IFSCC 2025 full paper (IFSCC2025-819)

"Exosomes Isolated from Iris germanica L. Rhizome Recovered Human Follicle Dermal Papilla Cells Impaired by Dihydrotestosterone"

Mujun Kim[1], Jung Woo[2], Jinsick Kim[1], Minah Choi[2], Hee Jung Shin[2], Youngseok Kim[2], Junoh Kim[2], and Dong Wook Shin[1,*]

[1]Research Institute for Biomedical and Health Science, Konkuk University, Chungju 27478, Republic of Korea

[2]Shinsegae International Inc., Seoul 06015, Republic of Korea

Abstract

Hair loss is commonly linked to oxidative stress and mitochondrial dysfunction in human follicle dermal papilla cells (HFDPCs), which impair cellular function and cause follicle degeneration. HFDPCs, mesenchymal cells located at the base of hair follicles with specialized functions, play a crucial role in regulating hair growth and follicular morphogenesis. Because of their ability to secrete growth factors that influence the hair cycle, HFDPCs have become essential in models for studying hair loss and assessing agents that promote hair growth. These models offer a controlled environment for exploring hair follicle biology and serve as an effective platform for screening potential therapeutic agents to regenerate hair follicles. Numerous studies have focused on natural plant-based remedies to prevent hair loss. After we screened out several plant-derived agents, we investigated the therapeutic potential of exosomes derived from the rhizomes of Iris germanica L. (Iris-exosomes) in HFDPCs damaged by dihydrotestosterone (DHT). Iris-exosomes significantly improved the migration of DHT-damaged HFDPCs. Iris-exosomes effectively reduced reactive oxygen species (ROS) levels, restored mitochondrial membrane potential, and enhanced ATP production in these cells, meaning that Iris-exosomes alleviated oxidative stress and improved mitochondrial function. These improvements were accompanied by enhanced cellular activities critical for hair follicle regeneration, including increased cell migration, elevated alkaline phosphatase activity, and three-dimensional (3D) spheroid formation, which mimics the follicle-like microenvironment and promotes inductive potential. Additionally, Iris-exosomes activated the Wnt/β-catenin signaling pathway by modulating glycogen synthase kinase-3β (GSK-3β), AKT, and extracellular signal-regulated kinase (ERK), resulting in β-catenin stabilization and nuclear translocation. This activation supported the expression of key genes involved in hair growth. In summary, these findings suggest that Iris-exosomes hold significant promise as an innovative solution for addressing hair loss.

1. Introduction

Exosomes are nanosized extracellular vesicles (30–400 nm) secreted by various animal and plant cells, playing pivotal roles in intercellular communication by transporting proteins,

lipids, mRNAs, and microRNAs. They are increasingly recognized for their therapeutic potential, especially in regenerative medicine, as they can modulate immune responses and promote tissue repair. Exosomes derived from mesenchymal stem cells (MSCs) and dermal papilla cells have shown promise in enhancing hair growth by stimulating follicular cells and growth factor secretion. Moreover, plant-derived exosomes, containing bioactive molecules such as mRNAs and secondary metabolites, have emerged as biocompatible candidates for antioxidative and anti-inflammatory therapies.

Androgenetic alopecia (AGA) is the most common type of hair loss, marked by progressive follicular miniaturization in androgen-sensitive scalp areas loss . AGA results from the interaction between genetic susceptibility and the androgen hormone dihydrotestosterone af(DHT), which binds to androgen receptors in hair follicles, disrupting the hair cycle and shortening the anagen phase. Although current treatments like finasteride and minoxidil (MIX) target DHT-related pathways, their limited efficacy and side effects necessitate alternative therapeutic strategies.

Human hair follicle dermal papilla cells (HFDPCs), located at the base of hair follicles, are key regulators of hair growth and serve as valuable in vitro models for investigating hair regeneration mechanisms and screening novel treatments. Central to follicular regeneration is the Wnt/β-catenin signaling pathway, which governs stem cell activation and dermal papilla cell proliferation. However, DHT impairs this pathway, contributing to AGA pathology. Consequently, strategies aimed at restoring Wnt/β-catenin signaling are considered promising for promoting hair regrowth.

Botanical compounds have recently gained attention for their potential to protect against hair loss by exerting anti-androgenic, antioxidant, and anti-inflammatory effects. Among them, Iris germanica L., rich in flavonoids, phenolics, and isoflavones, is well known for its pharmacological properties, including antioxidant and anti-inflammatory activities. Despite extensive research on Iris species, their potential impact on hair regeneration remains unexplored.

In this study, we aimed to investigate whether exosomes isolated from the rhizomes of Iris germanica L. (Iris-exosomes) could ameliorate DHT-induced damage in HFDPCs, thereby evaluating their potential as a novel plant-derived therapeutic approach for combating hair loss.

2. Materials and Methods

2.1. Preparation and characterization of Iris-exosomes

Iris-exosomes (ABio Materials, Suwon, Korea) were isolated according to a previously reported protocol by Kim et al. In brief, dried rhizomes of *Iris germanica* L. were subjected to ultrahigh-pressure processing, followed by mechanical extraction using a juicer. The resulting extracts were centrifuged at 10,000 × g for 10 minutes at 4 °C to remove debris. The collected supernatant was frozen at −80 °C for 20 hours and subsequently freeze-dried under vacuum for 100 hours. The dried material was rehydrated with distilled water and partitioned using an aqueous two-phase system composed of 3.3 % polyethylene glycol and 1.7 % dextran. After centrifugation at 1,000 × g for 10 minutes at 4 °C, the supernatant was removed, and the lower-phase fraction, enriched in exosomes, was collected. This two-phase extraction procedure was

repeated three times to enhance the purity of the exosomes. The final exosome preparation was obtained in freeze-dried form and stored for further experimental use.

2.2. Cell culture

HFDPCs were purchased from PromoCell (Heidelberg, Germany) and maintained in follicle dermal papilla cell growth medium supplemented with 1% penicillin–streptomycin at 37 °C in a humidified incubator containing 5 % CO_2.

2.3. Cell viability assay

HFDPCs were incubated with Iris-exosomes at concentrations of 10^6, 5×10^6, 10^7, 5×10^7, and 10^8 particles/mL for 24 hours. Following incubation, the cells were washed with Dulbecco's phosphate-buffered saline (DPBS) (Welgene Inc., Gyeongsan, Republic of Korea), and EZ-Cytox reagent (DoGenBio, Seoul, Republic of Korea) was added to each well. After an additional 1-hour incubation at 37 °C, the absorbance was measured at 450 nm.

2.4. Wound healing assay

HFDPCs were seeded into 6-well plates and maintained for 24 hours at 37 °C in a humidified 5 % CO_2 incubator. A straight-line scratch was introduced at the center of each well using a sterile 1 mL pipette tip. After removing the culture medium, the cells were treated with 1 μM DHT (Sigma-Aldrich, St. Louis, MO, USA), 1 μM MIX (Sigma-Aldrich), or Iris-exosomes at concentrations of 10^7 and 10^8 particles/mL. Phase-contrast images were captured at 0, 24, and 48 hours post-treatment using a Nikon light microscope (Tokyo, Japan) to evaluate wound healing and cell migration.

2.5. Alkaline Phosphatase Staining assay

Alkaline phosphatase (ALP) staining was carried out using an ALP staining kit (Abcam, Cambridge, UK). HFDPCs were cultured for 24 hours in a humidified incubator containing 5 % CO_2. After incubation, the cells were treated with 1 μM DHT, 1 μM MIX, or Iris-exosomes at concentrations of 10^7 and 10^8 particles/mL, followed by incubation for another 24 hours at 37 °C. Following treatment, the cells were fixed with a fixing solution for 2 minutes and then stained with the ALP staining reagent for 24 hours. Purple-colored colonies indicative of ALP activity were observed and counted under a Nikon light microscope (Tokyo, Japan).

2.6. DCF-DA ROS assay

Reactive oxygen species (ROS) levels within HFDPCs were assessed using the Cellular ROS Assay Kit (Abcam, Cambridge, UK). Cells were plated into confocal dishes and incubated for 24 hours at 37 °C in a 5% CO_2 incubator. Cells were exposed to 1 μM DHT, 1 μM MIX, or Iris-exosomes at a final concentration of 10^8 particles/mL and further incubated for 24 hours. After treatment, the cells were loaded with 10 μM 2′,7′-dichlorofluorescin diacetate (DCF-DA) and maintained at 37 °C for 20 minutes in the absence of light. Fluorescence signals were captured using a Nikon Eclipse Ti2 fluorescence live-cell imaging microscope (Tokyo, Japan).

2.7. Measurement of Mitochondrial Membrane Potential

Mitochondrial membrane potential was evaluated using the JC-1 Mitochondrial Membrane Potential Assay Kit (Abcam, Cambridge, UK). HDMECs were seeded in a confocal dish and cultured for 24 hours at 37 °C in a 5% CO_2 incubator. Cells were treated with 1 μM DHT, 1 μM MIX, or Iris-exosomes (10^8 particles/mL) for 24 hours. Cells were incubated with 2 μM JC-1 dye for 30 minutes at 37 °C, followed by washing with DPBS. Fluorescence signals

were observed using a Nikon Eclipse Ti2 fluorescence live-cell imaging microscope (Tokyo, Japan).

2.8. Immunofluorescence Analysis

HFDPCs were cultured for 24 hours at 37 °C in a 5% CO_2 incubator. The cells were treated with 1 μM MIX or Iris-exosomes at a concentration of 10^8 particles/mL for 24 hours, and 1 μM DHT for 6 hours. After treatment, the cells were fixed with 4 % paraformaldehyde for 10 minutes and permeabilized with 0.1% Triton X-100 for 15 minutes at room temperature. Following blocking with 3 % bovine serum albumin (BSA) for 1 hour, the cells were incubated overnight at 4 °C with a primary antibody against phosphorylated Nrf2. The next day, cells were incubated with an HRP-conjugated secondary antibody for 1 hour at room temperature. The nuclei were counterstained with DAPI for 15 minutes, and fluorescence images were captured using a Nikon Eclipse Ti2 live-cell fluorescence microscope (Tokyo, Japan).

2.9. ATP assay

Mitochondrial ATP levels in HFDPCs were evaluated using ATP Red™ and MitoLite™ Green FM dyes (AAT Bioquest, Pleasanton, USA) following the manufacturer's protocol. Cells were seeded into confocal dishes and cultured for 24 hours at 37 °C in a 5 % CO_2 incubator. Cells were treated with 1 μM DHT, 1 μM MIX, or Iris-exosomes at a concentration of 10^8 particles/mL for 24 hours. Cells were incubated with ATP Red™ working solution for 30 minutes at 37 °C. After washing with DPBS, the cells were further stained with MitoLite™ Green FM solution for 30 minutes. The stained cells were washed again with DPBS, and fluorescence images were captured using a Nikon Eclipse Ti2 live-cell fluorescence microscope (Tokyo, Japan).

2.10. Western blot analysis

HFDPCs were cultured in a 5 % CO_2 incubator at 37 °C for 24 hours. Following incubation, the cells were treated with 1 μM DHT, Iris-exosomes (at final concentrations of 10^7 and 10^8 particles/mL), and 1 μM MIX for 24 hours. After treatment, cells were rinsed twice with DPBS and lysed using RIPA lysis and extraction buffer on ice. Protein concentrations were quantified using the Pierce™ BCA Protein Assay Kit (Thermo Fisher Scientific, Waltham, MA, USA). Thirty micrograms of total protein were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) at 120 V for 2 hours. Separated proteins were transferred overnight onto a polyvinylidene fluoride (PVDF) membrane (Roche, Mannheim, Germany). Membranes were blocked with blocking solution, followed by overnight incubation at 4 °C with primary antibodies against p-AKT (4060S), AKT (9272S), p-ERK (9101S), and ERK (9102S) (Cell Signaling Technology, Beverly, CA, USA), each diluted 1:1000 in blocking solution. Primary antibodies against β-catenin (SC-59737) and p-GSK-3β (SC-373800) (Santa Cruz Biotechnology, Dallas, TX, USA) were diluted 1:500 in blocking solution. After primary antibody incubation, membranes were washed three times with TBS-T buffer (Bio-Rad Inc., Hercules, CA, USA). HRP-conjugated secondary antibodies (Cell Signaling Technology, Beverly, CA, USA) were applied for 1 hour at room temperature. Protein bands were visualized using an ECL detection reagent (Cytiva, Marlborough, MA, USA), and images were captured with an Invitrogen iBright 1500 imaging system (Thermo Fisher Scientific, Waltham, MA, USA). Band intensities were analyzed using Fiji ImageJ software (Win 64-bit), version 1.53e.

2.11. 3D Spheroid Formation of HFDPCs

HFDPCs were plated and cultured for 24 hours at 37 °C in a 5 % CO_2 incubator. Cells were exposed to 1 μM DHT, Iris-exosomes (at 10^7 and 10^8 particles/mL), or 1 μM MIX under standard culture conditions. Spheroid structures were observed, and their diameters were measured using a Nikon light microscope (Tokyo, Japan).

3. Results

3.1. Effect of Iris-Exosome Treatment on the Viability of HFDPCs

The cytotoxic effects of Iris-exosomes on HFDPCs were evaluated using MTT assays conducted over a range of concentrations for 24 hours. This viability assay was performed to determine the optimal concentration of Iris-exosomes for subsequent experiments. No cytotoxic effects were observed at the tested concentrations, which ranged from 10^6 to 10^8 particles/mL (data not shown).

3.2. Iris-Exosomes Promote the Migration of DHT-Damaged HFDPCs

The proliferative and migratory capacities of dermal papilla cells are essential for hair follicle regeneration and maintenance. These cellular activities play a pivotal role in sustaining and prolonging the anagen phase of the hair growth cycle. To investigate the effects of Iris-exosomes on the migration of DHT-damaged HFDPCs, a wound healing assay was performed. Cells treated with DHT alone showed a significant delay in wound closure compared with the untreated control group, indicating that DHT impaired cell motility. However, treatment with Iris-exosomes and MIX accelerated wound closure after 48 hours compared to the DHT-treated group. These findings suggest that Iris-exosomes enhanced the migration of HFDPCs (Figure 1).

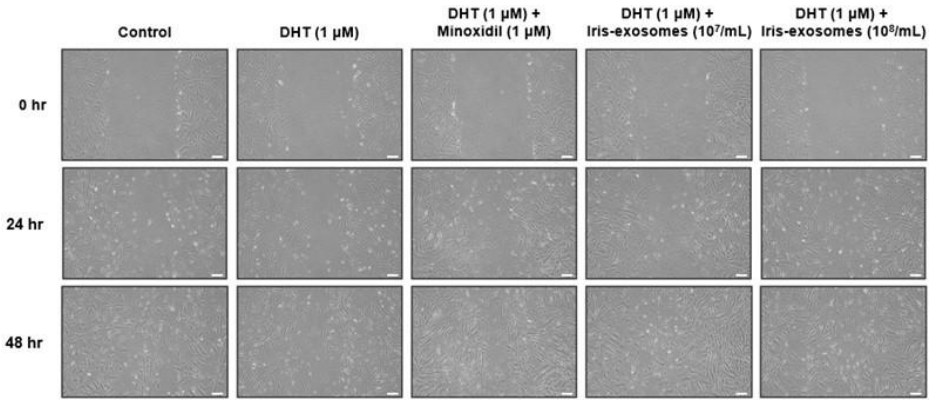

Figure 1. Wound healing effects of Iris-exosomes on HFDPCs stimulated with 1 μM DHT. Cells were treated with Iris-exosomes (10^7 and 10^8 particles/mL) or 1 μM MIX for 48 hours. Wound closure was assessed by phase-contrast microscopy at 24 and 48 hours (scale bar 20 μm).

3.3. Iris-Exosomes Promote Alkaline Phosphatase Expression in DHT-Damaged HFDPCs

ALP is abundantly expressed in HFDPCs, with maximal enzymatic activity observed during the initial stage of anagen. Given its role in advancing the hair follicle cycle, ALP activity is widely recognized as a marker of hair inductivity. In this study, HFDPCs treated with DHT

displayed a reduction in ALP expression compared to untreated controls, indicating that DHT impairs important indicators related to the hair growth cycle. Conversely, previous reports have demonstrated that MIX treatment enhances ALP activity, thereby facilitating the progression of the hair cycle. Consistent with these findings, 1 μM MIX treatment restored ALP expression in DHT-damaged HFDPCs. Likewise, Iris-exosome treatment also significantly increased ALP levels compared to the DHT-only group (Figure 2).

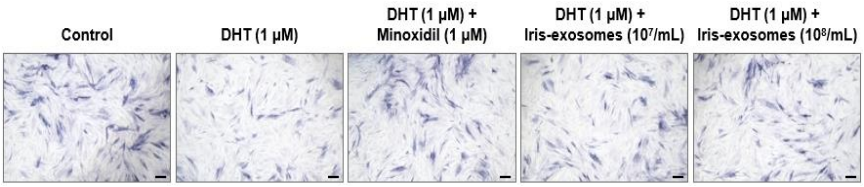

Figure 2. Effects of Iris-exosomes on ALP expression in HFDPCs stimulated with 1 μM DHT. Cells were treated with Iris-exosomes (10^7 and 10^8 particles/mL) or 1 μM MIX for 24 hours (scale bar 20 μm).

3.4. Iris-Exosomes Attenuate ROS Levels in DHT-Damaged HFDPCs

Both intrinsic factors and extrinsic environmental stressors influence skin aging. Notably, extrinsic aging is largely attributed to the generation of ROS from environmental sources such as UV radiation, psychological stress, and pollution. While ROS is involved in normal cellular redox regulation, excessive ROS accumulation can induce cellular damage and contribute to the development of various pathological conditions. In HFDPCs, ROS exposure impairs hair growth and maintenance, ultimately leading to hair loss.

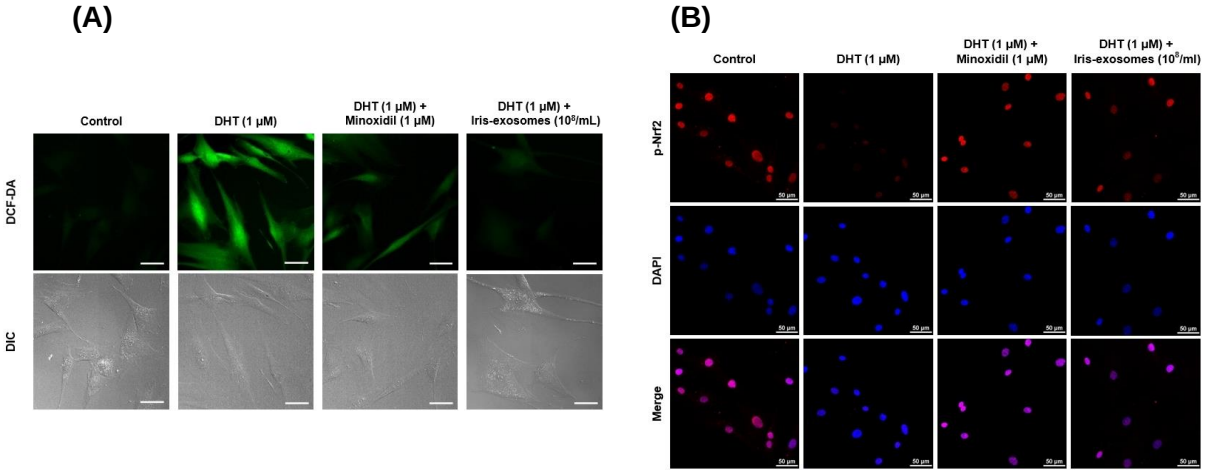

Figure 3. Effects of Iris-exosomes on ROS levels in HFDPCs stimulated with 1 μM DHT. (A) DCF-DA fluorescence images showing green fluorescence as an indicator of intracellular ROS levels (scale bar 50 μm). (B) Immunofluorescence analysis of p-Nrf2 expression (scale bar 50 μm).

To investigate the potential of Iris-exosomes in mitigating ROS accumulation, DCF-DA staining was performed. Consistent with previous findings, DHT-treated HFDPCs exhibited elevated ROS levels compared to untreated controls. However, treatment with MIX effectively reduced ROS accumulation in DHT-exposed cells. Similarly, Iris-exosome treatment significantly attenuated DHT-induced ROS levels, restoring them close to control levels (Figure 3A). Nuclear factor erythroid 2-related factor 2 (Nrf2) serves as a key regulator of the antioxidant defense system by controlling the expression of various antioxidant enzymes. To

further examine this mechanism, we evaluated the nuclear translocation of phosphorylated Nrf2 (p-Nrf2) using immunofluorescence staining. Our results demonstrated that Iris-exosomes promoted Nrf2 activation and enhanced catalase expression, both of which were downregulated following DHT exposure (Figure 3B).

3.5. Iris-Exosomes Recover Mitochondrial Membrane Potential in DHT-Damaged HFDPCs

Mitochondria are critical for cellular energy production, and mitochondrial dysfunction has been implicated in the progression of various diseases. The anagen phase of the hair cycle requires substantial energy and metabolic activity to support follicular regeneration. Disruption of mitochondrial function can impair the hair cycle and ultimately contribute to hair loss. To assess changes in mitochondrial membrane potential following Iris-exosome treatment, we conducted a JC-1 assay in DHT-damaged HFDPCs.

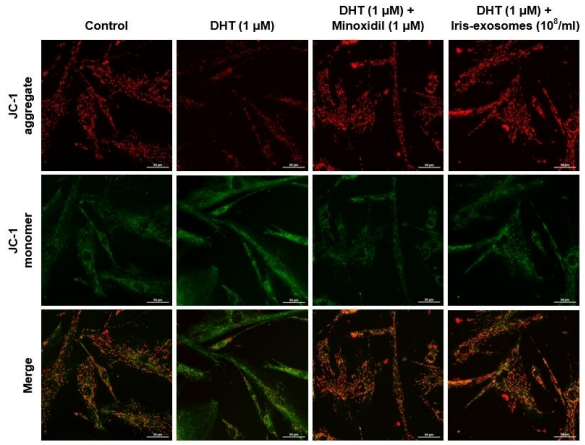

Figure 4. Effects of Iris-exosomes on mitochondrial membrane potential in HFDPCs stimulated with 1 μM DHT. JC-1 assay was performed on HFDPCs treated with 1 μM DHT, followed by treatment with Iris-exosomes (10^8 particles/mL) or 1 μM MIX for 24 hours. Green fluorescence indicates depolarized mitochondria, while red fluorescence represents hyperpolarized mitochondria (scale bar 50 μm).

In this assay, green fluorescence indicates a loss of membrane potential, whereas red fluorescence reflects intact and healthy mitochondrial function. As anticipated, DHT-treated cells displayed increased green fluorescence compared to the control group, indicating mitochondrial impairment. Conversely, treatment with Iris-exosomes promoted red fluorescence intensity in DHT-damaged HFDPCs, suggesting a restoration of mitochondrial membrane potential (Figure 4).

3.6. Iris-Exosomes Improve ATP Production in DHT-Damaged HFDPCs

ATP is the primary energy currency of the cell, supporting critical biological processes such as cell proliferation, migration, and the secretion of signaling molecules. In dermal papilla cells, a reduction in ATP availability compromises these essential functions, ultimately weakening hair growth and contributing to hair loss. Moreover, mitochondrial dysfunction exacerbates this problem by impairing ATP synthesis, further hindering hair follicle regeneration. We performed fluorescence microscopy analysis in DHT-damaged HFDPCs to evaluate ATP production following Iris-exosome treatment. In this assay, red fluorescence

reflects ATP presence, while green fluorescence indicates mitochondrial structures. As expected, red fluorescence intensity was markedly decreased in the DHT-treated group compared to the control, suggesting ATP depletion. However, treatment with Iris-exosomes significantly restored red fluorescence intensity, indicating recovery of ATP levels (Figure 5).

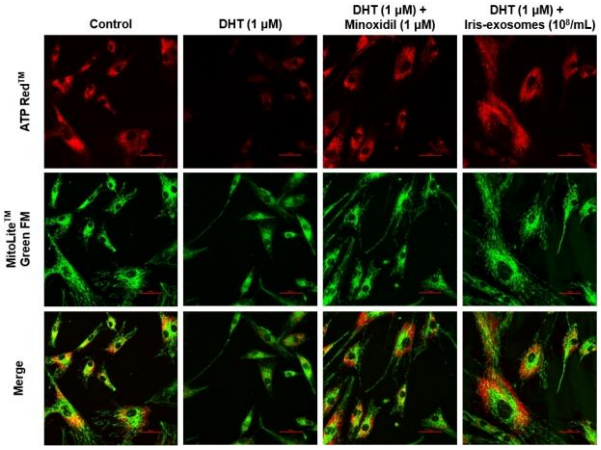

Figure 5. Effects of Iris-exosomes on ATP levels in HFDPCs stimulated with 1 μM DHT. ATP assay was performed on HFDPCs treated with 1 μM DHT, followed by treatment with Iris-exosomes (10^8 particles/mL) or 1 μM MIX for 24 hours. Fluorescence images. Red fluorescence indicates ATP levels, while green fluorescence represents mitochondria (scale bar 50 μm).

3.7. Iris-Exosomes Promote Phosphorylation of AKT, ERK, and GSK-3β and Increase β-Catenin Expression in DHT-Damaged HFDPCs

The Wnt/β-catenin signaling pathway plays a crucial role in regulating hair follicle development and regeneration. Wnt ligands bind to frizzled receptors and low-density lipoprotein receptor-related proteins, or receptor tyrosine kinases. Activation of receptor tyrosine kinases enhances GSK-3β phosphorylation through the upstream activation of ERK and AKT, facilitating the nuclear translocation of β-catenin. This nuclear localization subsequently triggers the expression of target genes involved in hair growth and proliferation.

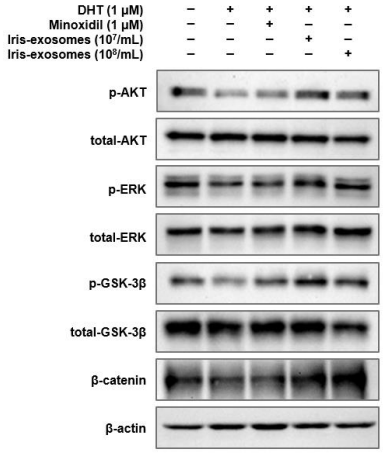

Figure 6. Effects of Iris-exosomes on the phosphorylation of AKT, ERK, and GSK-3β, and the expression of β-catenin in DHT-damaged HFDPCs. Western blot images showing the relative protein expression levels.

To evaluate the impact of Iris-exosomes on these signaling pathways, we performed western blot analysis to assess the phosphorylation levels of AKT, ERK, and GSK-3β, as well as the expression of β-catenin. DHT-damaged HFDPCs exhibited reduced phosphorylation of AKT, ERK, and GSK-3β, along with decreased β-catenin expression, compared to untreated controls. In contrast, treatment with Iris-exosomes restored the phosphorylation of AKT, ERK, and GSK-3β and upregulated β-catenin expression relative to the DHT-treated group (Figure 6). These results suggest that Iris-exosomes may ameliorate hair loss by activating the AKT/ERK and Wnt/β-catenin signaling pathways in DHT-damaged HFDPCs.

3.8. Iris-Exosomes Promote 3D Spheroid Growth in DHT-Induced Damaged HFDPCs

Three-dimensional (3D) spheroid culture systems have emerged as an alternative in vitro strategy to enhance the trichogenic capacity of HFDPCs. This culture method restores critical cell–cell interactions and improves the hair-inductive potential of HFDPCs in vivo. Compared to conventional two-dimensional (2D) cultures, 3D spheroid models provide deeper insights into complex cellular behavior. In our study, DHT-treated HFDPCs exhibited a notable reduction in spheroid size compared to the untreated control group.

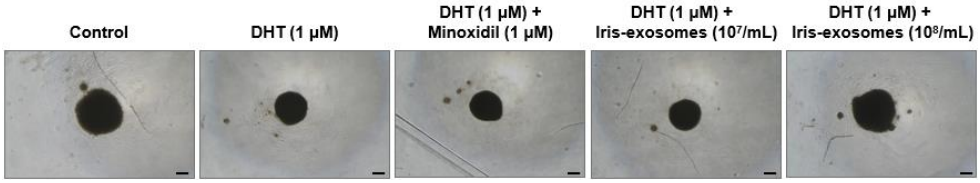

Figure 7. Effects of Iris-exosomes on 3D spheroid formation in DHT-damaged HFDPCs. HFDPCs were treated with 1 μM DHT, 1 μM MIX, or Iris-exosomes (10^7 and 10^8 particles/mL) at 2-day intervals. Images were captured after 21 days of culture. Phase-contrast images of 3D spheroids (scale bar 20 μm).

Conversely, treatment with Iris-exosomes significantly increased spheroid size relative to DHT-damaged HFDPCs, suggesting that Iris-exosomes confer beneficial effects on the regenerative properties of HFDPCs (Figure 7).

4. Discussion

HFDPCs are critical for hair follicle development and cycling, and their functional state is commonly assessed by markers such as ALP activity, cell migration capacity, and spheroid formation ability. In our study, Iris-exosomes were shown to improve these key functional attributes in DHT-damaged HFDPCs. Specifically, Iris-exosomes significantly enhanced cell migration and ALP activity, both of which are essential for folliculogenesis and hair growth (Figures 1 and 2). Moreover, Iris-exosome treatment promoted 3D spheroid formation, suggesting an improvement in the intrinsic trichogenic potential of HFDPCs by mimicking the natural dermal papilla microenvironment (Figure 7).

Oxidative stress and mitochondrial dysfunction are recognized as major contributors to hair loss. Excessive ROS impairs hair follicle cell function, while mitochondrial defects reduce ATP production, further compromising cellular viability. Our results demonstrated that Iris-exosomes effectively reduced ROS levels and restored mitochondrial membrane potential in DHT-treated HFDPCs (Figures 3 and 4). Additionally, ATP production, a critical energy source

for active hair growth during the anagen phase, was significantly recovered following Iris-exosome treatment (Figure 5), highlighting their role in preserving mitochondrial integrity and cellular energy metabolism.

Activation of the Wnt/β-catenin signaling pathway is essential for hair follicle regeneration, promoting cell proliferation and maintaining the anagen phase. Key upstream regulators, such as AKT and ERK, enhance β-catenin stabilization by inhibiting GSK-3β activity. In this study, Iris-exosomes increased the phosphorylation of AKT, ERK, and GSK-3β, and concurrently elevated β-catenin expression (Figure 6). These findings suggest that Iris-exosomes may stimulate Wnt/β-catenin signaling by preventing β-catenin degradation, thereby promoting hair follicle cell proliferation and regeneration.

In summary, our study demonstrates that Iris-exosomes significantly restore the functional capacity of DHT-damaged HFDPCs through multiple mechanisms, including oxidative stress reduction, mitochondrial function recovery, and Wnt/β-catenin pathway activation. These results support the potential of Iris-exosomes as a novel, plant-derived therapeutic agent for hair loss. Future studies, including in vivo evaluations and clinical trials, are warranted to further validate their efficacy and therapeutic applicability.

5. Conclusion

Exosomes derived from *Iris germanica* L. rhizomes present a promising plant-based strategy for supporting hair follicle regeneration. By promoting key cellular activities and enhancing regenerative signaling, Iris-exosomes offer potential as an alternative approach for hair loss treatment. Further research is needed to explore their clinical applications and long-term benefits.

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