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# Secretome of Hypoxic Mesenchymal Stem Cells Improves Fluconazole-Induced Alopecia in Rats via Immunoregulatory Modulation of IL-15 and IFN-γ

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**Abstract.** Increased IL-15 and IFN-γ characterize fluconazole-related alopecia (FRA). The hypoxia mesenchymal stem cell (hMSC) secretome has anti-inflammatory capabilities that can potentially be used as alopecia therapy. Therefore, the study aims to determine the effect of topical administration of hMSCs secretome gel on reducing IL-15 and IFN-γ gene expression and baldness in male Wistar rats, a model of FRA. MSC was collected from rat umbilical cord, cultured under hypoxia for 24 hours, and yielded a sterile secretome formulated into a water-based gel ointment for treatment. A total of 24 male Wistar rats were randomly divided into four groups: K1 for health controls with placebo administration only; K2 for negative control which contain FRA rats by applying fluconazole from day 7$^{th}$ to 14$^{th}$ and continued placebo administration until day 29$^{th}$; treatments were conducted for FRA rat groups treated with 200 mg/day of topical gel contained with 10% of hMSCs secretome for K3 and 20% of hMSCs secretome for K4. Observations were made to analyze hair growth and IL-15 and INF-γ mRNA expression using qPCR. The analysis showed a significant decrease in IL-15 and IFN-γ mRNA expression ($p \leq 0.001$) and a reduction in baldness of up to 60% after topical hMSCs secretome gel administration. The prominent result was that the topical gel contained 20% of hMSCs secretome. Based on research results, a topical gel dose containing 20% hMSC secretome had the best effect on improving the condition of FRA. This research may help optimize doses and treatment methods in hMSC secretome therapy.

**Keywords**: alopecia; fluconazole; secretome; IL-15; INF-γ.

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## INTRODUCTION

Alopecia, in general, is a hair loss condition, which includes patchy, partial, diffuse, or complete, associated with significant psychological burden and physiological stress (Maloh et al.,2023). Prolonged fluconazole treatment has been linked to a substantial increase in hair loss, exceeding 25%, compared to the initial administration (Benitez & Carver, 2019; Thompson et al., 2019). However, the underlying mechanism behind fluconazole-induced alopecia (FRA) remains elusive, with speculation pointing toward inhibiting cytochrome P450 enzymes (Alammari et al., 2020; Ogawa et al., 2020). These enzyme blockers lead to a decline in testosterone levels, ultimately diminishing follicular cell health and triggering hair loss (Benitez & Carver, 2019b). Furthermore, FRA is also mediated by inflammatory cytokines and chemokines that regulate the hair follicle cell cycle and do not cause scar tissue because of excesses radical stress. Exposure to fluconazole also mediates the production of proinflammatory cytokines that cause follicle damage and the inability to grow hair (Thompson et al., 2019).

Recent studies also explain that there is a significant increase in the cytokine's interferon-gamma (IFN-γ) and interleukin (IL)-15 in individuals with alopecia compared to control individuals (Agamia et al., 2020). IFN-γ and IL-15 are proinflammatory cytokines that increase the immune response to infection and inflammation. Inhibition of the IL-15ß receptor (IL-15Rß) reduces the number of $NKG2D^+$ cells and $CD8^+$ T cells in the skin, thereby preventing hair loss in

animal models of alopecia (Xing et al., 2014; Agamia et al. 2020). In addition, IL-15 receptor inhibition also prevented the accumulation of $NKG2D^+$ and $CD8^+$ T cells in the skin and reduced the IFN-γ response in animal models of alopecia (Madras et al., 2020).

The current standard treatment, finasteride and minoxidil 2.5-5% takes a long time and causes skin irritation. One strategy for treating FRA is the secretome of hypoxia mesenchymal stem cells (hMSCs), which have the potential to modulate IL-15 and INF-γ (Yuan et al., 2020). The hMSCs secretome is a stem cell culture medium rich in anti-inflammatory cytokines and chemokines such as IL-10 and transforming growth factor (TGF-β), which helps division, regeneration, differentiation, homing, and engraftment (Damayanti et al., 2021; Eleuteri & Fierabracci, 2019; Rahimi et al., 2021). Nevertheless, research on the optimal secretome dosage of hMSCs, the mechanisms involved, and their direct effects on hair growth are poorly understood. Therefore, this study aims to analyze the impact of topical administration of hMSCs secretome gel on reducing IL-15 and IFN-γ gene expression and its effect on hair growth in Wistar strain rats with alopecia induced by fluconazole exposure. This was done to develop therapy for alopecia that is safer, more effective, and more efficient. It is also hoped that this research can become the basis for applying hMSC secretome as a safer therapy for inflammation-related diseases.

## METHOD

This study uses experimental research with a post-test-only control group design. The research object was male Wistar rats aged 2-3 months, weighing 200 g ± 10 g. A total of 24 homogenized Wistar rats were obtained from Stem Cell and Cancer Research (SCCR), Semarang City, Central Java, Indonesia. The cage conditions were set at 20-24 °C, 60% humidity, 12-14 hours of light exposure, and non-stop ventilation. The male Wistar rats were given only standard feed for 29 days from pretreatment to termination. The use of experimental animals in this research has received approval from the Ethical Clearance Commission, Faculty of Medicine, Universitas Islam Sultan Agung, Semarang, No. 298/VII/2023/Komisi Bioetika.

### Mesenchymal Stem Cell (MSC) Isolation Procedure from Umbilical Cord

Following established procedures, mesenchymal stem cells (MSC) were isolated in a class 2 biosafety room (Nugraha & Putra, 2018). In this study, MSCs were obtained from the umbilical cords of 21-day-old rat fetuses, which were collected aseptically and immediately stored in a 0.9% NaCl solution. The umbilical cord is cleaned with phosphate buffer solution (PBS), removing the blood vessels. The clean umbilical cord was then finely chopped and placed in a T25 culture flask (Corning, Tewksbury, MA, USA).

A mixture of Dulbecco's modified eagle medium (DMEM) (Sigma-Aldrich, Louis St, MO), 0.25% fungizon, 1% Pen-Strep, and 20% fetal bovine serum (FBS) (Gibco™ Invitrogen, NY, USA) was added to the in a culture tube until the tissue covers the surface. The culture tubes were then stored in an incubator at 37 °C with 5% carbon dioxide ($CO_2$) levels for two weeks (Nugraha & Putra, 2018). During the culture process, cell growth was observed, and the medium was replaced every three days. Cells were maintained until they reached 80% confluence. At the end of the culture period, flow cytometry validated MSC cells by microscopic observation and measurement of CD90, CD29, CD45, and CD31 expression (Sazli et al., 2023). Glycoprotein biomarker measurements were carried out using a BD Accuri C6 Plus flow cytometer (BD Bioscience, San Jose, CA, USA), and post-acquisition analysis was calculated using BD Accuri C6 Plus software (BD Bioscience, San Jose, CA, USA). In addition, the capacity of MSC cells to differentiate into osteocytes and adipocytes was also tested by Alizarin red and oil-red o-dye (Sigma-Aldrich, Louis St, MO) staining (Zukhiroh et al., 2022).

### Hypoxia Induction of MSCs and Secretome Extraction

The isolated MSCs were then incubated in hypoxic conditions to increase hMSCs secretome production (Fredianto et al., 2023). Hypoxic conditions are achieved by reducing oxygen ($O_2$) levels in the culture medium to 5%. Each MSC culture tube with 80% confluence was added with 10 ml of DMEM media and placed in a hypoxic chamber for 24 hours. After 24 hours, the culture medium was removed and filtered using tangential flow filtration (TFT) to produce the secretome of hMSCs.

### Analysis of secretome content

100 µL of extracted secretome was used for each test of five anti-inflammatory cytokines and chemokines, including VEGF, PDGF, bFGF, IL-

10, and TGF-β. Tests were carried out using the sandwich-ELISA kit for rats (Elabscience®, Texas, USA) according to the manufacturer's specifications and standard procedures. Optical density (OD) was measured spectrophotometrically at 450 nm ± 2 nm wavelength. The OD value is compared with a standard curve to measure the concentration according to the specifications of each target protein.

**Making Topical Gel Preparations**

The treatment in this study was carried out by applying 200 mg topical gel containing hMSCs secretome per day on the dorsal area of the rat with a size of 2 cm x 2 cm. Every 200 mg of the topical gel containing 20 µL of hMSCs secretome for group K3 and 40 µL of hMSCs secretome for group K4. The topical gel for treatment was prepared aseptically by mixing secretome from hypoxia MSC well with sodium chloride (NaCl) in a 1:1 ratio (µl: mg). The two components were then mixed homogeneously with a water-based gel until the weight reached 200 mg for each day of treatment.

**Maintenance, alopecia induction, and hMSCs secretome topical gel therapy treatment**

Rat acclimatization and adaptation were carried out in the first week without treatment. On the day 7$^{th}$, the dorsal hair of each rat was shaved, and they were randomly grouped into four groups with equal numbers: the K1 group was healthy rats as the positive control, treated with 0.8% physiological NaCl placebo; the K2 group consisted of the fluconazole-induced alopecia rat and treated with water-based topical gel without hMSCs secretome. Then, two treatment groups consisted of FRA-model rats treated using 200 mg/day of a topical gel containing 10% hMSCs secretome for the K3 group and 200 mg/day of a topical gel containing 20% hMSCs secretome for the K4 group. The FRA was conditioned by applying fluconazole to the shaved backs of rats in groups K2, K3, and K4 from day 8$^{th}$ to 14$^{th}$. The topical gel containing hMSCs secretome was used in K3 and K4 and applied in the morning from day 15$^{th}$ to day 29$^{th}$.

At the end of the study, the rats were sacrificed on day 29$^{th}$ by being injected with 10 ml of a lethal-dose cocktail containing 6 ml ketamine and 4 ml xylazine through intramuscular injections. The rats' back skin was taken according to the size of the shaved area and preserved in a tube containing RNA Iso Plus (Toyobo: Osaka, Japan) to avoid RNA destruction. The rat carcasses were destroyed by cremation in an incinerator at a temperature of 850 – 1150 °C for 30 minutes.

**RNA extraction and cDNA synthesis**

A 100 mg skin sample was then cut into small pieces and placed into a microtube filled with 50 ml of RNA Iso Plus (Toyobo: Osaka, Japan). The skin pieces were ground using a micro pestle, and 50 ml of RNA Iso Plus was added and kept at room temperature for 5 minutes until it formed a firm paste. Then, 20 ml of chloroform was added into the microtube and vortex until the solution became milky white.

The solution was incubated at room temperature for 2-3 minutes and then centrifuged using 15,000 rpm at 40 °C for 15 minutes. After that, three layers appear in the tube: the top layer is RNA (liquid phase), the second layer is DNA (semisolid phase), and the bottom layer contains cell debris. The top layer was transferred to a new centrifuge tube, and the volume was measured. A volume of isopropanol equal to the RNA taken from the top layer was added. The tube was shaken until white threads appeared, then centrifuged at 15,000 rpm for 10 minutes at 40 °C. The supernatant was discarded until a white pellet was visible at the bottom of the tube.

After drying, 100 ml of 70% ethanol in diethylpyrocarbonate (DEPC) solution was added, then turned repeatedly, and centrifuged at 15,000 rpm for 5 minutes at 40 °C. The supernatant was discarded, and 30-50 µm DEPC was added. The mixture was incubated at 55 °C for 10 minutes. Next, the total RNA solution was obtained and stored at -80 °C. RNA was quantified with NanoDrop™ 2000/2000c Spectrophotometers (Thermo Fisher: Massachusetts, USA). The quantification results were calculated to be 3000 ng.

cDNA synthesis using ReverTra Ace® qPCR RT Master Mix (Toyobo: Osaka, Japan) following the manufacturer's procedures. The cocktail was made by mixing the calculated RNA sample, 1 µl of OligoDT, and PCR water until it reached a volume of 10 µl, then incubated for 5 minutes at 70 °C. Mixture A was added with mixture B consisting of 5X buffer 4 µl, DEPC-Treated H2O 5 µl, and ReverTraAce 1 µl. The mixture was incubated at 25 °C for 5 minutes, 42 °C for 50 minutes, and 85 °C for 5 minutes.

**Reading IL-15 and IFNγ with Real Time-Polymerase Chain Reaction (qRT-PCR)**

IL-15 and IFNγ gene expression were analyzed using quantitative reverse transcription-polymerase chain reaction (RT-PCR). Mix 1 µl cDNA sample, 2x SensiFAST SYBR no ROX Mix (Meridian Bioscience: Ohio, US), 10 µl, 0.8 µl forward primer, 0.8 µl reverse primer and 7.4 µl NFW. The IL-15 primers used were F: 5′-CCATCTCGTGCTACTTGTG-3′ and R: 5′ -CTGTTTGCAAGGTAGAGCACG-3′. The IFN-γ primers used were F: 5′-GATCCA-GCACAAAGCTGTCA-3′ and R: 5′-GACTCCTTT-TCCGCTTCCTT-3′. The IL-15 and IFN-γ primers were generated using the primer blast feature from the National Center for Biotechnology Information (NCBI) accessed through https://www.ncbi.nlm.nih.gov/tools/primer-blast/index.cgi. The IL-15 gen was arranged from the *Rattus norvegicus* interferon-gamma (IFN-G) gene, complete cds accession number AH002184.2, then IFN-γ primers were created from the *Rattus norvegicus* interferon-gamma (IFN-G) gene, complete cds, accession number AH002184.2.

The qPCR process was carried out using temperatures of 95 °C for 2 minutes, 95 °C for 5 seconds, and 56 °C for 20 seconds for 40 cycles. The qPCR process is carried out by analyzing hydrolyzed probes at a wavelength of 520 nm. The increase in gene expression was analyzed in the ratio of increase to housekeeping genes using EcoStudy software.

**Secretome content validation**

The validation stage of secretome content was carried out by isolating hMSCs secretome components, which had previously been filtered using TFF with a filter cassette. The filtration results were divided based on molecular weight into three groups, namely 10-50 kDa as much as 50%, 50-100 kDa as much as 25%, and 100-300 kDa as much as 25%. The secretome was then analyzed using a sandwich enzyme-linked immunoassay (ELISA) Kit for rat vascular endothelial growth factor (VEGF), platelet-derived growth factor (PDGF), basic fibroblast growth factor (FGF), IL-10, and tumor growth factor-β (TGF-β) (Invitrogen: Maryland, USA).

**Data analysis**

The IL-15 and IFNγ gene expression data was analyzed for normality using the Shapiro-Wilk test and Levene's Test and was declared regular and homogeneous if the significance value was $p > 0.050$. Normal distributed data was analyzed using One-Way ANOVA with significant $p < 0.050$, followed by the least significant difference (LSD) post hoc test to determine the significance of differences between research groups. If the data distribution is abnormal ($p < 0.050$), the Kruskal-Walli's test is carried out, followed by the Mann-Whitney test with a significance value of $p < 0.050$. All statistical analyses were done using the SPSS 26.0 for Windows desktop applications.

## RESULTS AND DISCUSSION

**Isolation and differentiation of MSCs into osteocytes and differentiation into Adipocytes B**

Mesenchymal stem cells (MSCs) are widely used as a prospective alternative cell-based therapy for diseases related to immune disorders and inflammation (Du et al. 2016). Therefore, using MSCs to treat fluconazole-induced alopecia is considered quite promising. However, quality control must be carried out to ensure appropriate cell growth and potential in culturing and maintaining MSC cells. Based on microscopic observations, MSC cells experienced increased growth during two weeks of cell culture; apart from that, the cell shape was flat and spindle-shaped and filled the culture medium (Figure 1).

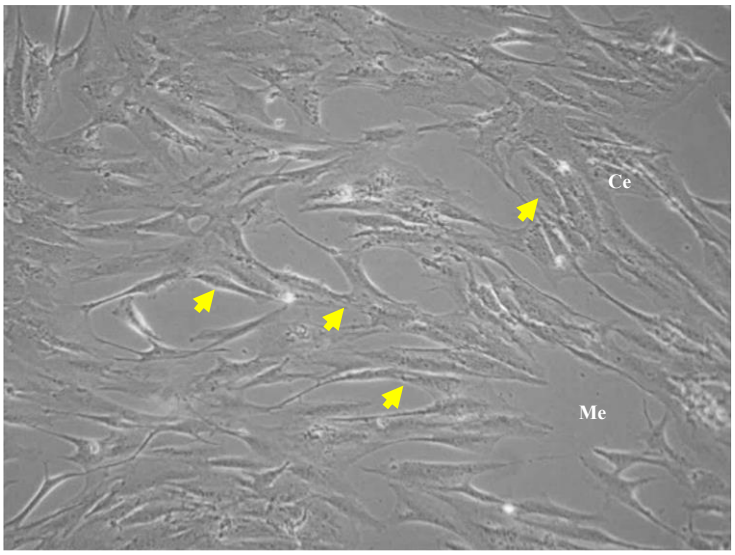


**Figure 1.** Phase-contrast microscopy image of mesenchymal stem cells (MSCs) isolated from rat umbilical cord. Spindle-shaped MSCs are evident, adhering to and populating the culture medium (yellow arrow). Ce: MSCs cells; Me: DMEM culture medium. Observations were made using 100x magnification.

Based on the validation results of MSCs isolation by examination via flow cytometry, the expression of the transmembrane glycoprotein marker CD90.1 was 97.6%, CD29 was 97.7%, while CD45 was only expressed around 1.5%, and CD31 was about 3.2% (Figure 2).

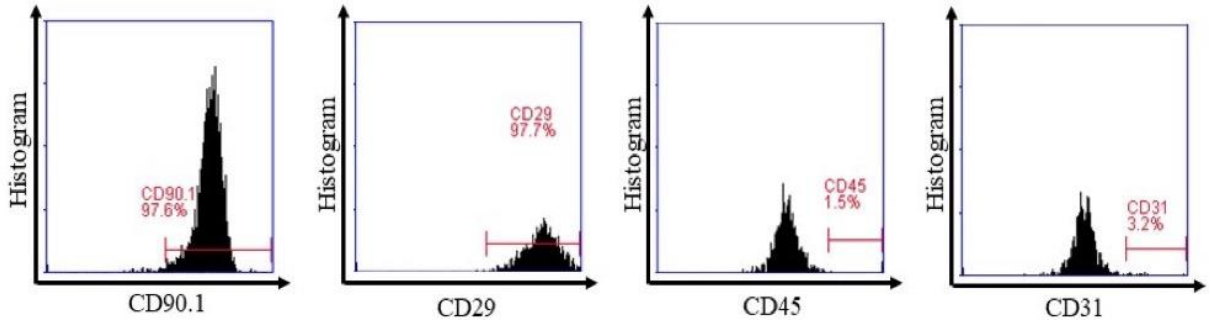


**Figure 2.** Flow cytometry analysis of CD90.1, CD29, CD45, and CD31 expression confirms high purity and minimal contamination of cultured mesenchymal stem cells (MSCs) for downstream research applications.

The appearance of CD90.1 and CD29 are considered positive markers for MSCs and are used to identify and isolate these cells. CD90.1, also known as Thy-1, is involved in cell adhesion, cell signaling, and immune modulation and is used to identify and isolate these cells from other cell types (Fang et al. 2019). Then CD29, also known as integrin β1, plays a crucial role in cell adhesion, cell signaling, and cell migration. CD29 also differentiates MSCs into various lineages, including keratinocytes, osteoblasts, adipocytes, and chondrocytes (Wen et al., 2023).

Furthermore, CD45 and CD31 are negative markers for MSCs and are used to exclude contaminating cells from MSC preparations. CD45, a leukocyte common antigen, is used to exclude hematopoietic cells from MSC preparations (Li et al., 2021). Then, CD31, also known as platelet endothelial cell adhesion molecule-1 (PECAM-1), is used to exclude endothelial cells from MSC preparations. This information shows that the MSCs have the purity, viability, and differentiation potential for treatment (Mahiddine et al., 2020).

This research also tested the ability of MSCs to differentiate into different adult cell types. The test results show that MSCs can differentiate into osteocytes and adipocytes based on the growth medium settings. The appearance of osteocytes can be identified through red calcium deposits due to alizarin red, while adipocyte cells are observed from red fat granules due to oil red dye staining (Figure 3).

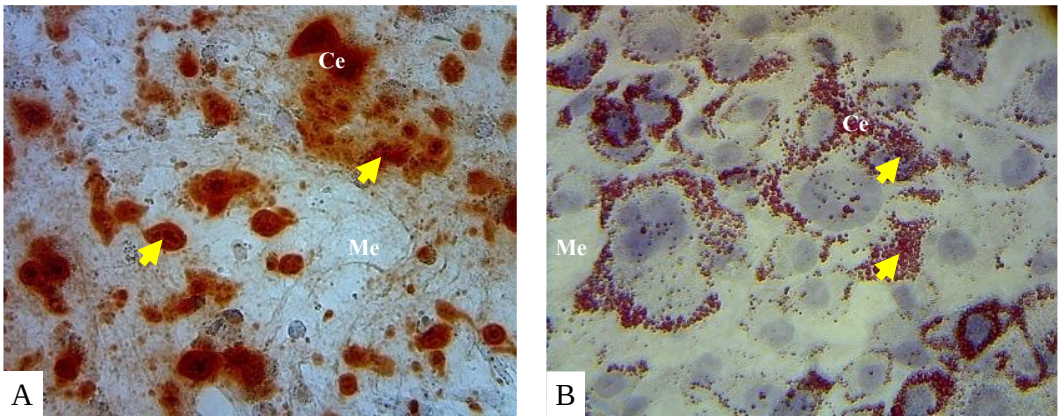


**Figure 3.** The differentiation ability of MSCs is shown by observing osteocytes after being replaced into medium conditioning alizarin red (A) and adiposity cells when placed in the medium containing oil red staining. The yellow arrow shows the differentiated cells. Ce: differentiated MSCs cells; Me: medium. Observations were made at 100x magnification

Based on the screening results, the concentrations of five anti-inflammatory cytokines and chemokines are essential in inhibiting inflammation (Table 1).

**Table 1.** Results of analysis of anti-inflammatory cytokine and chemokine content from the secretome of rat umbilical cord hMSCs

| Molecules | Cytokine (pg/ mL) | Potential function | Reference |
|---|---|---|---|
| VEGF-A | 1015.43 ± 56.95 | Angiogenic factor, proliferation, and migration of keratinocytes promote collagen fiber remodeling and reduce excess extracellular matrix. | (Andjelkov et al., 2022) |
| PDGF | 1093.51 ± 53.85 | Mitogen and chemoattractant agents that promote cell proliferation and migration, including fibroblasts and keratinocytes, stimulate the proliferation and migration of mesenchymal stem cells essential for hair follicle regeneration. | (González et al., 2017; Pazzaglia et al., 2019; Steward et al., 2020) |
| bFGF | 1167.31 ± 68.53 | Pleiotropic growth factor that promotes cell proliferation, differentiation, and migration, including fibroblasts, keratinocytes, and endothelial cells, stimulating their proliferation and migration | (Liu et al., 2022) |
| IL-10 | 675.31 ± 41.35 | An anti-inflammatory cytokine that suppresses proinflammatory cytokine production and promotes tissue repair.<br><br>Targeting various immune cells, including macrophages and T cells, modulating their activity to promote an anti-inflammatory environment, which is essential for hair follicle regeneration. | (Gautam et al., 2020; Torkestani et al., 2021) |
| TGF-β | 459.71 ± 20.03 | A multifunctional cytokine that regulates cell proliferation, differentiation, and matrix production.<br><br>Targeting includes fibroblasts, keratinocytes, and immune cells, regulating their activity to promote hair follicle regeneration and maintain hair follicle homeostasis. | (Alvandian et al., 2022) |

The therapeutic effect of MSCs is due to a secretome rich in growth factors, cytokines, extracellular vesicles, and exosomes (Eriani et al., 2018; Kosaric et al., 2020; Matthay et al., 2019). MSC media contains whole cell secretions and has been reported to reduce lung inflammation, improve functional recovery in multiple sclerosis, and accelerate skin wound healing (Lee et al., 2017). In addition, under short hypoxic conditioning of MSCs for ± 24 hours, they can increase the production of cytokines and chemokines, which can affect cell proliferation, differentiation, paracrine function, migration, and pro-angiogenesis (Gentile & Garcovich, 2019; Isik et al., 2021; Yu et al., 2021).

**Effect of topical hMSCs secretome on IL-15 and IFN-γ gene expression in fluconazole-related alopecia**

In this study, the administration of hMSCs secretome reduced IL-15 and IFN-γ gene expression in a rat model of FRA. The analysis shows that IL-15 and IFN-γ mRNA synthesis increases in alopecia conditions, indicating inflammation. Furthermore, this study also shows that hMSC secretome application can significantly decrease IL-15 and IFN-γ gene expression in the K3 and K4 groups compared to the K2 group ($p \leq 0.050$) (Figure 4).

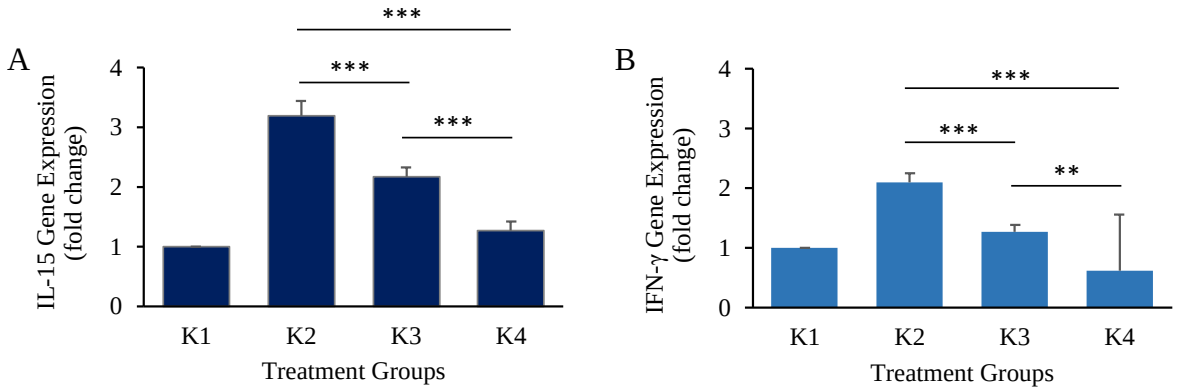


**Figure 4.** IL-15 (A) and INF-γ (B) mRNA expression in each treatment group significantly decreases as the hMSC secretome dose increases. The three superscript stars (***) indicate groups that are significantly different based on the LSD test ($p \leq 0.001$) for IL-15 mRNA expression, and two superscript stars (**) represent the Mann-Whitney Test significantly result ($p \leq 0.050$) for expression INF-γ mRNA.

Application of the hMSC secretome on K4 caused IL-15 expression of 1.26 ± 0.16 fold and IFN-γ of 0.61 ± 0.95 fold change, or the largest decrease, and was significantly different compared to K3 and K2 ($p \leq 0.050$). The results of this study are consistent with previous studies, which showed that using 200 µL/kgBW secretome hMSCs significantly reduced inflammation and accelerated wound recovery (Putra et al., 2022). MSCs secretome significantly reduced rats' hair loss and reduced inflammation around hair follicles in vitro and in vivo (Deng et al., 2021). Various studies have proven the role of inflammatory factors in the etiopathogenesis of FRA. Fluconazole triggers the production of radical oxygen species (ROS), thereby activating the nuclear factor kappa B (NF-κB) signaling pathway in epithelial cells and macrophages in the skin (Huang et al., 2022).

This signaling pathway triggers the expression of several proinflammatory cytokine genes, including IL-15, activating $NKG2D^+$ and $CD8^+$ T cells. The presence of ROS together with proinflammatory cytokines, including IL-15, triggers the activation of three inflammatory signaling pathways, namely mitogen-activated protein kinase (MAPK)-extracellular-signal-regulated kinase (ERK), Janus kinase (JAK)-signal transducer and activator of transcription (STAT), and Phosphoinositide 3-kinase (PI3K) which activates mammalian target of rapamycin (mTOR) (Clark et al., 2019). This intracellular signaling produces IFN-γ, which strengthens the positive feedback signaling loop and maintains inflation in the area exposed to fluconazole. Meanwhile, other inflammatory cells such as $CD4^+$ cells, NK T cells, mast cells, and eosinophils accumulate around the hair bulb, possibly causing pyrolysis (Byun et al. 2017). IFN-γ promotes the fragility of hair follicles, leading to exposure of

$CD8^+$ $NKG2D^+$ auto-antigens and facilitating autoimmunity in hair follicles (Uchida et al. 2021).

The presence of proinflammatory cytokines such as IL-15 and IFN-γ can be developed as inflammatory biomarkers in the etiopathology of FRA. This refers to the expression of IL-15 in skin tissue that is not influenced by other disease factors (Kamil et al., 2023), as well as IL-15 and IL-15 receptor, IL-15Rβ. Overexpression of IL-15 triggers a significant increase in inflammatory response, leading to the destruction of hair follicles (Kamil et al., 2023; Ragab et al., 2020). Initially, chemical exposure causes oxidative stress that epithelium and hair follicle cells increase their IL-15 gene and the IL-15α receptor, which triggers the activation of $CD8^+$ T cells and inhibits regulatory T lymphocyte ($T_{reg}$) cells (Ujiie, 2019). It also increases IL-6 production, inhibiting TGF-β-induced formation of $Foxp3^+$ $T_{reg}$ cells and induces differentiation of pathogenic $T_H17$ cells (Shin et al. 2013). IL-6 and IL-1β can also reverse the suppressive function of $T_{reg}$ cells that contribute to the imbalance of immunocyte populations, including $CD8^+$ T cells/$T_{reg}$ cells and $T_H17$ cells/$T_{reg}$ cells (Han et al., 2015; Wan et al., 2023). The presence of $T_{reg}$ cells in the skin is needed to maintain the homeostasis of the hair follicle environment and encourage HFSC maturation towards the anagen stage (Han et al., 2015; Loh et al., 2018; Ujiie, 2019)

The appearance of IFN-γ and $NKG2D^+$ $CD8^+$ T cells are involved in the development of alopecia by infiltrating hair follicle cells, initiating an IFNγ response, and upregulating γ-chain (γc) cytokines, especially IL-2 and IL-15. Simultaneously, several immune pathways are also responsible for autoreactivity in alopecia conditions, mainly modulated by NK and natural killer T (NKT) cells. In the hair bulb anagen phase, IFN-γ specifically promotes immune responses by increasing the expression of MHC I, NKG2D receptors, and chemokine (C-X-C motif) ligands (CXCLs) in hair follicles (Suchonwanit et al., 2021). IFN-γ inhibits the Janus kinase (JAK)/signal transducer and activator transcription (STAT) pathway. This inhibits angiogenesis, proliferation, and activation of stem cells in hair follicles, resulting in alopecia.

**Hair growth after administration of hMSCs secretome**

Inflammation in the hair follicle area due to exposure to fluconazole triggers a change in the hair follicle phase from anagen to telogen. Based on the results of hair growth analysis, the FRA model rats experienced delayed hair growth and had a reddish rash, which was thought to be due to localized inflammation in the exposed area. Furthermore, hair growth was observed to increase after administration of hMSCs secretome (Figure 5).

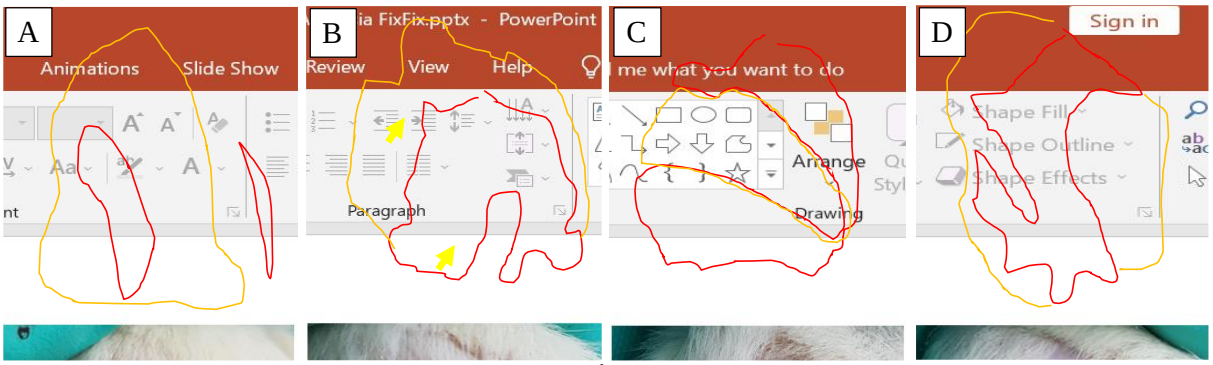


**Figure 5.** Comparison of baldness areas on day 29th (red line) and the appearance of differences in hair growth in healthy rats K1 (A), group K2 (B), group K3 (C), and group K4 (D) (orange line). A post-recovery rash appears faint (upper yellow arrow), and an abscess that has healed (lower yellow arrow) was observed in the K2 group.

Based on observations on day 29th, the impact of fluconazole on hair growth was still observed two weeks after cessation of exposure. Furthermore, hMSCs secretome therapy for one week accelerated hair growth, so the area of baldness in groups K3 and K4 decreased significantly compared to K2 ($p > 0.050$). The location of baldness measured using ImageJ software (https://imagej.net/ij/download.html) shows differences in hair coverage (Figure 6).

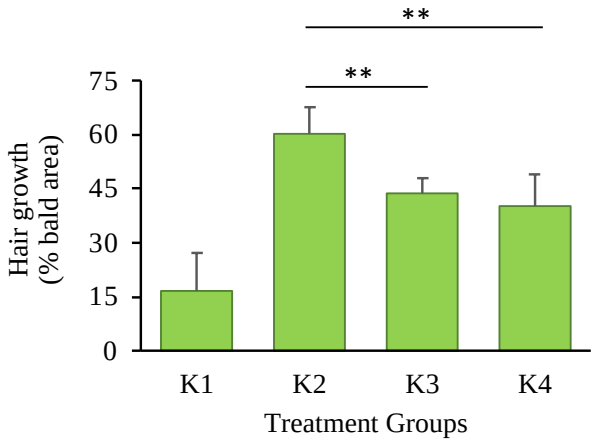


**Figure 6.** Calculate the percentage area suffering alopecia using Image J on a pixel camera. The superscript three stars (**) indicate groups that are significantly different based on the LSD test ($p < 0.050$).

The average percentage of baldness in the fluconazole group reached 68.26 ± 7.96%, while in the healthy group, it was only 16.77 ± 10.27%. The baldness area in the K3 group leaves 43.65 ± 4.42%, which is not significantly different from the K4 group, which leaves 40.14 ± 8.74% ($p > 0.050$), but significantly different from K2. This shows topical hMSCs secretome gel can improve hair growth in fluconazole-related alopecia rats.

Hypoxic treatment of MSCs (hMSCs) produces a secretome rich in growth components, cytokines, and anti-inflammatory chemokines with increased regenerative properties. In alopecia therapy, the hair follicle microenvironment experiences oxidative stress and inflammation, which disrupts the hair follicle cycle, causing hair loss. Previous research has also shown the hMSC secretome promotes hair follicle regeneration (Damayanti et al., 2021; Eleuteri & Fierabracci, 2019; Nandina et al., 2022). These factors stimulate HFSC proliferation and differentiation.

High composition of VEGF and PDGF play crucial roles in hair follicle development and growth. VEGF promotes angiogenesis, the formation of new blood vessels, which supply nutrients and oxygen to hair follicles. PDGF stimulates hair follicle proliferation and activates HFSC, the process by which hair follicle cells divide and create new hair shafts (Zhu et al., 2020). Studies have shown that fluconazole can disrupt the expression and signaling of VEGF and PDGF, leading to impaired angiogenesis and hair follicle proliferation. For instance, one study demonstrated that fluconazole downregulates VEGF expression in hair follicle endothelial cells, hindering blood vessel formation (Zhang et al., 2019). Another study found that fluconazole reduces PDGF levels in hair follicles, impeding hair follicle growth (Thompson et al., 2019). PDGF also works synergistically with activating the Wnt/β-Catenin and Sonic Hedgehog (Shh) signaling pathways where there is an interaction in hair follicle development and stem cell activation. Second, the Wnt/β-Catenin and Notch signaling pathways. These pathways also interact to regulate HFSC activation in regenerating hair follicles (Mysore et al., 2021)

Furthermore, bFGF is another important growth factor involved in hair follicle development. It promotes hair follicle proliferation, differentiation, and apoptosis. Fluconazole has been shown to alter bFGF expression and signaling in hair follicles, disrupting these processes. Studies have reported that fluconazole can downregulate bFGF expression in hair follicle keratinocytes comprising the hair shaft. This reduction in bFGF can impair hair follicle differentiation and apoptosis, leading to abnormal hair growth and hair loss (Roy et al., 2023). By giving hMSCs secretome containing a high amount of bFGF to the skin tissue, the keratinocyte is induced to actively produce a new hair shaft (Fredianto et al., 2023).

The hMSCs' secretome has IL-10, which comes from the hMSCs' secretomes activating the STAT3 pathway through the translocation of JAK-1 from the intracellular membrane to the cytoplasm. This impacts STAT3 phosphorylation and its translocation to the nucleus (Clark et al. 2019). Translocation of STAT3 to the nucleus can activate the SOCS3 gene for subsequent synthesis into a protein released into the cytoplasm. SOCS3

expression in the cytoplasm inhibits the IKK intracellular signaling pathway and prevents NF-κβ translocation to the nucleus so that proinflammatory gene expression does not occur. Through SOCS3 synthesis and NF-κB inhibition, IL-10 can suppress the expression of various proinflammatory molecules, including the cytokine IFN-γ (Kuntardjo et al. 2019).

On the other hand, TGF-β is a multifunctional cytokine that plays both proinflammatory and anti-inflammatory roles. In hair follicles, TGF-β can regulate the hair follicle cycle, differentiation, and apoptosis (Miao et al., 2022). However, excessive TGF-β signaling can also contribute to hair loss. Studies have shown that fluconazole can modulate TGF-β signaling in hair follicles, disrupting the hair follicle cycle and differentiation (Sheth & Dunaief, 2016). This suggests that TGF-β may play a role in FRA pathogenesis. However, further research is needed to elucidate the specific mechanisms involved.

Several studies show that HMSCT can activate anagen and trigger the growth of human follicle outer root sheath cells (hORSC) (Gentile & Garcovich, 2019; Kim et al., 2020). Immunomodulatory effects of MSCs on hORSEs increased viability, migration, and anagen-upregulated hORSC expression (Lee et al., 2021). hMSCs as an anti-alopecia therapy have the advantage of being minimally invasive compared to hair follicle transplantation, autologous, and multifunctional. Therefore, the secretome of hMSCs has excellent potential to be developed as a therapy for skin inflammation and alopecia due to exposure to chemical compounds. However, this study has limitations, especially in providing short-term treatment with two doses, so the optimal dose, potential toxicity, and effects of allergic reactions in the subject animals are unknown. Apart from that, the preparation process for cell culturing and harvesting the secretome requires a lot of time and money, so treatment with the secretome is estimated to be still expensive.

## CONCLUSION

The hMSCs secretome gel application significantly reduces IL-15 and IFN-γ gene expression, and it cures baldness in Wistar rats with FRA, where the best results are shown at a dose of 200 mg/day topical gel containing 20% hMSCs secretome. The best result of the hMSC secretome application is performed in the K4 group, lowering IL-15 gene expression to 1.26 ± 0.16 fold and IFN-γ to 0.61 ± 0.95 fold change, which is significantly different from the other group ($p \leq 0.050$). Besides that, the administration of hMSC secretome significantly decreases baldness by up to lower than 45%. Overall, hMSC secretome has promising potential as a novel alopecia therapy. Its ability to stimulate modulating immune responses, anti-apoptosis, and enhancing hair follicle regeneration makes hMSC secretome a potential candidate for research and development of alopecia therapy. Further research is needed to optimize hMSC secretome preparation, administration, and dosing for maximum efficacy and safety with the lowest cost. Additionally, research is required to investigate the long-term effects of secretome therapy and its potential for treating various types of alopecia.

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