenous infusion of MSC secretomes in rats.[23] Co-SMAD4 is also involved in promoting IL-10 T-cell production by directly activating IL-10 promoters.[24] MSCs strongly produce other cytokines or growth factors, including TGF-β, as evidenced by the release of IL-10, These findings support previous studies that reported IL-10 increased at 6 and 12 hours later decreased at 24 hours.[22]

In this study, researchers wanted to see the ability of topical gel secretome hypoxic mesenchymal stem cells doses of 20 µL and 40 µL on IL-10 levels and TNF-α levels with alopecia rats on day 6 post-inflammatory to see the picture of both IL-10 and TNF-α parameters in the process of accelerating hair healing and growth.

II. MATERIAL AND METHOD

Study Design and Experimental Animals

This research is an *in vivo* experimental study using *Post Test Only Control Group Design* research design. The study subjects used 24 male Wistar rats aged 2-3 months with a body weight of 200 g and according to inclusion and inclusion criteria, acclamatized for 7 days. This study used 4 groups, a healthy group without treatment (P1/K1), a negative control group, namely rat with alopecia smeared with base gel/day then on day 6 skin tissue was taken to be examined for IL-10 and TNF-α gene expression with the RT-qPCR method (P2/K2), the group with alopecia given MSC topical gel dose 20 µL in 100 mg gel daily as much as 1x in the morning, day 6 skin tissue was taken to check IL-10 and TNF-α gene expression by RT-qPCR method (P3/K3), and group tikus with alopecia given MSC topical gel dose 40 µL in 100 mg gel daily as much as 1x in the morning, Day 6 skin tissue was taken to check IL-10 and TNF-α gene expression by RT-qPCR method (P4/K4). The research subject has been declared fit by veterinarians from Animal House Stem Cell and Cancer Research (SCCR), Faculty of Medicine, Sultan Agung University.

Research Materials

The research materials used culture materials, namely mouse umbilical cord, *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. While the ingredients used for the treatment process are water-based gels, 70% alcohol, PBS, Ketamine, and Xylasine, and other ingredients such as Aquadest, NaCl 0.9%, Fungizon 0.5%, Streptomycin-penicillin 1% (penstrep), and Povidon iodine.

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Research Equipment

This study used equipment in the form of cell culture equipment consisting of *Biosafety Cabinet* (BSC), micropipettes, CO_2 incubators, *dissecting kits*, and 75T flaps. Hypoxic culture conditions were obtained using a hypoxic *chamber*. Oxygen meters are used to measure oxygen levels in the *hypoxic chamber*. In addition, this study also sterilized swabs to apply topical gel SH-MSCs. Tools used for IL-10 and TNF-α gene expression analysis such as *Centrifuges, Beaker glass, Cell counters*, Disposable pipettes, 1 cc syringes, Light microscopes, Shavers, Surgical tool sets, Micropipettes, Masks, *Handscoons*, Experimental animal cages, *Micropipette with tips* (blue tip, yellow tip, pink tip), Pipette filler, *Conical tube* (15 ml, 50 ml), *Biosafety, Inverted microscope*, *Cryotube* 1 ml, *Scissor*, and Tweezers.

Procedure of *Mesenchymal Stem Cell* Isolation from *Umbilical Cord*

Work on *biosafety cabinet class* 2, the equipment used has been sterilized first, The steps are as follows: **(1)** Umbilical cord is collected and placed in a sterile container containing 0.9% NaCl, **(2)** Umbilical cord is placed into a *petri dish* with tweezers, washed thoroughly using PBS **(3)** Separate *Umbilical cord* From the fetus of mice and blood vessels are removed. **(4)** Chop the *Umbilical cord* until smooth and place it on the 25T flask evenly and let stand for 3 minutes until the tissue adheres to the surface of the flask. **(5)** A complete medium consisting of DMEM, fungizon, penstrep, and FBS) is added slowly until it covers the network. **(6)** The explant is incubated in the incubator at a temperature of 37°C and 5% CO_2, cell culture will appear after 14 days. **(7)** The medium is replaced every 3 days by removing half of the medium and replacing it with a new medium. **(8)** Cell maintenance is carried out until the cell reaches 80% consequence.

Hypoxia Process

(1) MSC culture that reaches 80% confluence plus complete medium up to 10 mL to the flask then transfer it into the *hypoxic chamber*. **(2)** Nitrogen gas is channeled through the inlet valve and an oxygen meter is placed in the sensor hole to measure the oxygen concentration in the *chamber*.**(3)** Nitrogen addition until the indicator needle shows a concentration of 5% oxygen.**(4**) *The chamber* containing the flask is incubated for 24 hours at a temperature of 37^{0}C. **(5)** After 24 hours, the culture media is taken and filtered using TFF to obtain SH-MSC which is then mixed with gel according to the dose of P3/K 3 and P4/K4.

Topical Gel Preparation Manufacturing Process

(1) The preparation of SH-MSC gel is done by mixing 200 mg of gel with secretomes in P3 and P4. **(2)** Stirring is carried out under aseptic conditions to form a homogeneous mixture of physical characteristics of observations under a microscope.

Making Preparations and Giving Treatment to Test Subjects

(1) Rats that have been given topical gel every day for 6 days containing SH-MSC dose 40 µL. The negative control rats were given the base gel treatment. **(2)** Skin samples in all groups were taken to make histological preparations by paraffin method and Hematoxylin-Eosin (HE) staining. **(3)** Skin samples of mice in all groups were taken and fixed in a 10% NBF solution. **(4)** The skin sample is rinsed with 70% alcohol until clean from the remaining fixative solution. **(5)** Skin samples are put in 70%, 80%, 90%, 96%, and absolute alcohol for 30 minutes each for dehydration process. **(6)** The skin sample is inserted into the toluol until clear or transparent for 1 hour. **(7)** Infiltration into paraffin is carried out in the oven at a temperature of 56-60°C by inserting skin samples into a mixture of toluol and paraffin in a ratio of 3:1, 1:1, and 1:3 for 30 minutes each. Skin samples were put into pure paraffin I, pure paraffin II, and pure paraffin III each treatment for 30 minutes. **(8)** *Embedding*, a skin sample of pure paraffin is implanted into a paraffin block mold that has contained liquid paraffin and waited until the paraffin hardens. **(9)** Skin samples in paraffin blocks were sliced with a slice thickness of 6 µm using microtomes. **(10)** Slices of tissue samples are attached to the glass of objects by applying *Mayer's* albumin and dripping with a little aquade and then heated on a *hot plate* until the slices stick perfectly. **(11)** Histological preparations indeparafination by insertion into *xylol* for 24 hours. **(12)** *Staining* is done with hematoxylin-eosin dyes. The xylol content is absorbed with filter paper, then successively put into alcohol 96%, 90%, 80%, 70%, 60%, 50%, 40%, 30%, and aqueous each done for 1-2 minutes. The preparation is put in Hematoxylin dye for 5-10 seconds, then rinsed under running water for 10 minutes. Further process the preparation dipped in 30%, 40%, 50%, 60%, 70% alcohol for 3-5 minutes each. The preparation is infused into eosin for 5-10 minutes, then dipped in 70%, 80%, 90%, and 96% alcohol for 3-5 minutes respectively, then dried with filter paper. The preparation is put in xylol for 15 minutes, then the histological preparation is dripped *canada balsam*. **(13)** Preparations are covered with cover glass, labeled, and stored in preparation boxes.

RNA Extraction and cDNA Synthesis

(1) The skin sample is inserted in a tube containing 300 µL RNA later and stored at -20°C**. (2)** Skin samples of 50 mg were then inserted in a tube containing 1ml of trizol then homogenized with ultrasonicator and incubated at room temperature for 5 min**. (3)** Add 0.2 ml of *Choloform* and incubate at room temperature 2-3 minutes, centrifuge at a speed of 2000 rpm at 4 °C for 15

minutes**. (4)** The sample centificus results form 3 layers, the pink bottom side contains protein, the white middle side contains DNA and the clear upper side contains RNA (*aqueous phase*), separate the upper side into different tubes**. (5)** *Aqueous phase* sample plus 0.5 mL isopropanol then resuspensions and incubation for 10 minutes, then centrifuged at a speed of 2000 rpm with a temperature of 4ºC for 10 minutes**. (6)** Remove the supernatant and add 1 mL of 70% etanoh to be resuspensioned at 4 ºC for 5 minutes**. (7**) Add with 50 µL NFW (nuclease-free water)**. (8)** RNA samples were quantified using µDrop with a wavelength of 260 nm by spectrophotometer method**. (9)** RNA samples of 0.1 µL in 1 µL NFW were added 5 µL NFW for denaturation incubated at 65 ºC using *a thermal cycler* for 5 minutes**. (10)** Add RNA sample 2 µL 4x DN master mix then incubate at 37ºC with *thermal cycler* for 5 minutes**. (11)** Continue with the *reverse transcription* process by adding 2 µL 4x DN master mix then incubate at 37ºC with a thermal cycler for 15 minutes, at 50ºC for 5 minutes, and at 98ºC for 5 minutes using *a thermal cycler.* **(12)** Store the cDNA sample at -20ºC.

IL-10 and TNF-α Gene Expression Reading by *Real Time-Polymerase Chain Reaction* (RTq-PCR)

(1) IL-10 and TNF-α gene expression was analyzed on RTq-PCR. **(2)** Sequentially mix 1 µL of sample cDNA, 2x SensiFAST SYBR No-ROX Mix of 10 µL, *forward primer* 0.8 µL and reverse primary *0.8* µL *and NFW 7.4* µL. **(3)** *IL-10 primers used are F*: 5'-CTGTCACGGAGATCAATGTGG-3' and R: 5'-AAGGCGTAGCTGAACAAGGTG-3'. **(4)** TNF-α primers used are F: 5'-AGGCAATAGGTTTTGAGGGCCAT -3 and R: 5'-TCCTCCCTGCTCCGATTCCG-3' **(5)** The qPCR process is carried out at a temperature of 95ºC for 2 minutes, a temperature of 95ºC for 5 seconds and a temperature of 56ºC for 20 seconds as many as 40 cycles, the qPCR process is carried out to analyze the probe hydrolyzed at a wavelength of 520 nm. **(6)** Quantification of qPCR data is analyzed using EcoStudy software.

Statistical Analysis

The data in this study was processed, edited and tabulated, then descriptive tests were carried out including independent and bound variables using ratio data scales. then, data normality tests were carried out using the *Shapiro Wilk* test and data variance tests using *Levene's Test*. The results obtained the distribution of normal data (p>0.05) and the same data variant (p>0.05), then using the *One Way Anova* test difference test for IL-10 parameters. While the results for the TNF-α parameters obtained a normal data distribution (p > 0.05) and homogeneous data variants (p > 0.05), then using the *One Way Anova* test difference test.Next, both parameters were performed *Post Hoc LSD* tests to determine the most influential dose group. Data processing in research using SPSS 26.0 for Windows desktop application.

III. RESULT

Isolation of SH-MSCs from *Umbilical Cord*

The use of the umbilical cord of Wistar rats who were 19 days pregnant, SH-MSCs isolation was added media consisting of DMEM (*Dulbeccos Modified Eagle Medium*), fungizone, penstrep, and FBS, the isolation results were then cultured in a plastic flask. When the MSC culture results were examined under a microscope, cells with spindle-like cell shapes were attached to the base of the flask at 80% confluence, as shown in figure 1.

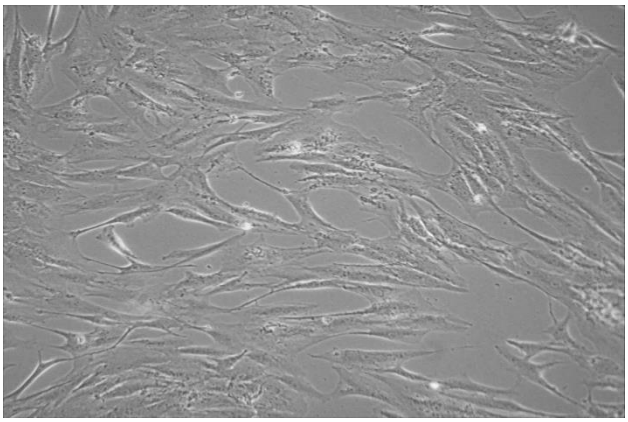

Figure 1. Result of MSC Culture.

Validation of the results of the MSC cell isolation process is carried out by looking at the ability of MSCs to develop into osteogenic and adipogenic cells. In adipogenic differentiation tests, *oil red O* staining is used to show the formation of red lipid droplets. The formation of calcium deposits is clearly visible in red and is shown by osteogenic differentiation test using *Alizarin red* staining.

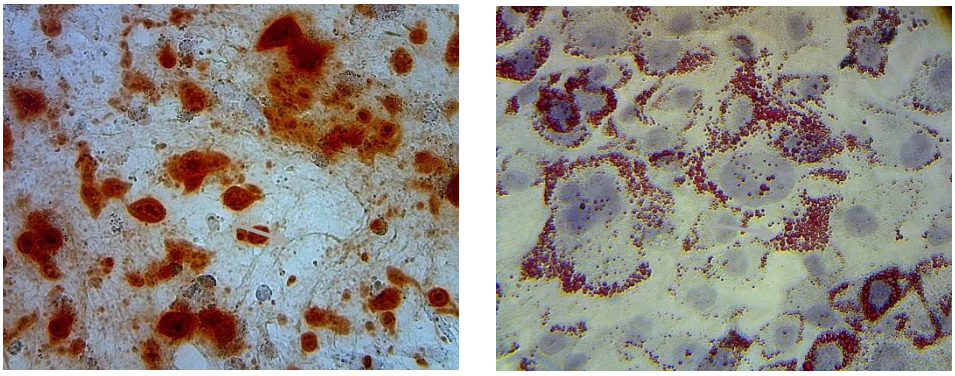

Figure 2. Adipogenic differentiation test, oil red staining O osteogenic differentiation test using Alizarin red staining.

The isolation findings of SH-MSCs demonstrate the ability to express various special surface markers, which is confirmed by *flow cytometry.*

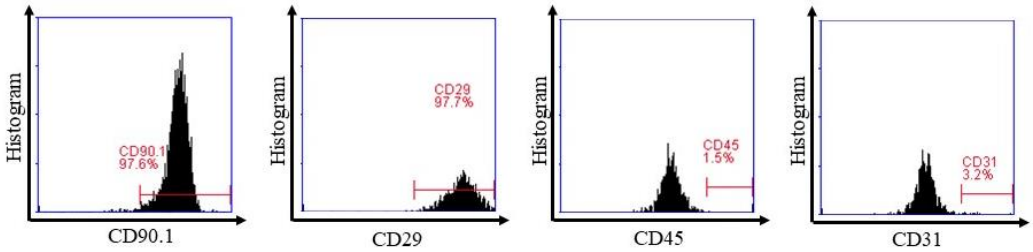

Figure 3. Confirmatory test with flow cytometry.

MSC expresses CD45 (97.6%), CD31 (97.7%), CD90 (1.5%), and CD29 (3.2%).

This study isolated *growth factors and anti-inflammatory cytokines contained in SH-MSCs to obtain pure SH-MSCs using secretomes isolased from MSCs isolated from MSCs preconditioned hypoxia 5% using* Tangential flow filtration *(TFF), SH-MSCs containing anti-inflammatory* cytokines and growth factors*, after the filtration process was analyzed the content* of growth *factor* cytokines and anti-inflammatory*,* measurement of *growth factor content* in SH-MSCs is carried out using the ELISA method. The results of SH-MSCs analysis are known to contain PDGF levels of 1093.51 ± 53.85 pg / mL, VEGF levels of 1015.43 ± 56.95 pg / mL, bFGF levels of 1167.31 ± 68.53 pg / mL, IL-10 levels of 675.31 ± 41.35 pg / mL, and TGF-β levels of 459.71 ± 20.03 pg / mL.

Table 1. Analysis of cytokine growth factor and anti-inflammatory content, in SH-MSCs.

Molecules	SH-MSCs Value ± SE (pg/mL)
VEGF	1015.43 ± 56.95
PDGF	1093.51 ± 53.85
bFGF	1167.31 ± 68.53
IL-10	675.31 ± 41.35
TGF-β	459.71 ± 20.03

Macroscopic Validation and Histopathological Staining of HE

Macroscopic Overview of Alopecia-like Rat Skin

The results of macroscopic observation on the skin of *alopecia-like* rats on day 14 after *administration of fluconazole* showed a slowdown in hair growth in rats when compared to the group of healthy mice, as shown in figure 4:

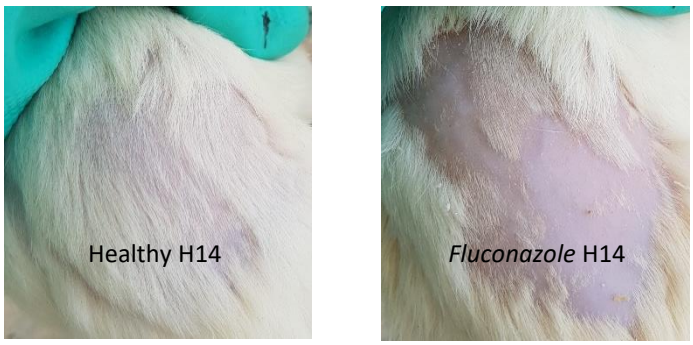

Figure 1. Comparison of fur growth of healthy rats with rats given fluconazole

The average percentage of baldness on day 14 in the healthy group obtained results of 51.11 ± 10.27 while in the *alopecia like group* obtained an average result of 68.26 ± 7.96, macroscopic observation of the results of precondition experimental rats validated to have impaired hair growth by administering *Fluconazole* 35 mg / kg BB,

Results of HE staining of Skin Tissue of Alopecia-like Rats

The readings of histopathological preparations with *hematoxylin-eosin* (HE) staining showed that the group of rats induced with *Fluconazole* 35 mg for 14 days caused interference with the follicle anagen resulting in hair loss, HE preparations showed the results of no finding of the anagen follicle phase *in the treatment group compared to a group of healthy mice that were microscopically different from having phases anagen follicle* in the field of view. The results of the HE staining analysis are shown in figure 5.

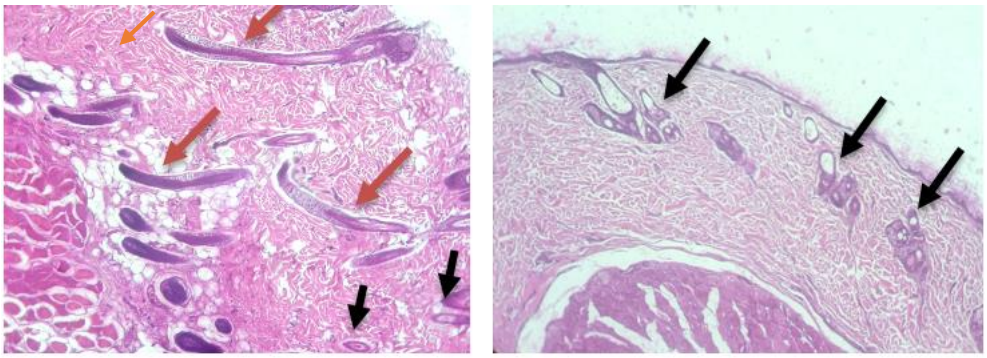

Figure 5. Validate hair follicles of healthy and alopecia-like mice using HE. Black arrows indicate telogen phase follicles, red arrows indicate anagen phase follicles

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

Research with the RT-PCR method using Illumina' s *Eco Real-Time PCR tool, SH-MSCs showed the results of* increasing IL-10 levels in alopecia-like mice. Described in the following table:

Table 2. IL-10 and TNF-α gene expression.

Variable	**Group**			**pvalue**
	P2/K2 n=6 Mean±SD	P3/K3 n=6 Mean±SD	P4/K4 n=6 Mean±SD	
Expressive has IL-10	0.79 ± 0.14	1.45 ± 0.11	2.61 ± 0.23	
Saphiro wilk	0,64	0,06	0,23	
Levene's Test				0,05
One way Anova				0,00
TNF-α gene expression	2.54 ± 2.0	1.40 ± 0.10	0.63 ± 0.20	
Saphiro wilk	0,26	0,47	0,38	
Levene's Test				0,77
One way Anova				0,00

Information:

- *Saphiro Wilk test* (p > 0.05 = normal)
- *Levene's Test* (p > 0,05 = homogen)
- *One way Anova* (p<0.05) = significant difference)

Effect of Topical Gel Administration of Secretome Hypoxia Mesenchymal Stem Cells (SH-MSCs) on IL-10 and TNF- α Gene Expression (In Vivo Experimental Study in Male Rats of Wistar Strains Model of Fluconazole-Induced Alopecia)

Based on table 2, the average IL-10 gene expression results in the negative control group (P2) were 0.79 ± 0.14, the mean SH-MSCs gel group dose 20 µL (P3) was 1.45 ± 0.11, and the mean value in the SH-MSCs gel group dose 40 µL (P4) was 2.61 ± 0.23. Statistical analysis for normality and homogeneity of IL-10 gene expression obtained normal distributed data results with the *Saphiro Wilk* test (p > 0.05), and homogeneity data obtained from *Levene's Test* test values of 0.05 (p > 0.05).
It can be concluded that the results of IL-10 gene expression data are normally distributed and homogeneous, followed by the One way Anova test with a value of 0.00 *(p < 0.05) so that significant differences in IL-10 gene expression between groups can be known in mice with alopecia-like models*.

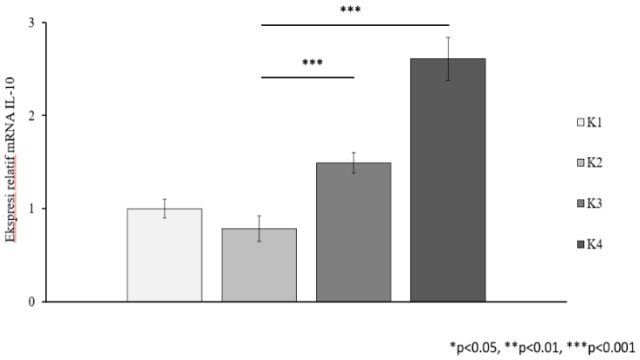

Figure 2. IL-10 gene expression graph per group

Based on table 2, the average results of TNF-α gene expression in the negative control group (P2) were 2.54 ± 2.0, the average SH-MSCs gel group dose 20 µL (P3) was 1.40 ± 0.10, and the mean value in the SH-MSCs gel group dose 40 µL (P4) was 0.63 ± 0.20. Statistical analysis for normality and homogeneity of TNF-α gene expression obtained normal distributed data results with the *Saphiro Wilk* test (p > 0.05), and homogeneity data obtained from *the Levene's Test* test value of 0.77 (p > 0.05).
It can be concluded that the results of TNF-α gene expression data are normally distributed and homogeneous, followed by the One way Anova test with a value of 0.00 (p < 0.05) so that significant differences *in TNF-α gene expression* between groups can be known in mice with alopecia-like models .

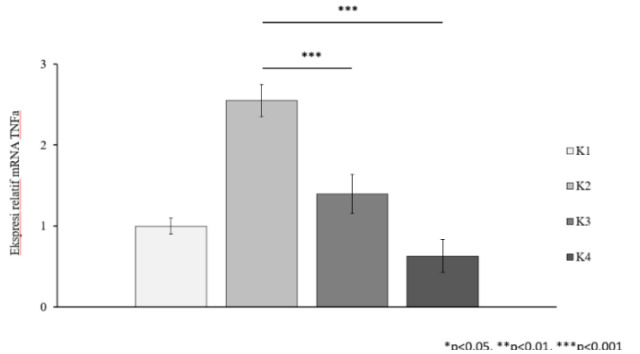

Figure 3. TNF-α gene expression graph for each group

Based on graph 6, the average IL-10 gene expression result was the highest value in the SH-MSCs group dose of 40 µL (P4) and decreased TNF-α gene expression with the lowest value in the SH-MSCs group dose of 40 µL (P4). It was concluded that in the SH-MSCs group, doses of 40 µL increased IL-10 gene expression and decreased TNF-α gene expression in Wistar mice with *fluconazole-induced* alopecia-like models.

Comparison of IL-10 gene expression between groups in alopecia-like mice with SH-MSCs gel.
Based on statistical tests, normally distributed and homogeneous data, a *One way Anova* statistical test was carried out followed by *a Post Hoc LSD* parametric test to determine the SH-MSCs topical gel dose group that had the most effect on increasing IL-10 gene expression in each group. Significant differences were obtained between all P2, P3, and P4 groups with a significance of p:0.000 (P <0.05).

Table 3. Comparison of IL-10 gene expression between groups with Post Hoc LSD assay

Group	Comparison Group	Significance	Confidence Interval 95% Border Below	Border Above
P2	P3*	0,00	-0,90	-0,49
	P4*	0,00	-2,04	-1,62
P3	P2*	0,00	0,49	0,91
	P4*	0,00	-1,34	-0,92
P4	P2*	0,00	1,62	2,04
	P3*	0,00	0,92	1,33

LSD Test: * *mean difference significant* P <0.05

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

Based on statistical tests, normally distributed and homogeneous data, a statistical test *of One way Anova* was carried out followed by *a Post Hoc LSD* parametric test to determine the SH-MSCs topical gel dose group that had the most effect on decreasing TNF-α gene expression in each group. Significant differences were obtained between all P2, P3, and P4 groups with a significance of p:0.000 (P <0.05).

Table 4. Comparison of TNF-α gene expression between groups with Post Hoc LSD assay

Group	Comparison Group	Significance	Confidence Interval 95% Border Below	Border Above
P2	P3*	0,00	0,88	1,41
	P4*	0,00	1,65	2,17
P3	P2*	0,00	-1,41	-0,88
	P4*	0,00	0,50	1,03
P4	P2*	0,00	-2,18	-1,65
	P3*	0,00	-1,03	-0,50

LSD Test: * *mean difference significant* P <0.05

IV. DISCUSSION

Alopecia is a dermatological disorder caused by bacterial or fungal infections, inflammation, or immune dysregulation.[1] The disorder is characterized by a shorter anagen phase and a longer telogen phase in the hair cycle.[1] The integrity of the ends of the hair is related to cortical hair, the texture and gloss of the hair is related to the quality of its surface.[23] Normally hair will detach from the scalp up to approximately 100 strands per day, it is considered abnormal if more hair falls out than 120 strands daily, diffuse and local hair loss may both occur unifocal or multifocal hair.[24]

This study aimed to determine the effect of topical gel administration of SH-MSCs on IL-10 and TNF-α gene expression in *Wistar rats with* fluconazole-induced *alopecia-like models*. A mouse model of fluconazole-induced alopecia, describing the type of hair loss in the telogen effluvium phase, the molecular mechanisms of development from anagen to catagen/telogen, and telogen effluvium deposition, changes in retinoic acid metabolism and retinoic acid accumulation as major contributors to the cause need to be analyzed further.[25]

Application of stem cells to engineered tissue to reconstitute damaged tissue and develop regenerative therapies for the skin.[16] Developments in stem cell research using MSCs in restoring and saving ischemic tissue through angiogenesis.[26] *MSC secretomes* induce angiogenesis in *vitro and* in vivo*, angiogenic factors expressed namely PDGF, BFGF, VEGF, TGF-β*, Angiopoietin-1 (ANG-1), *Placental Growth Factor* (PIGF), IL-6, and MCP-1. [27] MSCs contribute to angiogenesis through direct differentiation, interaction of cell-contact with endothelial cell lines, and paracrine release of pro-angiogenic molecules. Paracrine factor can increase the blood supply of damaged tissues through the activation and recruitment of stem cells and progenitor cells.[28]

The results of the IL-10 gene expression study experienced the highest increase in the SH-MSCs gel group dose of 40 µL (P4) of 2.61 ± 0.23 compared to the other groups P2, and P3, showing that molecularly SH-MSCs gel significantly increased IL-10 gene expression. Intravenously injected MSCs can prevent skin graft-induced AA, possibly through inhibition of IFNγ, CXCL9, and CXCL10

production and CD3 and CD8+NKG2D+ T cell infiltration, suggesting that MSC-mediated IFNγ inhibition and CD3 and CD8+NKG2D+ T cell infiltration protect against hair follicle collapse.[29] By secreting TGFb1, IL10, and PGE2, MSCs have the capacity to make iTreg cells and have immunomodulatory abilities that modulate the inflammatory environment.[17] In line with the research of Byun *et al*, (2016) Increased expression of proinflammatory Th1 cytokines, such as IFNγ, and CD8+NKG2D+ T cells, acting as cytotoxic effectors, responsible for autoimmune attacks on HF in AA pathogenesis. IFNγ expression is abnormally elevated in AA lesions through a Th1-mediated response. In addition, IFNγ produced from CD8+ T cells causes HF loss and promotes type I cellular autoimmunity. MSC-mediated immunosuppression requires early activation of immune cells through the secretion of proinflammatory cytokines, e.g. as IFNγ. Inflammatory diseases associated with cytokines have an advantage in successful treatment with MSCs.[25]

Pathological T cells express IFN-C and IL-17 early in the disease, with a significant increase in the production of cytokines IL-4 and IL-10.[30] Th1 and Th17 cytokines trigger attacks on hair follicles resulting in premature cessation of the hair growth cycle and induction of alopecia phenotype and further lead to disease occurrence, while Th2 pathways may play a role in the inhibition of cell-mediated immune attacks to delay disease progression.[31] Treg cells play an important role in inducing and maintaining tolerance to self-antigens and prevention of autoimmunity, producing anti-inflammatory cytokines (TGF-β and IL-10) and regulating the function of pro-inflammatory cytokines.[32] IL-10 has a regulatory effect rather than a stimulating effect on the immune process, inhibiting the production of activated T cell cytokines. Elevated IL-10 levels in alopecia areata compared to healthy controls, the role of IL-10 is difficult to present due to the separate form of Th2 and Treg pathways.[32]

The results of the study of the lowest TNF-α gene expression in the SH-MSCs gel group dose of 40 µL (P4) were 0.63 ± 0.20 compared to the other groups P2, and P3. showed that gel SH-MSCs significantly decreased TNF-α gene expression. Proinflammatory cytokines such as *tumor necrosis factor-α* (TNF-α) coordinate cyclical hair growth in the pathogenesis of alopecia.[2,3] AA patients had higher levels of IL-6 and TNF-α, IL-6 suppressed HFSC at rest through JAK STAT signaling (activating HFSC to renew damaged hair follicles). TNF-α has an important role in AA, suppression of TNF-α levels effectively treating *cases of alopecia Universalis* (severe stage AA) proves that suppression of TNF-α levels is effective in the treatment of AA. Decreased expression of IL-6 and TNF-α with HF-MSC affects hair follicles so that morphology remains intact.[15]

Research by Deng *et al*, (2021). showed that HF-MSCs promote hair follicle proliferation and reduce HF inflammation because CD8+ T cells are less observed in Vibrissa follicles treated with HF-MSC. What's more, in a mouse model of C3H/HeJ, HF-MSC injection suppresses AA and inhibits immune rights. Here we provide potential therapeutic methods for the treatment of AA, which are promising and beneficial for AA patients.[15] Stimulating factors can activate CD8+NKG2D+ T cells and produce IFN-γ via the JAK1 and JAK3 pathways. IFN-γ can increase IL-15 production in follicular epithelial cells through JAK1 and JAK2. IL-15 then binds to CD8+NKG2D+ T cells to produce more IFN-ÿ, which amplifies the positive feedback loop. IFN-γ promotes the collapse of hair follicle immune privilege, leading to the exposure of autoantigens to CD8+NKG2D+ T cells and facilitating autoimmune attacks on hair follicles. Meanwhile, other inflammatory cells, such as DC, CD4+T cells, NK T cells, mast cells, and eosinophils, accumulate around the hair bulbs.[33]

The stem cell therapy approach showed success, isolation of mononuclear cells derived from patients with *alopecia areata*, multipotent nature of stem cells derived from human cord blood and subsequently restored in the patient's circulation. Using this treatment, patients with severe *alopecia areata* experience improvements in hair growth and improved quality of life. The mechanisms are upregulation of cytokine T helper 2 (TH2) and restoration of balance of TH1, TH2 and TH3 cytokine production.[67] Side effects of many of these treatments are an important consideration, it is possible that combination with low doses and/or topical application will achieve results with an acceptable safety profile. [34]

V. CONCLUSION

Topical administration of SH-MSCs gel significantly increased IL-10 gene expression at a dose of 40 µL in *Wistar rats with* fluconazole-induced *alopecia-like models*. Topical administration of SH-MSCs gel significantly decreased TNF-α gene expression at a dose of 40 µL in *Wistar* mice with *fluconazole-induced* alopecia-like *models*.

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