Original Article

DOI: 10.5582/ddt.2024.01080

Baricitinib-loaded EVs promote alopecia areata mouse hair regrowth by reducing JAK-STAT-mediated inflammation and promoting hair follicle regeneration

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SUMMARY Alopecia areata (AA) is a common and recurrent type of hair loss. Despite oral administration of baricitinib exerts a good effect on refractory AA, the long-term administration of baricitinib carries significant side effects, poor compliance, and the efficacy is difficult to maintain after drug withdrawal. Therefore, the exploration of a safe and effective local administration of baricitinib to treat AA is of great clinical importance. However, baricitinib has a large molecular weight and is barely soluble in water, while the hair follicle lies deep, thus conventional topical dosage forms are ineffective. This study investigated the efficacy of local injection of baricitinib-loaded mesenchymal stem cell exosomes (EVs) in the treatment of AA. First, we constructed baricitinib loaded EVs (EV-B) and established AA mouse model by intravenously injection with murine INF-γ according to previous literature reports. The therapeutic effects of EV-B on hair regrowth were recorded and the underlying mechanism was also analyzed by Luminex protein biochip test and western-blot. Compared to control group, the baricitinib, EV and EV-B groups exhibited improved hair coverage in the AA mouse model. Besides, EV-B group achieved the optimal effect. The underlying mechanism might be attributed to the improvement of drug delivery efficiency as well as the synergistic effect of EVs, leading to better inhibition of JAK-STAT pathway and upregulation of the Wnt/β-catenin pathway. Our findings proved the effectiveness of EV-B on the treatment of AA, and might provide a new therapeutic approach for AA in future clinical application.

Keywords Alopecia areata, baricitinib, mesenchymal stem cell, exosomes

1. Introduction

Alopecia areata (AA) is a common inflammatory non-scarring type of hair loss that affects approximately 2% of the global population (*1,2*). The initial cause of AA remains elusive, while current research has shown that the loss of immune privilege in the hair follicle (HF) and the subsequent autoimmune response is a major precondition for the development of AA (*3,4*).

HFs are normally immune privileged sites under physiological conditions. However, in AA, this immune privilege is disrupted, leading to increased expression of major histocompatibility complex (MHC) I and II, along with the up-regulation of adhesion molecules and chemokines in anagen hair bulbs. This disruption exposes autoantigens and allows the infiltration of $CD8^+$ T cells (*1,3*). These $CD8^+$ T cells produce interferon-gamma (IFN-γ) and enhance the production of interleukin-15 (IL-15) *via* Janus kinases 1 (JAK1) and 2 (JAK2) signaling pathway. IL-15, in turn, binds to the surface of $CD8^+$ T cells and activate JAK1 and JAK3 to enhance the production of IFN-γ (*3*). The inflammation disrupts the hair cycle, inducing dystrophic anagen and premature catagen phases, ultimately leading to hair loss in AA (*3,5*).

Given the predominant role of the JAK-STAT signaling pathway in the initiation and progression of AA, Janus kinase inhibitors (JAKi) have emerged as promising drugs for the treatment of AA (*6,7*). As an effective JAK1 and JAK2 inhibitor, baricitinib is the first JAKi approved for the treatment of severe AA in

the USA and the EU (*4,8*). Despite oral baricitinib shows ideal therapeutic effects on severe AA, there are still concerns about long-term side effects such as increased infection or cancer risk (*9,10*). Besides, AA frequently recurs after the treatment cessation. Therefore, a topical formulation of baricitinib may be an ideal option for maintenance therapy (*11*). However, there is no such topical formulation of JAKi has yet been developed (*5*). Therefore, exploring an effective way for topical baricitinib administration is crucial for clinical practice.

Extracellular vesicles (EVs) are natural nanosized membrane vesicles derived from most cell types. As carriers, EVs carry and deliver various biomolecules and play an important role in cell-to-cell communication (*12*). EVs are valued for their stability, low immunogenicity, safety, and cell targeting capabilities, making them a promising drug delivery system (*13*). The approaches for EVs' loading can be divided into exogenous and endogenous loading. The endogenous loading methods include electroporation, simple incubation, sonication, extrusion and freeze-thawing (*14*). It's worth noting that EVs have been employed to deliver a variety of "cargos" such as nucleic acids, protein, metal nanoparticles as well as chemotherapeutic drugs, achieving ideal therapeutic effect (*15-18*).

In this study, we loaded the mesenchymal stem cells (MSCs)-derived EVs with baricitinib and evaluated its therapeutic effect on alopecia areata *in vivo*. The underlying mechanism was also explored. Our study may provide a theoretical basis for the topical administration of baricitinib in treating AA.

2. Materials and Methods

2.1. Cell culture

Human placentas-derived mesenchymal stem cells (dMSCs) were obtained from Shenyang Cell Center (Shenyang, China). These cells were cultured in Human Mesenchymal Cells Serum-free Culture Medium (NC0106, yocon, China). The supernatant of MSCs (passage 1-6) was collected for the subsequent experiments. Dermal papillary cells were purchased from Meissen CTCC (Hangzhou, China) and cultured in Dulbecco's Modified Eagle's Medium (DMEM) (12100, solarbio, China) supplemented with 10% fetal bovine serum (S9000, solarbio, China).

2.2. Isolation and characteristics of EVs

EVs were isolated from supernatant using a series of ultra-high-speed centrifugation procedures according to the published literatures. The morphology of EVs was observed by transmission electron microscope (TEM) (Hitachi, Japan), and the particle size was determined by nanoparticle tracking analyzer (Particle Metrix GmbH, Germany). The expression of EV positive markers CD63 (ab68418, abcam, USA), TSG101 (ab133586, abcam, USA), CD81 (ab232390, abcam, USA) and negative marker calnexin (ab133615, abcam, USA), were analyzed by Western blot.

2.3. Preparation of baricitinib-loaded EVs

Baricitinib was loaded into exosomes with electroporation. Briefly, 2E+11 particles of exosomes were co-incubated with 2 mL Gene Pulser Electroporation buffer and 1400 μg baricitinib (MedChemExpress, HY-15315) at 37℃ for 30 min. The mixture was then transferred to a cuvette and electroporated under the following conditions: 125 μF, 250 V, Max capacitance, 10 pulses with a 2 s interval. The electroporated exosomes were washed with PBS for 3 times in 100 kDa ultrafiltration tube, and then concentrated to 150 μL. This process was repeated until a sufficient amount of baricitinib-loaded EVs was collected. Baricitinib-loaded EVs were lysed with 1% Tween-20 and subjected to high-performance liquid chromatography (HPLC) testing. The procedure of HPLC are as follows. Baricitinib was dissolved to 10 mg/mL in DMSO and diluted to 1,000, 500, 250, 125, 62.5, 31.25, 15.6 μg/mL. 10 μL aliquots of each sample was injected into the HPLC system (Agilent 1260, USA). All data were acquired using a C18 column with the mobile phase H_2O: ACN (50:50, v/v) at a flow rate of 1 mL/min at 45℃. The elution of baricitinib was monitored by measuring absorbance at 254 nm, the standard curve was obtained by linear fitting with baritinib concentration as X axis and peak area as Y axis. The EV-B were transferred to 1% Tween and incubated at 4℃ for 2 hours and baricitinib was released. The solution was detected by HPLC and the concentration of baricitinib in EV-B was calculated according to the standard curve.

2.4. Uptake assay

The uptake assay was conducted to verify whether the baricitinib-loaded EVs could be endocytosed by dermal papillary cells (DPs). EVs was labeled with actin (cytoskeleton, red), and DP cells were counterstained by PKH67 (membrane, green) and DAPI (cell nucleus, blue). The internalization process was observed under confocal microscope (Leica, Germany).

2.5. Animal study

This study was approved by the Ethics Committees of the PLA General Hospital. Twenty 6-week-old female C57BL/6 mice purchased from the SPF Biotechnology (Beijing, China) were employed in this study. They were intravenously injected with murine INF-γ at a dose of 2×10^4 U (P00215, solarbio) for three consecutive days, followed by injections every 7 days (*19, 20*).

At 8.5 weeks after birth, the mice were shaved and randomly assigned into four groups. The control group was injected with 1 mL saline, the baricitinib group was injected with 1 mL of an 86.37 μg/mL baricitinib solution, the EV group was injected with 1 mL of a 2.48 × 10^{11} particles/mL EV solution, and the baricitinib + EV group was injected with 1 mL baricitinib-loaded EVs, which contained 86.37 μg baricitinib and 2.48 × 10^{11} EV particles in average. The solution was injected at 16 sites evenly within the shaved area in each group. The mice received those treatments above every day for two weeks. Photographs were taken at day 5, 10, 15, and day 20 after shaving to document the hair growth. At day 20, the mice were euthanized, and the entire dorsal skin was obtained for further histological analysis.

2.6. Western blot analysis

The skin tissue in each group was lysed by RIPA buffer (Invitrogen). Protein concentrations were measured by BCA Protein Assay Kit (PC0020, Solarbio, China). Equal amounts of heat-denatured protein samples were separated by BeyoGel™ TBE Precast PAGE Gel (D0171S, Beyotime, China) and then transferred onto PVDF membrane and blocked with 5% non-fat dried milk for 1 hour, followed by incubation with specific primary antibodies (4°C overnight), and secondary antibodies for 2 h. The specific antibodies include IFN-γ (# 8455S, Cell Signaling Technology, USA), Jak-2 (ab108596, abcam, USA), Stat-1 (ab109457, abcam, USA), IL-15 (ab52816, abcam, USA), β-catenin (ab32572, abcam, USA). The blots were finally detected by chemiluminescence.

2.7. Detection of inflammatory cytokines

Skin tissues from each group were collected, and proteins were extracted using RIPA buffer (Invitrogen). Inflammatory cytokines were detected by Luminex protein biochip testing system (Bio-Plex MAGPIX System, Bio-Rad) and a test kit (Bio-Plex Pro Mouse Cytokine Grp, #M60009RDPD), following the manufacturer's instructions.

2.8. Statistical analysis

Data are presented as the mean ± SD, and all the experiments were conducted at least three times. Statistical analysis was performed using one-way analysis of variance (ANOVA) for comparison of group means. Two-way ANOVA was employed for comparison among groups at different time points. $P < 0.05$ was considered statistically significant.

3. Results

3.1. Characteristics of EVs

The characteristics of EVs were determined by TEM, nanoparticle tracking analyzer (NTA) and Western blotting. The ultrastructure of EVs was presented in Figure 1A, which exhibited a typical cup shape and a smooth double-layer structure. Particle diameters were measured by NTA (Figure 1A). The average diameter of particles was 109.7 nm for the EV group and 120.6 nm for the EV-B group. The morphological characteristics in both groups were consistent with those described for EVs in previous studies. The positive markers of EVs including CD63, TSG101 and CD81 were detected by western blot, but the negative marker calnexin was undetectable in both groups (Figure 1B). The concentration of baricitinib was detected by HPLC. The result showed that the concentration of baricitinib is 86.37 μg/mL in the supernatant from sample of EV-B group (Figure 1C). The concentration of EV is 2.48 × 10^{11} particles/mL (Figure 1D). Uptake assay revealed that after 12 hours, the EVs in EV-B group (labeled with Phalloidin, red fluorescence) could be internalized by dermal papillary (DP) cells (the nucleus was labeled with DAPI, blue fluorescence, and cytoskeleton was labeled with PKH-67, green fluorescence) (Figure 2).

3.2. EV-B promote hair regrowth in mice

To assess hair growth in C57BL/6 mice, photographic images were captured at intervals of 5, 10, 15 and 20 days. 10 days after the treatment, the skin color of baricitinib, EV and EV-B groups shifted from pink to light gray. Besides, the EV-B group began to show some hair growth. By day 15, the baricitinib, EV and EV-B groups exhibited improved hair coverage compared to the control group. In addition, the EV-B group achieved most significant improvement among groups. By day 20, the entire depilated area on the backs of the mice in the EV-B group was covered with new hair (Figure 3). The above results showed that both baricitinib solution and EV could promote hair growth in AA mouse model, while EV-B had a more significant effect. Compared to baricitinib group, the better efficacy of EV-B may be attributed to the improvement of drug delivery efficiency as well as the synergistic effect of EVs.

3.3. EV-B exerts its role by inhibiting JAK-STAT pathway and activating Wnt/β-catenin pathway

Luminex protein biochip testing system and Western-blot were employed to illustrate the regulatory mechanism by which EV-B promotes hair regrowth. As is shown in Figure 4, compared to control group, EV-B significantly down-regulated the expression of IFN-γ and IL-2 in mice tissue. In addition, the expression levels of IFN-γ, Jak-2, Stat-1, IL-15 and β-catenin in mice tissue were also detected by Western-blot. The results indicated that EV-B down-regulated the expression of IFN-γ, Jak-2, Stat-1 and IL-15, and up-regulated the expression of β-catenin

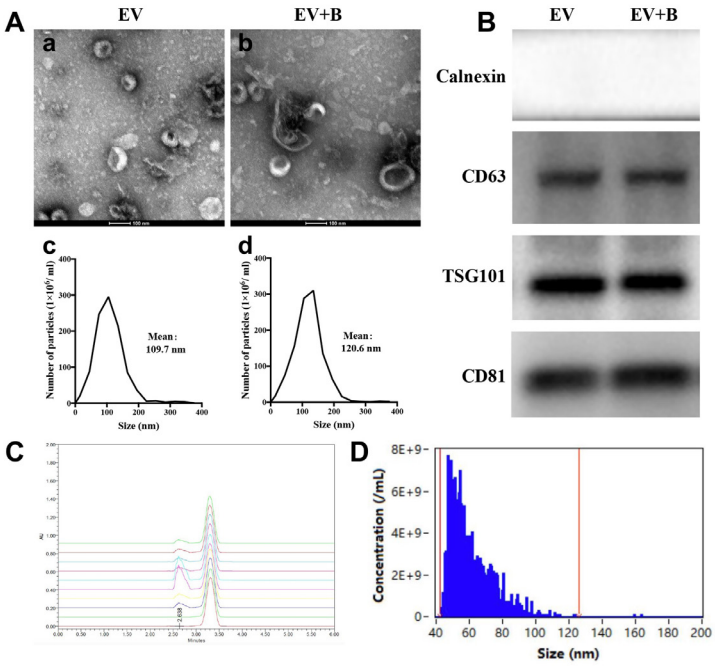

Figure 1. Characteristics of EVs. (**A**) (a-b) Ultrastructure of EVs, scale bar = 100 nm. (c-d) particle size distribution measured by nanoparticle tracking analyzer. (**B**) The expression level of exosome negative marker Calnexin and positive maker CD63, TSG101 and CD81 measured by western-blot. (**C**) The solution of EV-B was detected by HPLC, the average peak area was 466823 and the concentration of baricitinib in EV-B was 86.37 μg/mL. (**D**) The number of EV-B particles was measured by nanoflow, there is 2.48×10^{11} particles/mL in EV-B solution. These results provide the standard concentration of baricitinib and EVs in control group in the following experiments (EVs: extracellular vesicles, EV-B: baricitinib loaded EVs; HPLC: high-performance liquid chromatography).

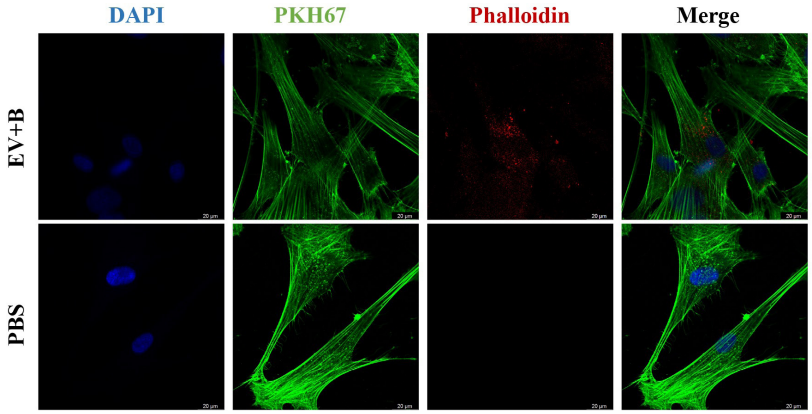

Figure 2. Uptake assay. The membrane of EVs were labeled with phalloidin (red). DPs cytoskeleton was counterstained by PKH67 (green) and nucleus was labeled by DAPI (blue). (EVs: extracellular vesicles, DPs: dermal papillary cells, PKH67: a kind of green fluorescent cell membrane dye, DAPI: a kind of blue fluorescent cell nucleus dye).

(Figure 5). Compared to baricitinib group, the expression of Jak-2, Stat-1, IL-15 in EV-B group were significantly down-regulated ($P < 0.05$), which achieved a better effect of inhibiting the inflammation. As is known, IFN-γ, Jak-2, Stat-1, IL-15, β-catenin and IL-2 are key elements of JAK-STAT pathway. β-catenin is also one of the important effector molecules of the Wnt/β-catenin pathway as well as a common marker for anagen hair follicles. These results indicate that EV-B promotes hair growth by inhibiting inflammation and promoting hair follicle regeneration (Figure 6).

4. Discussion

Alopecia areata (AA) is a common chronic autoimmune disease characterized by the loss of the immune privilege of hair follicle (*1*). Although the exact pathobiology remains elusive, the JAK-STAT signaling pathway plays a pivotal role in the initiation and development of AA (*5*). Baricitinib is a small molecule (371.41 Da) adenosine

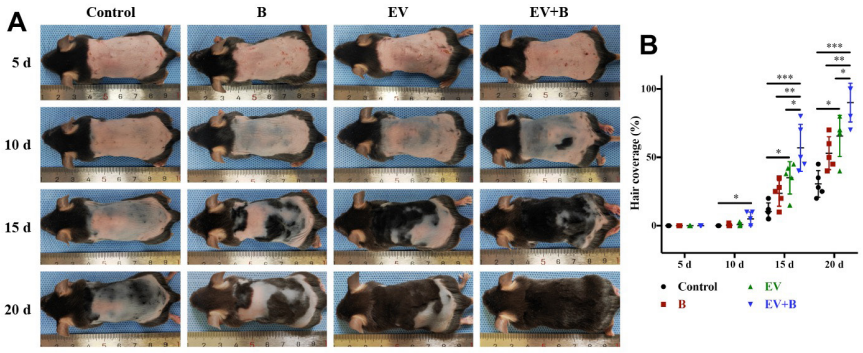

Figure 3. EV-B promotes hair regrowth *in vivo*. (**A**) Hair regrowth progression from day 5 to day 20. (**B**) The statistical analysis of hair coverage rate in each group ($n = 5$). *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

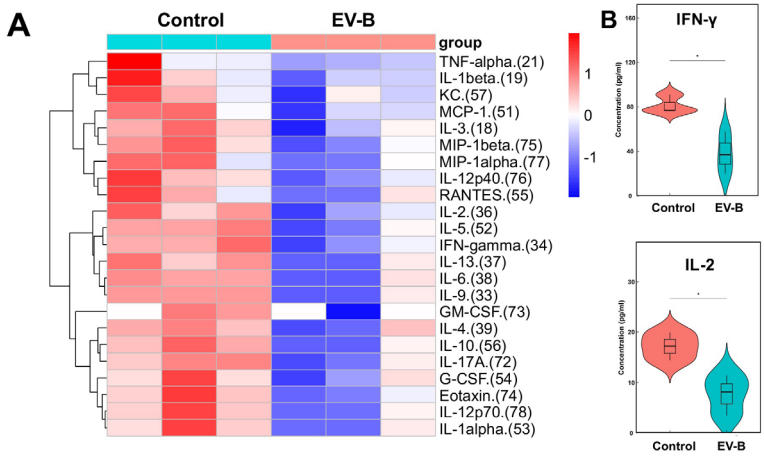

Figure 4. EV-B reduces the inflammation response. (**A**) Luminex protein biochip test between control group and EV-B group ($n = 3$). (**B**) The concentration of IFN-γ and IL-2 between control group and EV-B group ($n = 3$) *$P < 0.05$; (EV-B: baricitinib loaded EVs, IFN-γ: interferon-γ, IL-2: interleukin-2).

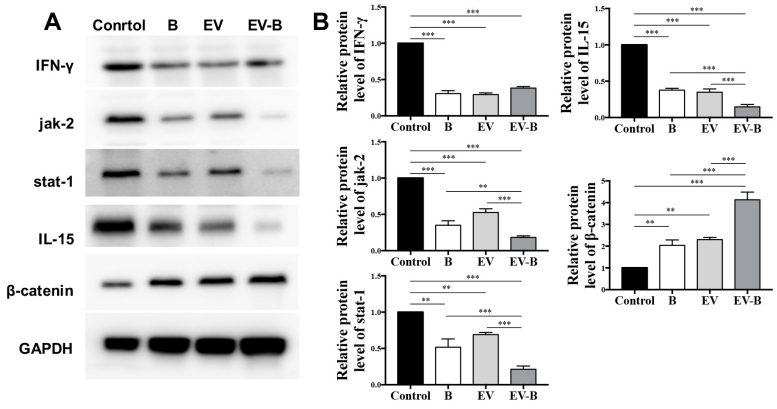

Figure 5. EV-B exert its role by up-regulating Wnt/β-catenin pathway and down-regulating JAK-STAT pathway. (**A**) The expression level of IFN-γ, jak-2, stat-1, IL-15, β-catenin in each group. (**B**) The relative protein level in each group. The result was normalized to GAPDH expression. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$. (EV-B: baricitinib loaded EVs, IFN-γ: interferon-γ, jak-2: Janus Kinase 2, stat-1: signal transducer and activator of transcription 1, IL-15: interleukin-15).

triphosphate competitive inhibitor that selectively inhibits the JAK1 and JAK2 enzymes. The *in-vitro* half maximal inhibitory concentrations (IC_{50}) for JAK1 and JAK2 are 5.9 nM and 5.7 nM, respectively (*6*). Oral baricitinib has demonstrated high efficacy in patients with moderate to severe alopecia areata and is approved for the treatment of severe AA in many countries. While systemic treatments can lead to unwanted long-term

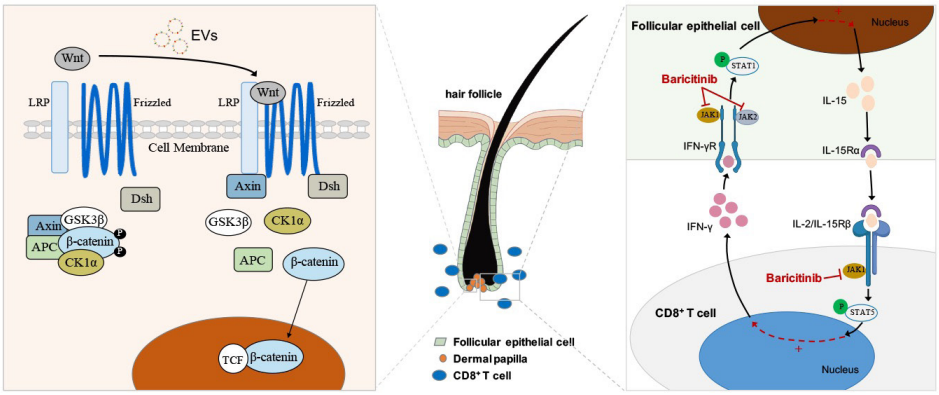

Figure 6. The therapeutic mechanism of EV-B in the treatment of AA. The therapeutic mechanism of EV-B in the treatment of AA may be the inhibition of JAK-STAT pathway and the upregulation of the Wnt/β-catenin pathway. (EV-B: baricitinib loaded EVs, AA: alopecia areata).

side effects, making topical application of baricitinib a potentially desirable treatment option for AA (*5*). Despite this, baricitinib has high molecular weight and very poorly water solubility (0.357 mg/mL to 0.46 mg/mL at 25°C) present challenges for its effective delivery as a topical treatment (*21*). Therefore, it is important to explore a proper drug carrier to improve the efficacy of topical baricitinib.

Exosomes have been investigated as delivery vesicles for various drugs. The exosome delivery system has specific advantages such as stability, a long-circulating half-life, specificity for targeting tissues, low immunogenicity and satisfactory biocompatibility. For example, Kim *et al*. (*17*) used exosome to deliver a RAGE-binding peptide for the treatment of acute lung injury. Wang *et al*. (*22*) used HEK-293T-derived exosomes loaded with adriamycin to improve the efficacy of tumor radiotherapy and chemotherapy. Zhu *et al*. (*16*) used ESC-exos as a therapeutic carrier to deliver paclitaxel and treat brain glioma.

MSCs are cell populations known for their self-renewal activity, which has broad prospects in tissue repair and regeneration. Lee *et al*. (*23*) reported that MSCs not only up-regulated the Wnt/β-catenin pathway to promote hair follicle growth, but also antagonized IFN-γ-induced hair follicle inflammation by down-regulating the JAK-STAT pathway in outer hair root sheath cells. While the safety of cell therapy remains controversial, exosome provides a major breakthrough to overcome the therapeutic limitations of MSCs. Therefore, MSC-derived EVs can promote the growth phase of hair follicles as well as inhibit inflammation. Their unique structure and properties make them an ideal drug carrier to load baricitinib.

In conclusion, we built baricitinib-loaded EVs and demonstrated that they can effectively promote hair growth in mice. The underlying mechanism may be the inhibition of JAK-STAT pathway and the upregulation of the Wnt/β-catenin pathway. Compared to baricitinib group, the better efficacy of EV-B may be attributed to the improvement of drug delivery efficiency as well as the synergistic effect of EVs. Our findings proved the effectiveness of EV-B, which could possibly be of practical use in the field of AA treatment.

Funding: This work was supported by grants from the Beijing Natural Science Foundation (L244027), Beijing Natural Science Foundation (7234388) and Youth Independent Innovation Science Fund of PLAGH (22QNFC013).

Conflict of Interest: The authors have no conflicts of interest to disclose.

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Received November 15, 2024; Revised December 7, 2024; Accepted December 8, 2024.

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Released online in J-STAGE as advance publication December 11, 2024.