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Exosome Therapy from Hypoxia-treated Mesenchymal Stem Cells Reduces TNF-α and Increases VEGF Levels in Fluconazole-Induced Alopecia Model

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Background: Alopecia is a condition with partial or complete hair loss, leading to psychological distress. Current treatments, such as minoxidil and finasteride, have limited efficacyand side effects. Recent studies suggest that mesenchymal stem cells (MSCs)-derived exosomes offer regenerative potential by modulating inflammation and enhancing hair follicle regeneration, though optimal dosage remains unclear. Tumor necrosis factor-alpha (TNF-α) inhibits hair follicle growth, while vascular endothelial growth factor (VEGF) promotes hair regrowth. This study evaluates exosome therapy from hypoxia (Hypo-Exo)-treated MSCs in modulating TNF-α and VEGF in a fluconazole-induced alopecia-like model.

Materials and methods: An experimental post-test only control group design was used with 30 male Wistar rats, divided into five groups: Healthy group, 0.9% NaCl-treated group, 5% Minoxidil-treated group, 100 µg/mL Hypo-Exo MSCs-treated group, and 200 µg/mL Hypo-Exo MSCs-treated group. TNF-α and VEGF levels were analyzed using ELISA on day 14 post-treatment.

Results: The highest TNF-α level was found in the 0.9% NaCl-treated group (307.46 ± 20.68 pg/mL) and significantly reduced (p<0.05) in 100 µg/mL Hypo-Exo MSCs-treated group (65.38±15.05 pg/mL) and 200 µg/mL Hypo-Exo MSCs-treated group (37.16±7.14 pg/mL). VEGF levels were the highest in the 200 µg/mL Hypo-Exo MSCs-treated group (189.11±9.75 pg/mL) and 100 µg/mL Hypo-Exo MSCs-treated group (158.50±5.33 pg/mL), compared to the 0.9% NaCl-treated group (69.60±15.39 pg/mL).

Conclusion: Hypo-Exo MSCs significantly reduced TNF-α and increased VEGF levels, supporting their potential as a novel regenerative therapy for alopecia.

Keywords: *alopecia, TNF-α, VEGF, exosome, hypoxia, mesenchymal stem cells*

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Introduction

Alopecia is hair loss that can be partial or complete, temporary or permanent.[1] It affects all ages, caused by factors such as genetics, nutrition, stress, diseases, or medications.[2] Globally, androgenic alopecia (AGA) (37.6%), alopecia areata (18.2%), and telogen effluvium (11.3%) are the most common types.[2] In Indonesia, similar trends are seen, with androgenic alopecia accounting for 39.7% of cases.[3]

Alopecia impacts psychological well-being, increasing the risk of depression, anxiety, and social isolation.[4,5] Children with alopecia also exhibit higher rates of anxiety and lower self-esteem compared to their peers.[6] Moreover, both androgenic alopecia and primary cicatricial alopecia (PCA) are associated with reduced quality of life.[7,8]

Current first-line treatments, including topical minoxidil, oral finasteride, and corticosteroids, offer limited efficacy.[9] These treatments require prolonged use, often exceeding 12 months, to show results and are associated with side effects like irritation, libido loss, and gynecomastia.[10] Therefore, alternative therapies are needed.

Regenerative therapies, particularly mesenchymal stem cells (MSCs) and their derived exosomes, show promise in alopecia treatment. Exosomes, cell-free vesicles derived from MSCs, have advantages such as non-immunogenicity, ease of administration, and the absence of tumorigenic risks.[10,11] Exosomes also contain therapeutic molecules like growth factors, cytokines, and miRNAs that stimulate hair growth and regulate the hair cycle.[12,13] However, limited research exists on the optimal dosage and mechanisms of umbilical cord-derived MSC exosomes, which offer benefits like lower graft rejection risk.[14]

Tumor necrosis factor-alpha (TNF-α) is a proinflammatory cytokine that also influences non-immune responses in tissues, such as cell proliferation and differentiation by modulating intracellular signaling pathways, such as NF-κB and MAPK.[15] In some tissues, TNF-α promotes apoptosis and inhibits proliferation, whereas in others, it stimulates cell survival, differentiation, and tissue remodeling. Specifically, in hair follicles, TNF-α has been shown to disrupt the hair growth cycle by inducing premature catagen phase, inhibiting keratinocyte proliferation, and impairing stem cell differentiation, contributing to hair loss. Previous research demonstrated that TNF-α completely halts hair growth in cultured human hair follicles. Additionally, TNF-α induces rod-shaped follicular structures resembling the catagen phase of hair follicles.[16] In *in vivo* studies, elevated serum levels of TNF-α have been observed in alopecia areata (AA) patients, suggesting a systemic inflammatory response associated with the disease. A previous study reported significantly higher serum TNF-α levels in AA patients compared to healthy controls, supporting the cytokine's role in the pathogenesis of AA.[17]

Vascular endothelial growth factor (VEGF) is a crucial mediator of vascular regeneration, enhancing vascular permeability, endothelial cell proliferation, and capillary formation.[18] VEGF has been reported to reduce hair loss by promoting new blood vessel formation and improving blood circulation around hair follicles. Previous research revealed that overexpression of VEGF in follicular keratinocytes accelerates hair regrowth and increases follicle size, providing direct evidence that enhanced angiogenesis can improve hair growth and thickness.[5,19]

The hair growth cycle includes the anagen (growth) and telogen (resting) phases. Anagen is marked by active follicular proliferation, while telogen is a dormant stage.[20] Fluconazole-induced alopecia-like rats showed more telogen-phase follicles, confirming hair growth disruption.[21] Exosomes, cell-free vesicles derived from MSCs, have advantages such as non-immunogenicity, ease of administration, and the absence of tumorigenic risks.[22]

Hypo-Exo MSC treatment increased anagen-phase follicles, indicating improved hair regeneration. Hypoxic conditions are commonly used in mesenchymal stem cell (MSC) cultures to enhance their regenerative potential.[23] Under low oxygen levels, MSCs exhibit increased secretion of bioactive molecules, including exosomes enriched with growth factors, cytokines, and microRNAs that promote tissue repair and angiogenesis.[24,25] This study was conducted to address the knowledge gap regarding the therapeutic mechanisms of exosomes from hypoxia-treated (Hypo-Exo) MSCs in managing alopecia by exploring the expression of VEGF and TNF-α in a fluconazole-induced alopecia-like model in Wistar rats.

Material and methods

Study Design and Animal Model

This study was conducted using a randomized post-test-only control group design to evaluate the effects Hypo-Exo MSCs on TNF-α and VEGF levels in a fluconazole-induced alopecia-like model in Wistar rats. The population consisted of male Wistar rats aged 6-8 weeks, with a mean weight

of 225±15 grams. Thirty rats were randomly assigned to five groups, with six rats per group to account for potential dropouts. Inclusion criteria required normal anatomy, successful alopecia induction, and no prior experimental use. Exclusion criteria included failure to induce alopecia or health complications during the study. The study setup consisted of five groups. The group 1 received no treatment, serving as the baseline control (Healthy group). The group 2 had fluconazole-induced conditions and was treated with a 0.9% NaCl injection to assess the effects of the fluconazole-induced alopecia without additional treatment (0.9% NaCl-treated group). The group 3 also had fluconazole-induced conditions but was treated with 5% minoxidil to serve as a positive control (5% Minoxidil-treated group). The group 4 received Hypo-Exo MSCs at a concentration of 100 µg/mL (100 µg/mL Hypo-Exo MSCs-treated group), while the group 5 received Hypo-Exo MSCs at 200 µg/mL (200 µg/mL Hypo-Exo MSCs-treated group), allowing for a comparison of the effects of different doses of Hypo-Exo MSCs on TNF-α and VEGF levels in the fluconazole-induced alopecia-like model. This experimental design allowed of treatment effects while minimizing potential bias. The study was approved by the Ethics Committee of the Faculty of Medicine, Universitas Islam Sultan Agung (No.18/I/2025/Komisi Bioetik).

Isolation of MSCs from Umbilical Cords of 21-Day Pregnant Rats and Their Differentiation into Osteocytes and Adipocytes

MSCs used in this study were isolated from the umbilical cords of 21-day pregnant rats. Under strict sterile conditions within a biosafety cabinet, umbilical cords were cleaned, chopped into small sections, and cultured in Dulbecco's modified eagle medium (DMEM) (#11965092 Gibco, Billings, MT, US) supplemented with fetal bovine serum (FBS) (#26140079 Gibco), penicillin-streptomycin (#15140122 Gibco), and fungizone (#A2942 Sigma-Aldrich, St. Louis, MO, US). The cultured cells were maintained in a 37°C incubator with 5% CO_2 (#51033557 Heracell VIOS 160i Thermo Fisher Scientific, Waltham, MA, US) until reaching 80% confluence. MSCs were validated through flow cytometry using surface markers CD90 and CD29 (positive) and CD45 and CD31 (negative) (#554894, 551262, 561796 and 561867 BD Biosciences, San Jose, CA, US). The cells were also tested for their ability to differentiate into osteocytes and adipocytes, confirmed through Alizarin Red (#10043-44-8 Sigma-Aldrich) and Oil Red O (#O0625 Sigma-Aldrich) staining.

Production and Characterization of Hypo-Exo MSCs

Validated MSCs were incubated under hypoxic conditions (5% oxygen) for 24 hours to produce Hypo-Exo MSCs. The culture medium was subsequently collected, and exosomes were isolated using tangential flow filtration (TFF) with 100-500 kDa membrane filters (#VF20P2 Sartorius, Göttingen, Germany). Exosome characterization was performed via flow cytometry using CD63 and CD9 markers, confirming the presence of exosomes.

Fluconazole-Induced Alopecia Model and Administration of Hypo-Exo MSCs

The alopecia-like model was induced by orally administering 35 mg/kgBW of fluconazole (#F8929 Sigma-Aldrich) orally for 14 days to shaved rats. Validation of alopecia-like status was conducted macroscopically by visual inspection for hair regrowth and microscopically through hematoxylin-eosin (HE) staining (#HHS32 Sigma-Aldrich) to confirm the absence of anagen phase follicles. On day 15, treatment groups received subcutaneous injections targeted at the alopecia-induced dorsal area of each rats. The fluconazole-only group received NaCl injections, the minoxidil group receivied 5% minoxidil (Rogaine, Skillman, NJ, US), and the Hypo-Exo MSCs groups received either 100 µg/mL or 200 µg/mL exosomes. The treatments were administered once daily for 14 consecutive days to ensure consistent exposure and maximize therapeutic effects on hair follicle regeneration and inflammation reduction.

Sample Preparation and Analysis for TNF-α and VEGF

Samples for TNF-α and VEGF analysis were collected on day 29. All rats were euthanized using a cocktail of ketamine (60 mg/kg) and xylazine (20 mg/kg). Skin tissues were harvested under sterile conditions, snap-frozen in liquid nitrogen, and stored at -80°C until analysis. Tissue homogenates were prepared with 300 µL of RIPA buffer (#R0278 Sigma-Aldrich), centrifuged at 13,000 rpm in 20 minutes, and the supernatants analyzed using ELISA kits for rat TNF-α and VEGF.

Study instruments included a high-quality ELISA system for precise quantification of TNF-α and VEGF levels, with kits specific to these markers (#E-EL-H0109 and E-EL-H0111 Elabscience, Houston Texas, US). The

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accuracy and reliability of histological assessments were ensured through HE staining, conducted to confirm alopecia induction and evaluate hair follicle changes.

Alopecia Induction and ELISA Analysis of TNF-α and VEGF

Alopecia induction was achieved by administering fluconazole (35 mg/kg) orally for 14 days. The success of alopecia induction was confirmed macroscopically (absence of hair regrowth) and histologically (lack of anagen-phase follicles in HE-stained skin samples). Skin tissue samples were then collected under sterile conditions, snap-frozen in liquid nitrogen, and stored at -80°C.

For TNF-α and VEGF level analysis, ELISA was performed according to the manufacturer's protocols (Elabscience). Briefly, 100 µL of standards, blanks, and samples were added to assigned wells of a 96-well plate, which was then sealed and incubated at 37°C for 90 minutes. Following incubation, the liquid was discarded, and 100 µL of Biotinylated Detection Antibody (#E-EL-R0019, Elabscience) was added to each well. The plate was resealed and incubated for one hour at 37°C, after which the wells were washed three times with Wash Buffer (#E-EL-WB-001, Elabscience).

Next, 100 µL of HRP Conjugate (#E-EL-HRP-001, Elabscience) was added to each well, and the plate was incubated for 30 minutes at 37°C. After five additional washes, 90 µL of Substrate Reagent (TMB Solution, #E-EL-TMB-001 Elabscience) was added, followed by a 15-minute incubation in the dark at 37°C. The reaction was stopped with 50 µL of Stop Solution (#E-EL-STOP-001, Elabscience), and the optical density (OD) was measured at 450 nm using a spectrophotometer.

Statistical Analysis

Statistical analysis was performed using SPSS (version 25.0; IBM Corp., Armonk, NY). Normality and variance were assessed using the Shapiro-Wilk and Levene's tests, followed by One-Way ANOVA and Post Hoc Tamhane for normally distributed data, or Kruskal-Wallis and Mann-Whitney for non-normally distributed data. This approach ensured a reliable evaluation of Hypo-Exo MSCs in reducing inflammation and promoting angiogenesis in an alopecia-like model.

Results

Hypo-Exo MSCs MSCs Modulated Surface Marker Expression and Induced Osteogenic and Adipogenic Differentiation

Flow cytometry analysis revealed that the cultured cells strongly expressed CD90 (100%) and CD29 (99.32%), while the expression of CD45 (0.02%) and CD31 (0.4%)

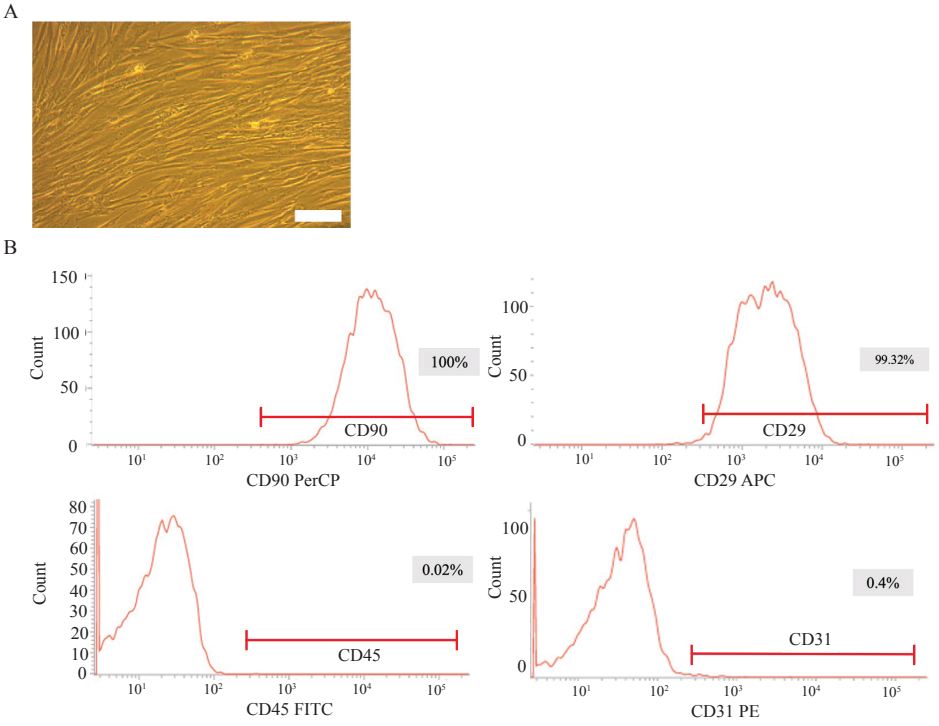

Figure 1. Flow cytometry analysis confirmed MSC identity. A: Microscopic appearance of the cultured cells at 40x magnification. B: Flowcytometry results. The cells strongly expressed CD90 and CD29, with minimal expression of CD45 and CD31. White bar: 100µm.

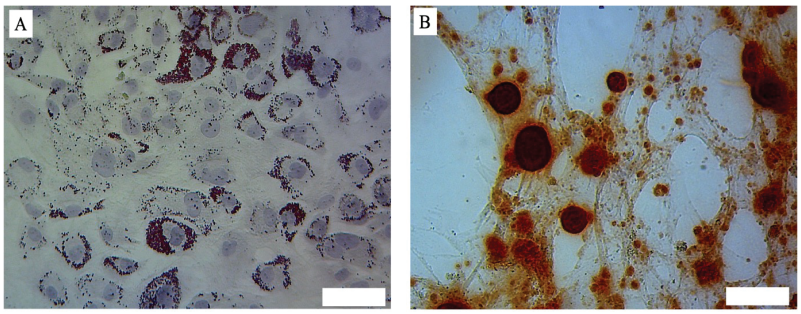

Figure 2. Staining results demonstrated MSC differentiation into adipocytes (Oil Red O staining) (A) and osteocytes (Alizarin Red staining) (B). White bar: 100µm.

was minimal (Figure 1), confirming their MSC identity. The staining results also verified the ability of the MSCs to form osteogenic and adipogenic lineages, further validating their multipotent characteristics (Figure 2).

Fluconazole Induces Hair Loss and Modulates Hair Follicle Phases in Rats

Alopecia induction using fluconazole was validated through macroscopic and histological assessments. Macroscopically, treated rats exhibited significant hair loss in the shaved dorsal region after 14 days (Figure 3). Histological validation using HE staining confirmed the absence of anagen-phase hair follicles and an increase in telogen-phase follicles, supporting successful alopecia induction (Figure 4).

Hypo-Exo MSCs Increased Hair Regrowth and Reduced Skin Inflammation in Fluconazole-Induced Alopecia-Like Model

Macroscopic evaluation of dorsal skin in fluconazole-induced alopecia-like rats revealed distinct differences across treatment groups (Figure 5). The 0.9% NaCl-treated group which received no treatment, exhibited patchy hair loss and sparse regrowth, confirming the alopecia-like condition (Figure 5A). The 5% Minoxidil-treated group, treated with 5% minoxidil, showed partial hair regrowth, but areas of skin irritation and small lesions were observed (Figure 5B). The 100 µg/mL Hypo-Exo MSCs-treated group demonstrated more pronounced hair regrowth, with fewer visible skin lesions (Figure 5C). The 200 µg/mL Hypo-Exo MSCs-treated group exhibited the most extensive hair regrowth, closely resembling the healthy control group, with minimal signs of skin inflammation (Figure 5D).

Hypo-Exo MSCs Reduced TNF-α Levels

The 0.9% NaCl-treated group exhibited the highest TNF-α levels (307.46±20.68 pg/mL), indicating heightened inflammation due to fluconazole-induced alopecia-like conditions (Figure 6). Treatment with Hypo-Exo MSCs significantly reduced TNF-α levels in the 100 µg/mL Hypo-Exo MSCs-treated group (65.38±15.05 pg/mL) and the 200

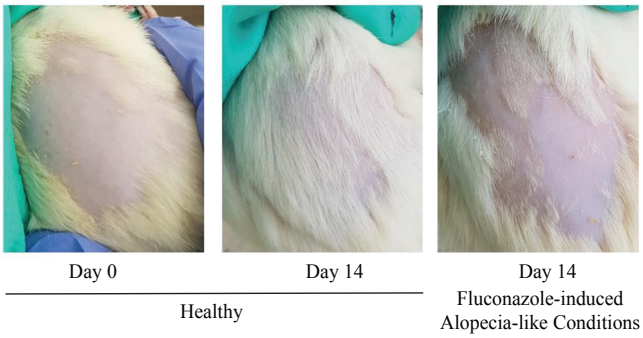

Figure 3. Macroscopic validation of the alopecia-like model, showing significant hair loss in the shaved dorsal region of treated rats after 14 days.

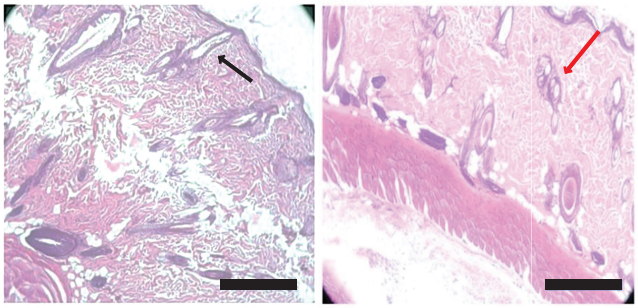

Figure 4. The absence of anagen-phase follicles and an increase in telogen-phase follicles in fluconazole-induced alopecia-like rats after 14 days. Black arrow: anagen. Red arrow: telogen. Black bar: 150 µm.

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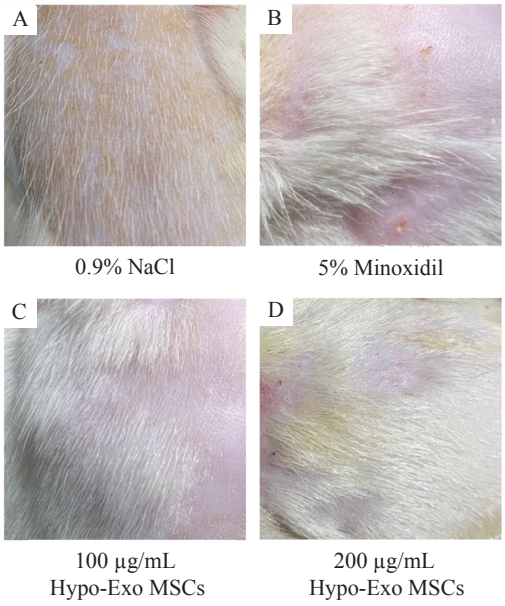

Figure 5. Macroscopic appearance of dorsal skin in fluconazole-induced alopecia-like rats. A: The 0.9% NaCl-treated group showed patchy hair loss with minimal regrowth. B: The 5% Minoxidil-treated group showed partial hair regrowth with skin irritation. C: The 100 µg/mL Hypo-Exo MSCs-treated group showed an increased hair regrowth with reduced skin damage. D: The 200 µg/mL Hypo-Exo MSCs-treated group showed most extensive hair regrowth, resembling normal hair coverage.

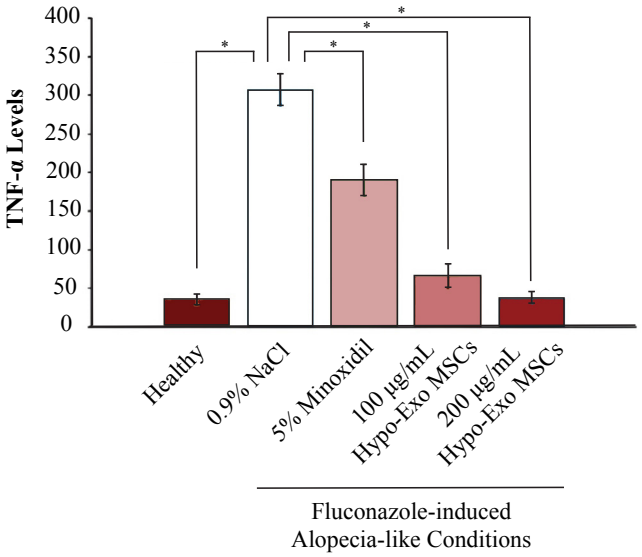

Figure 6. Hypo-Exo MSCs Reduced TNF-α levels. The TNF-α levels in fluconazole-induced alopecia-like rats were measured via ELISA after treatment with 100 µg/mL and 200 µg/mL Hypo-Exo MSCs. The 0.9% NaCl-treated group had the highest TNF-α levels and the 200 µg/mL Hypo-Exo MSCs-treated group showing levels close to the Healthy group. *significant (Post Hoc Tamhane test, $p<0.05$)

µg/mL Hypo-Exo MSCs-treated group (37.16±7.14 pg/mL) compared to 0.9% NaCl-treated group. Notably, TNF-α levels in the 200 µg/mL Hypo-Exo MSCs-treated group were similar to the Healthy group (35.43±7.51 pg/mL).

Hypo-Exo MSCs Increased VEGF Levels

For VEGF, the Healthy group had the highest levels (194.00±7.65 pg/mL), followed by the 200 µg/mL Hypo-Exo MSCs-treated group (189.11±9.75 pg/mL) and the 100 µg/mL Hypo-Exo MSCs-treated group (158.50±5.33 pg/mL) (Figure 7). VEGF levels in the 5% Minoxidil-treated group (123.90±6.18 pg/mL) and the 0.9% NaCl-treated group (69.60±15.39 pg/mL) were significantly lower, suggesting reduced angiogenesis.

Discussion

This study demonstrated that subcutaneous administration of Hypo-Exo MSCs significantly reduced TNF-α levels, particularly at the 200 µg/mL dose, which restored TNF-α concentrations to near-normal levels. Macroscopic observations revealed minimal hair regrowth in the 0.9% NaCl-treated group, confirming hair loss due to fluconazole-induced inflammation. The 200 µg/mL Hypo-Exo MSCs-treated group showing the most significant recovery of hair regrowth, resembling Healthy group. These findings correlated with previous research indicating that MSC-derived exosomes mitigated inflammation and promoted hair follicle regeneration by modulating cytokine activity.[26,27]

The macroscopic evaluation further supported these biochemical findings, as the 200 µg/mL Hypo-Exo MSCs-treated group exhibited the most extensive hair regrowth with minimal skin irritation, while the 0.9% NaCl-treated group had sparse regrowth with patchy alopecia. These results reinforced the therapeutic potential of Hypo-Exo MSCs in reducing inflammation and enhancing vascularization, thereby restoring hair follicle homeostasis in alopecia-like conditions.[28] The 200 µg/mL dose exhibited superior efficacy compared to the 100 µg/mL dose, as evidenced by greater reductions in TNF-α and increased VEGF levels.

These findings align with previous studies highlighting that higher exosome doses provided increased bioactive molecules, including microRNAs, cytokines, and growth

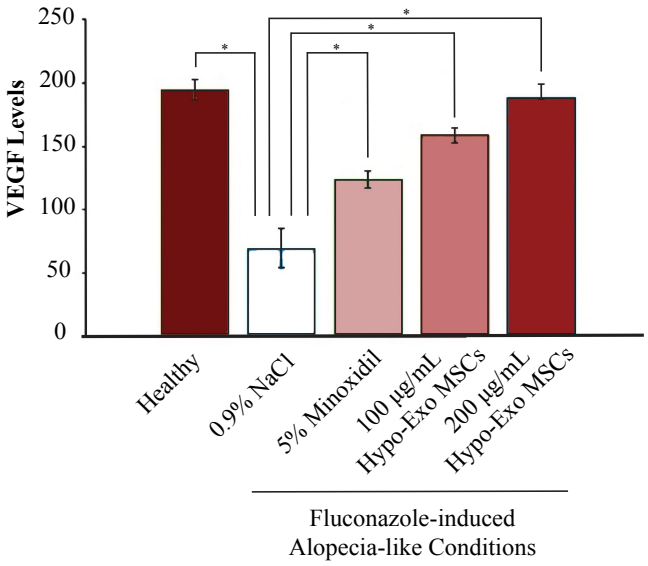

Figure 7. Hypo-Exo MSCs Reduced VEGF levels. The VEGF levels were measured in fluconazole-induced alopecia-like rats treated with 100 µg/mL and 200 µg/mL Hypo-Exo MSCs. The Healthy group had the highest VEGF levels, followed by the 100 µg/mL Hypo-Exo MSCs-treated group and 200 µg/mL Hypo-Exo MSCs-treated group. *significant (Post Hoc Tamhane test, $p<0.05$)

factors, which worked collectively to modulate inflammation and promote tissue repair, included reduce TNF-α.[29,30] In the treatment group, Hypo-Exo MSCs were administered at 100 µg/mL and 200 µg/mL doses in alopecia-like mouse models to evaluate their effects on TNF-α and VEGF levels and their implications for immune regulation and alopecia progression. Results showed that the 200 µg/mL dose significantly reduced TNF-α levels while increasing VEGF concentrations to levels comparable to healthy controls. In contrast, the 100 µg/mL dose failed to induce significant changes, suggesting that higher doses may be necessary to maximize therapeutic benefits.

The observed reduction in TNF-α with the 200 µg/mL dose highlighted its efficacy in activating biological pathways to resolve inflammation in hair follicle regions. TNF-α, alongside interleukins IL-1α and IL-1β, induced matrix cell vacuolization, reduces matrix size, and disrupts melanocyte organization in hair follicles. It also leads to abnormal keratinization in precortical cells and inner root sheath cells, characteristic of alopecia areata pathology.[31] By reducing TNF-α levels, Hypo-Exo MSCs mitigated these pathological processes. Conversely, VEGF elevation promotes angiogenesis, enhancing blood circulation around hair follicles and reducing hair loss. Increased VEGF expression had been linked to accelerated hair regrowth and follicle size enlargement, providing direct evidence that angiogenesis supports hair thickness and regrowth.

This finding aligned with prior research demonstrating that enhanced VEGF activity improves follicular health and function. Additionally, the administration of Hypo-Exo MSCs at the 200 µg/mL dose likely affected regulatory T cells (Tregs), crucial mediators of immune homeostasis. Enhanced Treg activity helped suppress immune attacks on hair follicles, a phenomenon often observed in autoimmune conditions like alopecia areata. By increasing Treg numbers, Hypo-Exo MSCs may have counteracted immune-mediated follicle destruction, offering a potential mechanism to mitigate alopecia progression.[28,32]

The superior efficacy of the 200 µg/mL dose compared to the 100 µg/mL dose could have been attributed to the higher concentration of exosomes carrying bioactive molecules, including miR-122 and miR-221. miR-122 promoted TGF-β production, which suppresses pro-inflammatory immune cell activity and reduces inflammation, while miR-221 enhanced VEGF expression to support angiogenesis and hair follicle repair. These findings underscored the dose-dependent therapeutic potential of Hypo-Exo MSCs in managing alopecia-like conditions.[33-39]

The results of this study aligned with previous findings demonstrating the therapeutic potential of MSC-derived exosomes in inflammatory and regenerative conditions. It's highlighted the regenerative capacity of MSC-derived exosomes in alopecia models, emphasizing their safety and efficacy.[40] However, this study provides additional insights by focusing on specific miRNA-mediated pathways, particularly the roles of miR-122 and miR-221, in modulating TNF-α and VEGF levels. This mechanistic understanding enhanced our knowledge of the underlying processes driving alopecia progression and recovery.

Differences also emerged when comparing these findings to prior studies. While many studies explored MSC-derived exosomes in general contexts, this study specifically evaluated the dose-dependent effects of umbilical cord-derived Hypo-Exo MSCs on alopecia.[30] The use of umbilical cord-derived MSCs offered unique advantages, including higher yields of naive, youthful cells with lower immunogenicity and graft-versus-host disease risks, making them suitable for broader applications. However, the precise dosing and long-term effects remained

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underexplored, warranting further investigation.

Despite its promising findings, this study had several limitations. First, the sample size was limited to an experimental animal model, necessitating caution when extrapolating results to humans. Second, the study focused primarily on TNF-α and VEGF as biomarkers, potentially overlooking other relevant inflammatory and regenerative pathways. Third, the long-term effects of Hypo-Exo MSC therapy, including sustained efficacy and safety, remained unknown and require further research. Finally, the specific interactions between miRNAs and other molecular pathways in the context of alopecia needed more detailed investigation to optimize treatment strategies.

Conclusion

Administration of Hypo-Exo MSCs at 100 µg/mL and 200 µg/mL significantly reduced TNF-α levels and increased VEGF levels in a fluconazole-induced alopecia-like model. These results suggest that Hypo-Exo MSCs have the potential to modulate inflammation and promote angiogenesis, supporting their use as a novel regenerative therapy for alopecia.

Authors' Contributions

SMS, SPM, AP, and ES were involved in conceptualizing and planning the research. SMS performed the data acquisition/collection, calculated the experimental data, analyzed the data, drafted the manuscript, designed the figures, and interpreted the results. All authors contributed to the critical revision of the manuscript.

Conflict of Interest

The authors declare that they have no conflicts of interest or competing interests related to the content of this manuscript.

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