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RESEARCH ARTICLE

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Adipose mesenchymal stem cell-derived exosomes promote cell proliferation, migration, and inhibit cell apoptosis via Wnt/β-catenin signaling in cutaneous wound healing

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Abstract

Cutaneous wounds, a type of soft tissue injury, are difficult to heal in aging. Differentiation, migration, proliferation, and apoptosis of skin cells are identified as key factors during wound healing processes. Mesenchymal stem cells have been documented as possible candidates for wound healing treatment because their use could augment the regenerative capacity of many tissues. However, the effects of exosomes derived from adipose-derived stem cell (ADSC-exos) on cutaneous wound healing remain to be carefully elucidated. In this present study, HaCaT cells were exposed to hydrogen peroxide (H_2O_2) for the establishment of the skin lesion model. Cell Counting Kit-8 assay, migration assay, and flow cytometry assay were conducted to detect the biological function of ADSC-exos in skin lesion model. Finally, the possible mechanism was further investigated using Western blot assay. The successful construction of the skin lesion model was confirmed by results of the enhanced cell apoptosis of HaCaT cells induced by H_2O_2, the increased Bax expression and decreased Bcl-2 expression. CD9 and CD63 expression evidenced the existence of ADSC-exos. The results of functional experiments demonstrated that ADSC-exos could prompt cell proliferation and migration of HaCaT cells, and repress cell apoptosis of HaCaT cells. In addition, the activation of Wnt/β-catenin signaling was confirmed by the enhanced expression of β-catenin at the protein level. Collectively, our findings suggest that ADSC-exos play a positive role in cutaneous wound healing possibly via Wnt/β-catenin signaling. Our study may provide new insights into the therapeutic target for cutaneous wound healing.

KEYWORDS
adipose-derived stem cell, exosomes, Wnt/β-catenin, wound healing

Ma, Fu, and Yang have contributed equally to this study.

1 | INTRODUCTION

Cutaneous wound healing is a significant factor among most soft tissue injuries and involves highly orchestrated multiple processes, including hemostasis, inflammation, proliferation, and remodeling.[1,2] Wound healing has a long healing cycle and nonhealing cutaneous wounds that can lead to severe clinical burdens without effective therapies.[3] Ineffective skin wound healing is a significant source of morbidity and mortality. Importantly, an increased risk of chronic nonhealing cutaneous wounds occurs with aging.[4] During cutaneous wounds healing processes, integration of differentiation, migration, proliferation, and apoptosis of skin cells play critical roles in skin tissue repair.[5] A healed wound is characterized by re-epithelialization associated with two basic functions of keratinocytes, namely proliferation and migration.[6] Several growth factors, such as epidermal growth factor (EGF), transforming growth factor β (TGF-β), fibroblast growth factor, and platelet-derived growth factor (PDGF), are utilized to stimulate cell proliferation, differentiation, and migration.[7] Applications of these growth factors in clinical studies have further confirmed their effect of acceleration in wound healing.[8-10] Despite the considerable progress involved in wound healing, the specific mechanism of cutaneous wound healing has not been fully understood. In addition, it is imperative to identify effective therapies for wound healing.

Mesenchymal stem cells (MSCs) are one type of multipotent stem cells with differentiation abilities and the most extensively used stem cells in the field of regenerative medicine because of their essential role in augmenting the regenerative capacity of many tissues.[11,12] A large body of studies has identified the important role of MSCs in neovascularization in ischemic tissue, including wound healing.[13,14] Exosomes, secreted from numerous types of cells, are small intraluminal vesicles of multivesicular bodies that vary from 30 to 100 nm in diameter.[15] Exosomes have received much attention recently for the fact that they are important paracrine mediators between MSCs and target cells, functioning in intercellular communication via transferring RNA and proteins to target cells.[16,17] Adipose-derived stem cells (ADSCs) are a stem cell population with multiple differentiation potentials.[18] As confirmed by in vitro and in vivo experiments and clinical studies, ADSCs promote skin wound healing mainly by two mechanisms. On the one hand, differentiation of ADSCs to target cells that participate in skin wound healing. On the other hand, differentiation of ADSCs to cytokines paracrine to promote proliferation, migration of various cell lines that are mandatory to promote skin wound healing.[19,20] Several growth factors, such as PDGF and TGF-β, could be secreted by ADSCs.[21] Notably, these growth factors are related to angiogenesis in wound healing. However, it has not been fully investigated whether exosomes released from ADSCs are implicated in wound healing.

In this present study, we isolated exosomes from ADSCs and established a skin lesion model via exposure of HaCaT cells to hydrogen peroxide (H_2O_2), and then investigated the wound healing properties of exosomes derived from ADSC-exos. Concretely, the functional roles of ADSC-exos in wound healing were examined by means of Cell Counting Kit-8 (CCK-8) assay, migration assay, and flow cytometry assay, and the potential molecular mechanism was assessed by Western blot assay. The results indicated the ADSC-exos' facilitating roles in cell proliferation and migration, and their inhibitory role in cell apoptosis. Furthermore, Wnt/β-catenin signaling was preliminarily identified as the potential mechanism of ADSC-exos in wound healing.

2 | MATERIALS AND METHODS

2.1 | Isolation and culture of ADSCs

Human facial adipose tissues were acquired from the Department Plastic Surgery of Zhongnan Hospital of Wuhan University (Wuhan, China) and then digested by collagenase type I for 2 hours. The digestion was terminated using DME/F12 complete media and the cell-debris pellet was obtained by centrifugation at 1000 rpm/min for 5 minutes. A small amount of adipose MSCs was observed after 1 week, and a large amount was observed after 1 month. Images of representative fields were visualized via a microscope (Olympus Corporation, Tokyo, Japan).

2.2 | Exosomes isolation and characterization

For exosomes isolation, exosomes were extracted from ADSCs cell supernatants without cell-debris using exosome isolation reagent (RiboBio, Guangzhou, China) in accordance with the manufacturer's guidelines. Final exosomes were obtained and stored at −80°C for use for the following study.

For exosomes characterization, the morphology of the isolated exosomes was monitored by means of transmission electron microscopy (TEM; JEOL Ltd., JEM2010-HT; Tokyo, Japan). Micrographs were used for quantifying the diameter of exosomes. Subsequently, a bicinchoninic acid (BCA) Protein Assay Kit (Beyotime Biotechnology, Shanghai, China) was applied for assessing the protein concentration of the exosomes. Finally, Western blot assay was conducted to detect the expression levels of CD63 and CD9.

2.3 | Cell culture

HaCaT cells were purchased from the China Center for Type Culture Collection (Cell Bank), College of Life Sciences at Wuhan University (Wuhan, China). Cells were cultured in Dulbecco's modified Eagle's medium (DMEM; HyClone, Logan) and 10% fetal bovine serum without exosomes (Gibco, Carlsbad, CA) and maintained in a humidified incubator at 37°C with 5% CO_2.

2.4 | CCK-8 assay

On the one hand, CCK-8 (Beyotime Biotechnology) was used to investigate whether H_2O_2 influenced cell inhibition. HaCaT cells were planted into 96-well plates (5000 cells/well) containing culture medium. Subsequently, HaCaT cells were exposed to different concentrations of H_2O_2 (200 to 2800 μM) and treated for different times (2 to 8 hours).

On the other hand, the effects of exosomes on cell viability of HaCaT cells were measured by means of CCK-8. HaCaT cells were planted into 96-well plates (2000 cells/well) containing culture medium. The ADSC-exos, H_2O_2-ADSC-exos, H_2O_2 or an equivalent volume of exosome diluent (phosphate-buffered saline [PBS]) was separately added into HaCaT cells and treated for 1, 2, and 3 days. Next, CCK-8 (10 μL/well) solution was added into each well to measure cell viability. The optical density (OD) at 450 nm wavelength was measured using the Multiskan FC (Thermo Fisher Scientific, Inc, Waltham, MA).

2.5 | Migration assay

The migratory properties of HaCaT cells were analyzed by two complementary methods. (1) For the scratch wound healing assay, HaCaT cells were seeded into plastic six-well plates at the density of 5×10^5 cells per well and cultured for 12 hours. Cells were treated with 800 μM H_2O_2 for 4 hours when they reached a confluence of 80%. After discarding the culture medium, uniform scratch wounds were scraped by a sterile pipette tip. Each well was washed with PBS, and then supplemented with basal DMEM containing ADSC-exos, H_2O_2-ADSC-exos, H_2O_2 or PBS, respectively. Images for each scratch were observed by microscope and captured at 0, 6, and 12 hours after scratching. (2) For the transwell assay, transwell chambers (8 μM, Corning Incorporated, Corning, NY) without Matrigel were applied following the manufacturer's protocol. Cells were treated with ADSC-exos, H_2O_2-ADSC-exos, H_2O_2 or PBS, respectively. Subsequently, the harvested cells were suspended in 200 μL serum-free media and were added into the upper chamber, and the lower chamber was filled with normal growth media. After 24 hours incubation, the migrated cells were fixed with 4% formaldehyde and stained with 0.1% crystal violet solution. The number of migrated cells were counted in random fields using a microscope with ×100 magnification.

2.6 | Flow cytometry assay

Cell apoptosis was evaluated using Annexin/V-FITC Apoptosis Kit (Beyotime Biotechnology) by flow cytometry based on the manufacturer's protocol. (1) To explore the effects of H_2O_2 on HaCaT cells, different concentrations of H_2O_2 (200 to 2800 μM) were added into HaCaT cells and incubated for 4 hours. (2) To explore the effects of ADSC-exos on HaCaT cells, ADSC-exos, H_2O_2-ADSC-exos, H_2O_2 or PBS were separately added into HaCaT cells and cultured for 24 hours. Cell apoptosis rate of HaCaT cells was determined with flow cytometry assay (BD Biotechnology, San Jose, CA).

2.7 | Western blot assay

Western blot assay was conducted in accordance with the standard protocols. Protein concentration was quantified with the BCA Protein Assay Kit (Beyotime, Thermo Fisher Scientific). The concentration of the inhibitor for exosomes (Dynasore; Sigma-Aldrich, Burlington, MA) used in this study was 50 μM. The primary antibodies were as follows: CD9 (Beyotime Biotechnology) at a 1:1000 dilution, CD63 (Beyotime Biotechnology) at a 1:1000 dilution, Bax (ABclonal, Wuhan, China) at a 1:1000 dilution, Bcl-2 (ABclonal) at a 1:1000 dilution, Wnt4 (ABclonal) at a 1:1000 dilution and β-catenin (ABclonal) at a 1:1000 dilution. While the corresponding secondary antibodies were GAPDH (goat anti-mouse, AS003, 1: 10000; ABclonal), and CD9, CD63, Bax, Bcl-2, Wnt4, β-catenin (goat anti-rabbit, AS014, 1: 10000; ABclonal). Signals were monitored by the enhanced chemiluminescence detection system (Millipore, Bedford, MA). GAPDH served as a loading control.

2.8 | Statistical analysis

All experiments were independently performed in triplicate. All data were presented as mean ± standard deviation and the differences among multiple groups (>2) were analyzed by one-way analysis of variance. The differences were considered to be statistically significant as a result of $P < 0.05$.

3 | RESULTS

3.1 | Construction of the skin lesion model

To establish the skin lesion model, HaCaT cells were exposed to H_2O_2. As shown in Figure 1A, the inhibition

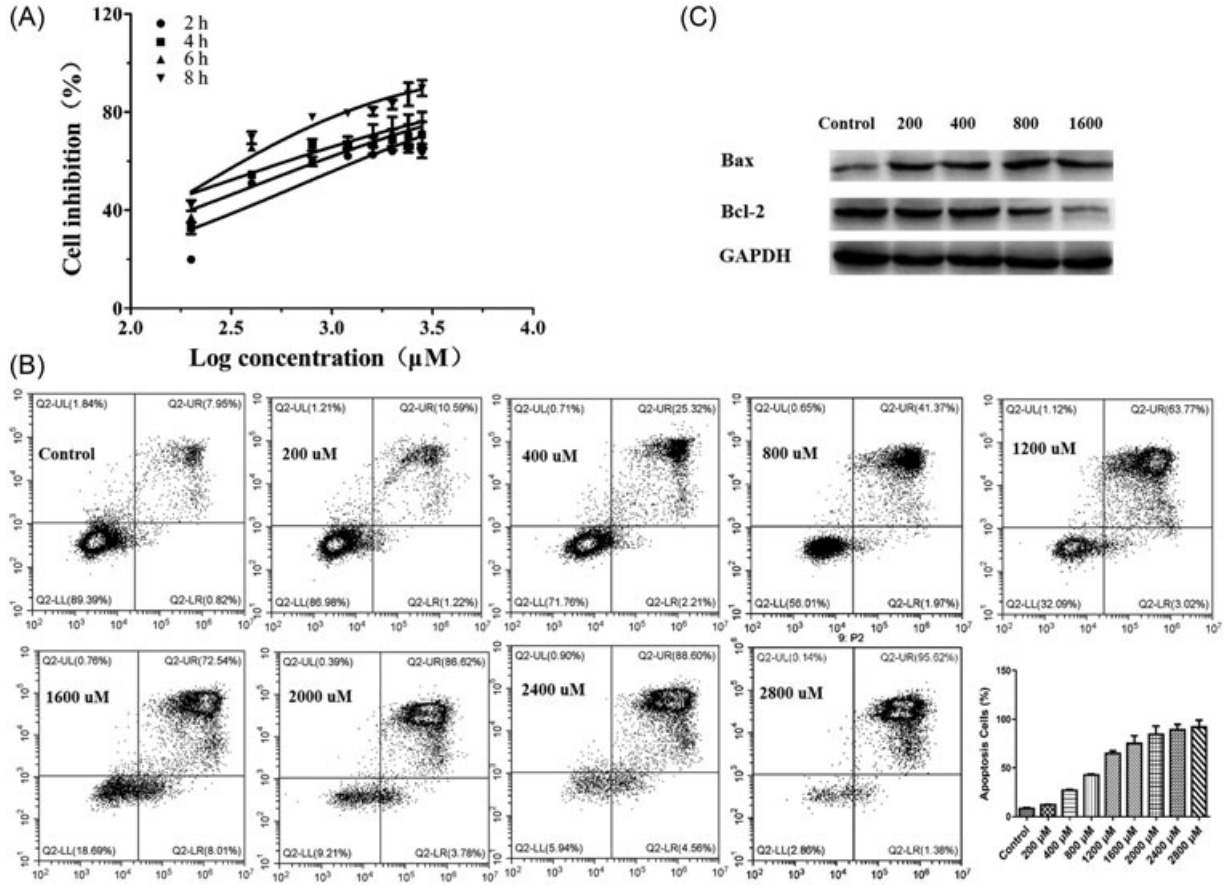

FIGURE 1 Construction of the skin lesion model. A, With the condition of increasing concentrations of H_2O_2 and extension of time, the results obtained from CCK-8 assay displayed the gradually enhanced cell inhibitions of HaCaT cells. B, With the treatment of the increasing concentrations of H_2O_2 (200 to 2800 μM) for 4 hours, the results obtained from flow cytometry assay showed that apoptosis rate of HaCaT cells was increased. C, With the improved concentration of H_2O_2 (200 to 1600 μM), the increased Bax expression and the decreased Bcl-2 expression were detected by Western blot assay. CCK-8, Cell Counting Kit-8; H_2O_2, hydrogen peroxide

rate of HaCaT cells was gradually enhanced with the increasing concentration of H_2O_2 and extension of time, suggesting that H_2O_2 could induce skin lesions. The results obtained from flow cytometry assay (Figure 1B) showed that apoptosis rate of HaCaT cells was increased in experimental group (11.81% at 200 μM, 27.53% at 400 μM, 43.34% at 800 μM, 66.79% at 1200 μM, 80.55% at 1600 μM, 92.4% at 2000 μM, 93.16% at 2400 μM, and 97.00% at 2800 μM) compared with the control group (8.77% at 0 μM). With the enhanced concentrations of H_2O_2, the increased expression of Bax and the decreased expression of Bcl-2 were detected (Figure 1C). Combining with the above results, it was confirmed that H_2O_2 could be used to construct the skin lesion model.

3.2 | Isolation and characterization of ADSC-exos

After primarily isolated and cultured for 24 hours, ADSC cells were observed via a microscope (×40 magnification) and most of the adherent cells were in spindle-like shape during cell culture (Figure 2A). Exosomes purified from ADSCs culture supernatants were characterized by TEM, and the results showed that exosomes were round membrane-bound vesicles with 30 to 100 nm diameter (Figure 2B). The protein concentration was determined as 0.2 μg/μL using BCA Protein Assay Kit. As demonstrated in Western blot assay, CD9 and CD63, namely the exosomes marker protein, were presented in the exosomes as expected (Figure 2C).

3.3 | ADSC-exos promote cell proliferation, migration, and inhibit cell apoptosis of HaCaT cells impaired by H_2O_2

To investigate the functional roles of exosomes isolated from ADSCs, cell proliferation, migration, and apoptosis assay were conducted. Compared with the PBS group, the OD at 450 nm was increased in ADSC-exos group. Similarly, the OD at 450 nm was increased in H_2O_2-ADSC-exos group in comparison with the H_2O_2 group. Notably, ADSC-exos could more efficiently influence

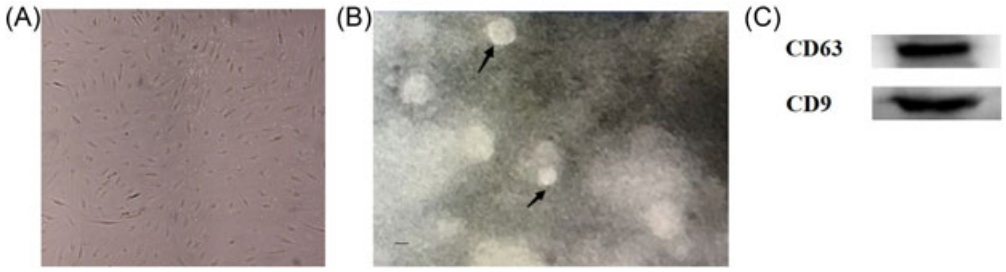

FIGURE 2 Isolation and characterization of ADSC-exos. A, Spindle ADSCs were observed through the microscope. ×40 magnification. B, TEM photomicrographs of exosomes. Scale bar = 50 nm. C, The expression levels of CD9 and CD63 in exosomes were examined by Western blot assay. ADSCs, adipose-derived stem cells; ADSC-exos, exosomes derived from an adipose-derived stem cell; TEM, transmission electron microscopy

the proliferation of HaCaT cells impaired by H_2O_2 (Figure 3A; $^*P < 0.05$, $^{**}P < 0.01$). The results of scratch wound healing assay displayed that the migratory ability of HaCaT cells cultured with ADSC-exos was enhanced both in the presence or absence of H_2O_2, but the effects of ADSC-exos on the migration of HaCaT cells impaired by H_2O_2 were more remarkable (Figure 3B; $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$). Similar results were obtained from transwell assay (Figure 3C; $^{**}P < 0.01$, $^{***}P < 0.001$). Flow cytometry assay showed the evidently inhibited apoptosis of HaCaT cells in ADSC-exos and H_2O_2-ADSC-exos group (Figure 3D). As shown in Figure 3E, Bax was markedly elevated and Bcl-2 was markedly reduced at the protein levels in the ADSC-exos group compared with the PBS group ($^*P < 0.05$). In addition, ADSC-exos significantly inhibited the increment of Bax ($^{***}P < 0.05$) and the reduction of Bcl-2 ($^{**}P < 0.05$) caused by H_2O_2. However, Dynasore partially decreased the effect of ADSC-exos on the Bax and Bcl-2 protein levels ($^*P < 0.05$). Taken together, these results indicated that ADSC-exos play a promoting role in cell proliferation and migration and play an inhibitory role in cell apoptosis.

3.4 | ADSC-exos activates Wnt/β-catenin signaling to prompt wound healing

It has been reported that Wnt/β-catenin signaling is related with wound healing,[22] and therefore we performed

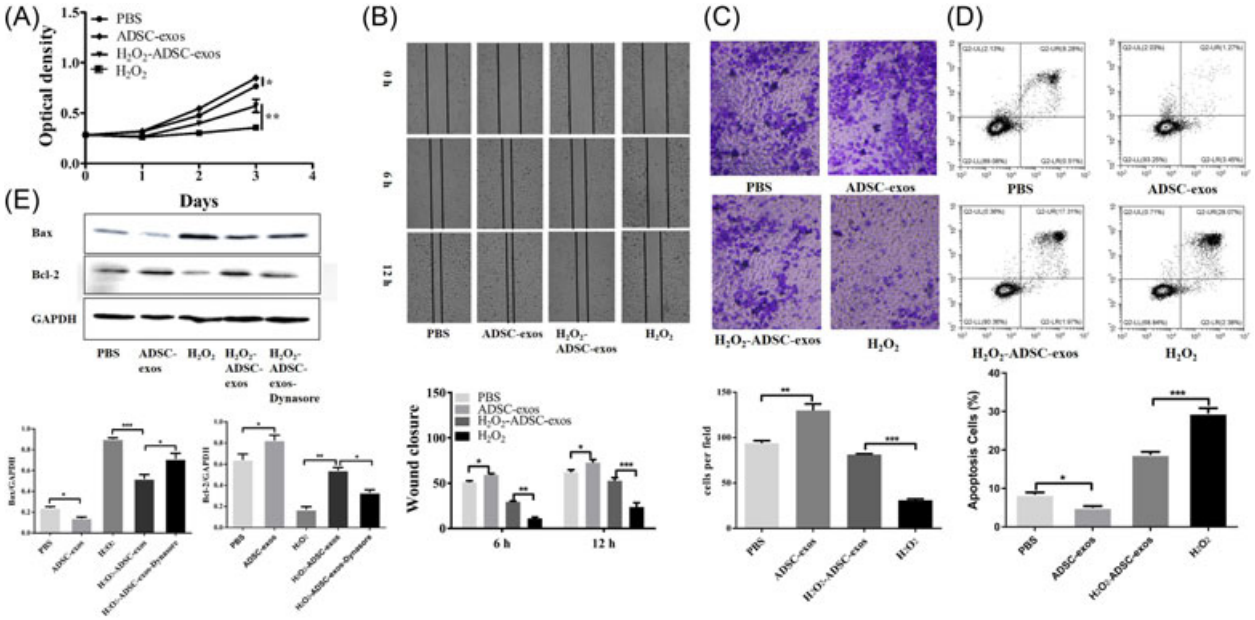

FIGURE 3 ADSC-exos promoted proliferation, migration, and inhibited apoptosis of HaCaT cells impaired by H_2O_2. A, Proliferation of HaCaT cells treated with ADSC-exos, H_2O_2-ADSC-exos, H_2O_2 or PBS was evaluated by CCK-8 assay. (B,C) Migration of HaCaT cells treated with ADSC-exos, H_2O_2-ADSC-exos, H_2O_2 or PBS was analyzed by the scratch wound healing assay and transwell assay. D, Apoptosis of HaCaT cells treated with ADSC-exos, H_2O_2-ADSC-exos, H_2O_2 or PBS was monitored by flow cytometry assay. E, Bax and Bcl-2 protein levels in HaCaT cells treated with PBS, ADSC-exos, H_2O_2, H_2O_2-ADSC-exos or H_2O_2-ADSC-exos-Dynasore was evaluated by Western blot assay $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$. ADSC, adipose-derived stem cell; ADSC-exos, exosomes derived from adipose-derived stem cell; CCK-8, Cell Counting Kit-8; H_2O_2, hydrogen peroxide; PBS, phosphate-buffered saline

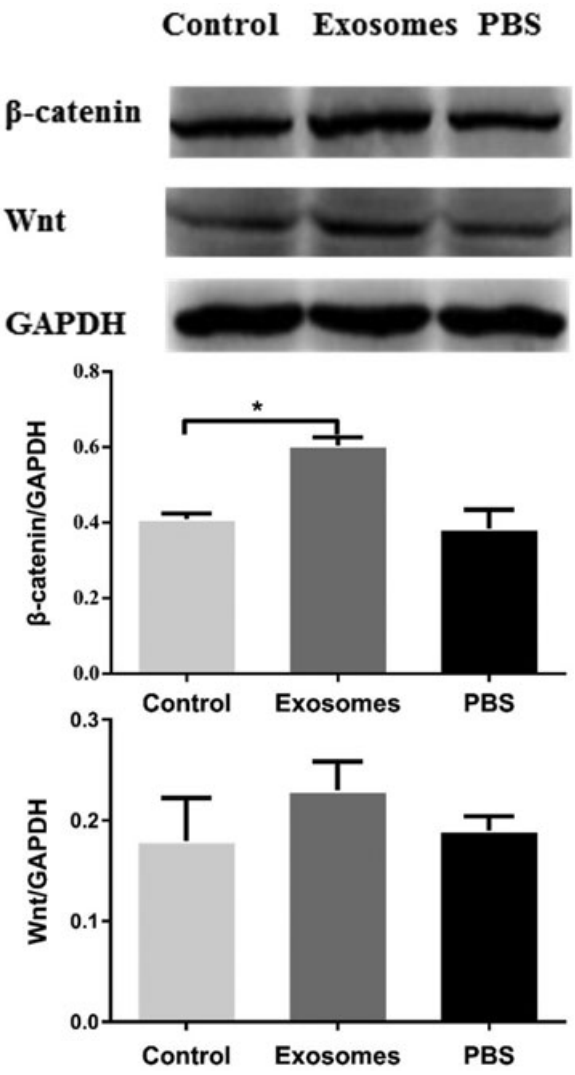

FIGURE 4 Western blot assay for the expression levels of Wnt and β-catenin in HaCaT cells. Compared with the PBS group, β-catenin expression was increased in exosomes group. $^*P < 0.05$. PBS, phosphate-buffered saline

Western blot assay for the purpose of investigation of the potential molecular mechanism of ADSC-exos in wound healing. We found that β-catenin expression was noticeably enhanced in exosomes group compared with PBS group (Figure 4; $^*P < 0.05$).

4 | DISCUSSION

Exosomes are released from cells and implicated in many biological and pathological process.[23] A novel role of exosomes as a cell-cell communicator has been identified in many research fields due to its capacity of carrying messenger RNA, microRNA, and proteins, which could be transferred into target cells.[24] The facilitating role of exosomes in the proliferation, migration, and angiogenesis process has been demonstrated.[25] The beneficial role of exosomes secreted from MSCs in wound healing has been found via activating Wnt/β-catenin signaling.[26] Remarkable progress has been made in the application of ADSCs in skin wound healing.[27] For example, it has been reported that application of ADSCs could improve ischemia-reperfusion (I/R) injury of skin flaps via interleukin-6 expression.[21] Bax is identified as a proapoptotic protein and Bcl-2 is identified as an antiapoptotic protein.[28] However, little is known regarding the roles of ADSC-exos in wound healing. Thus, we constructed the skin lesion model by exposing HaCaT cells to H_2O_2, aiming to investigate the biological function of ADSC-exos in wound healing. The obtained findings of the accelerated cell apoptosis, the increased expression of Bax and the decreased expression of Bcl-2 both confirmed the validity of the model.

The creation of the multilayered tissue for skin reparation is closely related to the integration of the differentiation, migration, proliferation, and apoptosis of skin cells.[5] Bai Y et al[29] demonstrate that ADSC-exos could promote neovascularization and alleviate inflammatory reactions and apoptosis in the skin flap in I/R injury. In this study, the existence of exosomes derived from ADSC was evidenced by CD9 and CD63 expression. Interestingly, we found that ADSC-exos promoted proliferation and migration, and inhibited apoptosis of HaCaT cells after exposure to H_2O_2, indicating the active role of ADSC-exos in wound healing.

Accumulating evidence has confirmed that the activation of Wnt/β-catenin signaling pathway plays an important role in the proliferative phase of wound healing.[22] The specific role of Wnt in the regulation of β-catenin has also been found. β-Catenin, a subunit of the cadherin protein complex, exerts as an integral component of the canonical Wnt signaling pathway.[30] Exosomes are an emerging platform and a critical factor in facilitating Wnt secretion and transport.[28] In this study, the elevated expression of β-catenin indicated that Wnt/β-catenin signaling may be implicated in the underlying mechanism of ADSC-exos in wound healing.

Taken together, these results suggested the positive role of ADSC-exos though Wnt/β-catenin signaling in cutaneous wound healing. However, more studies should be carried out in the aspect of the association between Wnt/β-catenin signaling and cutaneous wound healing, and we would like to further investigate it in our future research.

5 | CONCLUSIONS

In summary, this study may give a new insight that ADSC-exos serve as a significant factor in cutaneous

wound healing via mediating Wnt/β-catenin signaling. Future research should pay attention to the specific mechanism of ADSC-exos in cutaneous wound healing.

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CONFLICTS OF INTEREST

The authors declare that there are no conflicts of interest.

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