

# hucMSC Exosome Ameliorates Pressure Ulcers Through Inhibition of HMGB1.

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## Research Article

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# Abstract

**Background:** Pressure ulcers (PU) are a chronic wound for elderly populations. Previous works have shown that exosomes from stem cells contain cytokines and growth factors that play a role in tissue repair and can represent a therapeutic strategy for wound healing. Thus, as a new cell-free treatment model, fully understanding the extraction of exosomes and its mechanism of action can help promote the management of clinically chronic refractory wound healing.

**Material and Methods:** In this study, we initially isolated exosomes from human umbilical cord mesenchymal stem cells (hucMSC-Exo) and examined their roles in wound healing. Different time points were evaluated for 15 mice which were randomly divided into three groups to serve three I-R circles and took different dose of hucMSC-Exo. Quantitative real-time polymerase chain reaction (qRT-PCR) was applied to analyze collagen mRNA levels in tissue samples. HMGB1 content was explored by western blot and immunohistochemistry. Comparing  $\alpha$ -SAM, CD34, HMGB1 were used to investigate the potential mechanisms.

**Results:** We found that hucMSC-Exo could be taken up by fibroblasts and significantly regulate and improve fibroblast fibrotic status and in-vivo PU wound healing. Further, we observed that hucMSC-Exo treatment of PU wound was able to downregulate the expression of HMGB1 previously shown to have a deleterious role in the wound healing process.

**Conclusion:** Our study indicates that hucMSC-Exo regulates the repair of pressure ulcer wounds in part by inhibiting HMGB1. Exosome treatment has opened up a new perspective in regenerative medicine and trauma management.

## 1. Background

Pressure ulcers (PU) are local tissue injuries caused by long-term pressure on the skin and/or underlying tissues of bony protrusion body areas, blood circulation disorders, continuous ischemic/hypoxia, and malnutrition. Pressure or shear damaging the integrity of epidermis, dermis, adipose tissue, muscle and bone can lead to ischemia-reperfusion injury (I-R) and represent chronic refractory ulcer of skin [1–2]. Repeated I-R cycle has been shown to be more deleterious to skin tissue than simple long-term ischemia, and the reperfusion period impacts the tissue damages [3]. During repeated I-R events the leukocyte infiltration, oxidative stress, infection, inflammation, create an imbalance between pro- and anti-inflammatory factors leading to the reduction of tissue perfusion and necrosis [4]. The repetitive I-R events create unfavorable conditions for growth factor delivery necessary for the repair and homeostasis of damaged tissues [5]. PU afflict particularly elderly populations and the treatment of PU remains a challenge, suggesting a real need of new therapy development. Recent studies have shown the important implication of paracrine signal from tissue stem cells for promoting wound healing and damaged tissue regeneration [6]. Exosome are secreted vesicles (50-150nm) containing proteins, lipids or nucleic acid that can deliver paracrine signals between cells and may represent a therapeutic opportunity for tissue repair

and regeneration. Interestingly, exosomes derived from human umbilical cord mesenchymal stem cells (hucMSCs) have emerged as an accepted source and feasible delivery system of a large number of cytokines and growth factors [6] and can affect tissue response to injury promoting wound healing [7]. The high biodegradability of exosomes aside from their lower toxicity, immunogenicity and protection of their internalized cargo, is of high interest for usage of exosomes as therapeutic molecule delivery system [8–10].

Our previous work has demonstrated that exosomes derived from hucMSCs decreased the number of inflammatory cells and the level of High Mobility Group Box 1 (HMGB1) [11] which activates the release of pro-inflammatory cytokines in wounds of pressure ulcers. To our knowledge, this study was the first to investigate the effect of hucMSCs-derived exosomes on PU microenvironment and levels of HMGB1. However, the mechanisms underlying the therapeutic effects of exosome in excessive inflammation of PU are unclear. The immunomodulatory effects of HMGB1 have been linked to tissue repair and fibrosis [12], chronic inflammation and increase I-R severity [13–15]. Thus the effect of hucMSCs-derived exosomes on HMGB1 levels could be of interest for improvement of PU wound healing. In this study, we evaluate the effect of hucMSCs derived exosomes on PU wound healing *in-vitro* and *in-vivo* and the potential mechanism of HMGB1 regulation by hucMSCs-derived exosomes treatment. We show that hucMSCs derived exosomes can be taken up by fibroblasts and changed their activation status and led to increased wound healing *in vivo* while decreasing expression of HMGB1. The result presented in this study reinforce the interest of hucMSCs derived exosomes usage for PU wound healing treatment and indicate HMGB1 as a candidate target.

## 2. Materials And Methods

### 2.1 Animals:

Fifteen 6-week-old, male, BALB/c mice were purchased from the Guangdong Medical Laboratory Animal Center, All experimental and animal care procedures were approved by the Animal Care and Use Committee of Shenzhen people's hospital. Animals were randomly divided into three groups to serve three I-R circles (Fig. 2A). Three time points, 1, 7 and 14 days, were evaluated for a total of 15 mice for the entire study. Before all procedures, the mice were allowed to acclimatize to their environment for 1 week.

### 2.2 Mice skin wound model and treatment

The mice were anesthetized with isoflurane inhalation (1.8%). To create the pressure ulcers wound, the back dorsal of the mice was shaved and cleaned with 75% alcohol subsequently pinched between two circular magnets (Dongguan JinKun Magnet products, CO) that were 10 mm in diameter, 5 mm thick, and strength of 4000 G. The full-thickness skin was pulled up and placed between a pair of the magnet disks. The dorsal skin of mice was exposed to a 12h magnetic pressure and 12h rest (IR cycle). A total of 3 rounds of IR cycles were performed. The compressed area was left uncovered for the rest of the procedure after PU wound induction protocol.

Mice were randomly assigned to 3 different treatment groups and were left untreated (Control) or injected with either 200µL of PBS or 200ug of purified hucMSC exosomes (huCMSC-Exo) re-suspended in 200ul PBS around the wounds at 4 injection sites. All wounds were studied by histopathological analysis at everyday post-wounding. The wound-size reduction was calculated using the equation: wound-size reduction (%) =  $(AO - At)/AO \times 100$ , where AO is the initial wound area, and At is the wound area at everyday post-wounding. On days 1, 2, and 3 after injection of these DIR-labeled exosomes into the wounds, the fluorescence was measured every 12 hours by bioluminescence imaging.

## 2.3 Cell Culture

Human umbilical cords mesenchymal stem cells were purchased from Cyagen (stock number: HUXUC-01001 batch number: 160117131). MSCs were cultured immediately upon receipt using Cyagen's umbilical cord mesenchymal stem cell culture medium (Cyagen, Basal Medium, For Human: Umbilical Cord MSCs). Primary culture of MSCs and dermal fibroblasts were established using standard procedures. MSCs and fibroblasts were cultured in an incubator at 37 ° C, 5% CO<sub>2</sub>. MSCs were sub-cultured at a ratio of 1:3 every 2 or 3 days until passage 5. Medium was then replaced by TM-ACF PLUS Medium Human (MesenCult) supplemented with cell growth supplement (MesenCult TM-ACF PLUS 500X Supplement). MSCs were passaged at a ratio of 1:2 in T175 flasks, and the culture supernatant was collected for exosome purification when the culture confluency reached 90%.

## 2.4 Exosome purification and identification

At the end of the incubation period, the media were collected and centrifuged at 400× g for 10 minutes, 2000× g for 10 minutes, and 10000× g for 30 minutes to remove cell fragments and protein aggregates. Then the supernatant was centrifuged at 100000× g for 120 minutes to pellet the exosomes. In order to purify the exosome, the supernatant was discarded and the exosome pellet was washed with ice-cold PBS followed by centrifugation at 100000× g for 120 minutes. The washed exosome pellet was re-suspended with PBS and stored at -80 ° C, or directly added to sample loading buffer for purity analysis by Western Blotting. The exosomes were measured for their protein content using the BCA protein assay kit (Pierce Protein Biology; Thermo Fisher Scientific Life Sciences). Exosome purification was confirmed by western blotting detection of exosomal surface markers CD9/CD63/CD81. The shape and size of the exosomes were analyzed by transmission electron microscope (Tecnai G2 Spirit Biotwin with transmission electron microscopy).

## 2.5 *In vitro* exosome internalization and effects on fibroblast.

To examine the effect of hucMSC-Exo on fibrosis *in vitro*, primary fibroblast were treated with purified and DIR labelled hucMSC-Exo (100ul, 1mg/ml) for 12 h. Human fibroblasts and DIR labelled huCMSCs-exo were seeded on coverslips in 96-well plates at 37°C, 5% CO<sub>2</sub> in a humidified atmosphere and washed three times subsequently before counterstaining with Hoechst nuclear stain (Hoechst 33342, Germany) for immunofluorescence microscopy imaging. To analyze the effect of DIR labelled hucMSC-Exo on

fibroblast activation, fibroblasts were seeded into 24-well plates ( $5 \times 10^5$  cells/well) in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10 ng/ml fibroblast growth factor 2, 20% heat-inactivated fetal calf serum (FCS), 2 mM L-glutamine, 100 U/ml penicillin and 0.1 mg/ml streptomycin. Fibroblasts were cultured for 3 days either in presence of DIR labelled hucMSC-Exo (0.5mg/ml), PBS, or left untreated (control). Treated fibroblast were collected for mRNA expression analysis by RT-qPCR

## 2.6 Histology

Cutaneous wound bed tissues were dissected at 14 days and fixed in 4 % phosphate-buffered formalin (pH 7.4), embedded in paraffin, sectioned at 5 $\mu$ m, and mounted on glass slides. The sections were stained with hematoxylin and eosin (H&E) using standard procedures and observed under a light microscope (Olympus BX53)

## 2.7 Assessment of collagen

Masson's trichrome staining, which differentially stains collagen blue, was used to evaluate the wound bed for collagen deposition and to assess healing). The mRNA levels of *Type I, III collagen* and *TGF- $\beta$*  were examined by RT-qPCR. Expression levels were normalized to the housekeeping gene *GAPDH* and translated to relative values. The primers sequence used in this study are : Type I collagen: forward, 5'-TGACCGATGGATTCCCGTTC - 3', reverse, 5'- GCAGGGCCTTCTTGAGGTTG - 3', Type III collagen: forward, 5'- AAGCACTGGTGGACAGATTC-3', reverse, 5'- CGGCTGGAAAGAAGTCTGAG - 3', GAPDH: forward, 5'-AGCTTGTCATC AACGGGAAG - 3', reverse, 5'- TTTGATGTTAGTGGGGTCTCG - 3', TGF- $\beta$ : forward, 5'-ACAACCCACACCTGATCCTC-3', reverse, 5'-GTTCGTGGACCC ATTTCCAG-3'.

## 2.8 Immunohistochemistry

To evaluate inflammation and angiogenesis, biotinylated-antibodies against alpha-smooth muscle actin ( $\alpha$ -SAM) (Abcam), endothelial marker CD34 (Abcam) and HMGB1 (R&D Systems) were used to label PU wound sections. To assess the number of vessels, the entire area of the wound on the slide was observed under a microscope and the number of vessels was counted in each of the four quadrants of the wound and the average was calculated. Scoring of staining was done blindly by 4 independent experimenters.

## 2.9 HMGB1 content measurement

*HMGB1* were examined by RT-qPCR using the primer set: forward, 5'-CTTCCTCATCCTCTTCATCC-3', and reverse, 5'-GCCCTATGAGAA GAA AGC TG-3', and *GAPDH* as an internal standard. For Western blot analysis, the proteins from skin wound tissue were isolated using RIPA lysis buffer. Protein concentrations were measured with the BCA protein assay kit. Anti-HMGB1 (Sigma-Aldrich) antibody was used for western blot analysis. The blots were developed with Western Lightning Plus ECL.

## 2.10 Statistical Analysis

Group data were expressed as the mean and SD. Data were analyzed using GraphPad Prism version 6 (GraphPad Software). Statistical differences were determined by Student's *t*-test or analysis of variance, with *p*-values < 0.05 considered statistically significant.

## 3. Results

### 3.1 Isolation and characterization of hucMSC-Exo

To study the roles of exosomes in pressure ulcers, hucMSC-Exo were isolated and characterized (Fig. 1). Transmission electron microscopy (TEM) and nanoparticle tracking analysis (NTA) showed that exosomes purified from the hucMSCs culture supernatants were round membrane-bound vesicles with a size ranging from 30–150 nm in diameter (Fig. 1A and 1C). Western blotting analysis showed enrichment of the exosomal markers CD9, CD63, and CD81 in the hucMSC-Exo compare to hucMSC cell line extract (Fig. 1B). Figure 1D shows the exosome image tracked by NTA in real-time.

### 3.2 hucMSC-Exo promote cutaneous wound healing in mice

The experimental flow chart of our BALB/C wound healing model and representative wound pictures are shown in Fig. 2A and 2B. We showed that skin wound shrinkage rate was higher when wounds were treated by hucMSC-Exo in comparison of PBS or untreated wounds (Fig. 2C). The increased wound healing rate was observed from 7 days post wound treatment induction. Thus treatment of mouse PU wounds with hucMSC-Exo is able to improve wound healing *in-vivo*.

### 3.3 In vivo bioluminescence imaging

In order to evaluate if exosomes were taken up and localized in the wound after treatment, mice with induced PU wounds were treated with DIR-labeled hucMSC-Exo. As shown in Fig. 3, the signal that was detected immediately after hucMSC-Exo treatment remained detectable at least 48hr post-treatment, indicating that hucMSC-Exo can be taken up by cells present in the wounds and might deliver a continuous effect.

### 3.4 Effects of exosome treatment on scar formation

Reducing scar width, changes in collagen content, and tissue re-epithelialization are commonly used to assess wound healing. To evaluate the effect of hucMSC-Exo on PU wound healing, we analyzed trichrome stained tissue sections of wounds 14 days after exposure to hucMSC-Exo, PBS or untreated (Fig. 4A). The wound re-epithelialization analysis shows that the hucMSC-Exo group had smaller wound scar areas, regular epidermal and dermal structures. The dermal layer is densely packed with collagen fibers and the epidermis is arranged in parallel. No appendages such as sebaceous glands, sweat glands and hair follicles were observed in three groups (Fig. 4A). The collagen fibers in the hucMSC-Exo group are arranged neatly and densely, while the collagen fibers in the PBS group are disorganized (Fig. 4B). Those results confirm the beneficial effects of hucMSC-Exo treatment on PU wound healing and skin tissue proper regeneration.

### 3.5 hucMSC-Exo effect on wound fibrosis

As fibroblasts play a crucial role in wound healing and fibrosis, we evaluated if hucMSC-Exo can be taken and affect fibroblast activation. By tracking the fate of DiR-labeled exosomes in the fibroblast cultures, we observed the presence of numerous labeled particles inside fibroblasts. Internalized exosomes were mainly localized to the perinuclear zone (Fig. 5A). The expression of  $\alpha$ -SMA and CD34, indications of fibroblast activation and vasculature respectively, were evaluated by immunohistochemistry (Fig. 5B) *in vivo* and RT-qPCR (Fig. 5C) *in vitro* after treatment of either hucMSC-Exo, PBS or untreated. As expected,  $\alpha$ -SMA was significantly downregulated in the exosome-treated PU wounds. Expression of CD34 and vasculature structure were increased in wounds treated with hucMSC-Exo compared to PBS treated or untreated wounds. Taken together, our observations suggest that hucMSC-Exo can be internalized by fibroblasts and can accelerate PU wound healing and tissue regeneration in part by impacting fibrosis and activation of fibroblasts.

### 3.6 HMGB-1 localization in wounds

To assess the fibrosis and inflammation status in relation with HMGB1, collagen I (Col I), Col III, TGF- $\beta$ , and HMGB1 expressions were examined in wounds. We observed a significant decrease in *Col I*, *Col III* and *TGF- $\beta$*  mRNA expression after treatment by hucMSC-Exo (Fig. 6A). Interestingly, the expression of HMGB1 previously linked to bad prognostic of PU wound healing was also decreased in PU wounds after hucMSC-Exo treatment at the mRNA and protein levels (Fig. 6A-C). We also confirmed by western blot the decrease level of  $\alpha$ -SMA in hucMSC-Exo treated wound tissue (Fig. 6B). Thus, the results of this proof-of-concept study suggest that the use of hucMSC-derived exosomes for the treatment of pressure ulcers may be an interesting and feasible approach. The effect of hucMSC-Exo treatment on HMGB1 is of particular interest and need to be further investigated.

## 4. Discussion

Pressure ulcers are a skin lesion that has been identified as the primary cause of ischemia-reperfusion (I/R), an anti-inflammatory phase characterized by severe defects in cell-mediated immunity [16]. Many cellular elements and complex molecular interactions are involved in this inflammatory reaction. Exosomes derived from hucMSCs can communicate through its contents which contain various bioactive molecules including mRNAs, microRNAs [16, 17]. Exosomes have shown an important effect on wound repair by paracrine mode. Exosome can promote cell migration and metastasis and reduce negative effects in the processes of wound healing inflammation [18]. In the present study, we successfully isolated and purified exosomes from hucMSCs. Besides demonstrating that DiR-labeled exosomes could be internalized to the cytoplasm of fibroblast, we further showed that hucMSC-Exo decreased  $\alpha$ -SMA expression *in vitro* and *in vivo*. These results indicate that hucMSC-Exo can directly act on fibroblast and modulate its fibrotic activity.

HMGB1 is a chromatin-bound nuclear protein that can also act as cytokines under stress conditions or cell death [11]. Extracellular HMGB1 is a key factor in the induction of sterile immunity and activates the inflammatory pathway released by ischemic cells. HMGB1 is released in large quantities outside the cell

and plays a cytokine-like function during the injury process [19, 20]. HMGB1 has also been implicated as a factor stimulating fibrosis and scar formation [21–23] and inhibition of HMGB1 inhibits fibroblast migration and collagen synthesis which can sharply reduce the scar. In the present study, we found that the expression levels of the HMGB1 decreased in PU wound upon hucMSC-Exo treatment in parallel with decreased expression of the fibrosis markers Col I, Col II and TGF-beta. Based on the previous works showing the deleterious implication of HMGB1 in wound healing and chronic inflammation and our present results, we suggest that downregulation of HMGB1 by hucMSC-Exo treatment can have a direct implication in accelerating PU wound healing. How hucMSC-Exo impact HGMB1 remains to be studied. As exosome have been shown to transport microRNA, direct inhibition by RNA interference is one possible mechanism. Previous work has shown presence of miRNA221 in hucMSC-Exo [24]. Interestingly, miRNA221 might be involved in regulation of FAK pathway [25] which is involved in HGMB1exspression control. Thus the study of the implication of miRNA221 in regulation of HGMB1 expression and PU wound healing is of high interest and can represent a potential therapeutic strategy. The positive effect of hucMSC-Exo on decreasing fibrosis markers expressions was confirmed *in vivo* in our PU wound models. Treatment of PU wounds in mice with hucMSC-Exo accelerates wound shrinkage and tissue organization during the wound healing process. Remarkably, organization of collagen and re-vascularization of repaired tissue were expedited after hucMSC-Exo treatment. Thus hucMSC-Exo treatment appears to act first on improvement of fibrosis which can lead to better tissue regeneration. It is also possible that hucMSC-Exo treatment will directly assert positive impact on tissue re-epithelialization which remain to be investigated in our model.

## 5. Conclusions

In this study, we firstly confirm that the exosome can promote the healing of pressure ulcers by inhibiting the secretion of HMGB1 and scar hyperplasia while promoting tissue re-epithelialization and vascularization. Compared with stem cell therapy, exosomes have unique advantages in alleviating many safety problems and it is becoming a promising strategy for the treatment of pressure ulcers.

## Declarations

**Author's contribution** All the authors have substantial contributions to the conception or design of the work, revising and drafting the work.

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**Data availability** All data and materials of the work are available behind the corresponding author.

## Declarations

**Conflict of interest** The authors declare that they have no conflict of interest.

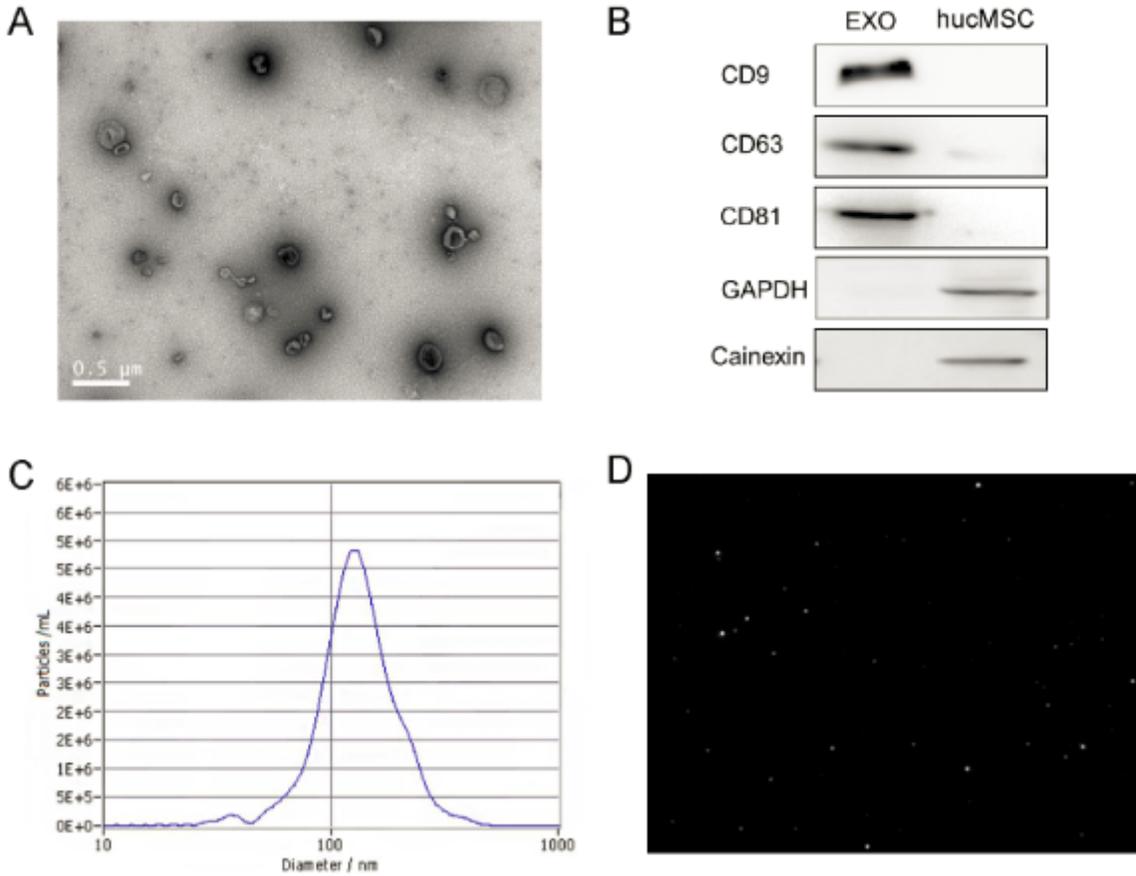
**Ethical approval** This work was approved by ethical committee of the Medical Ethical Committee of Shenzhen People's Hospital.

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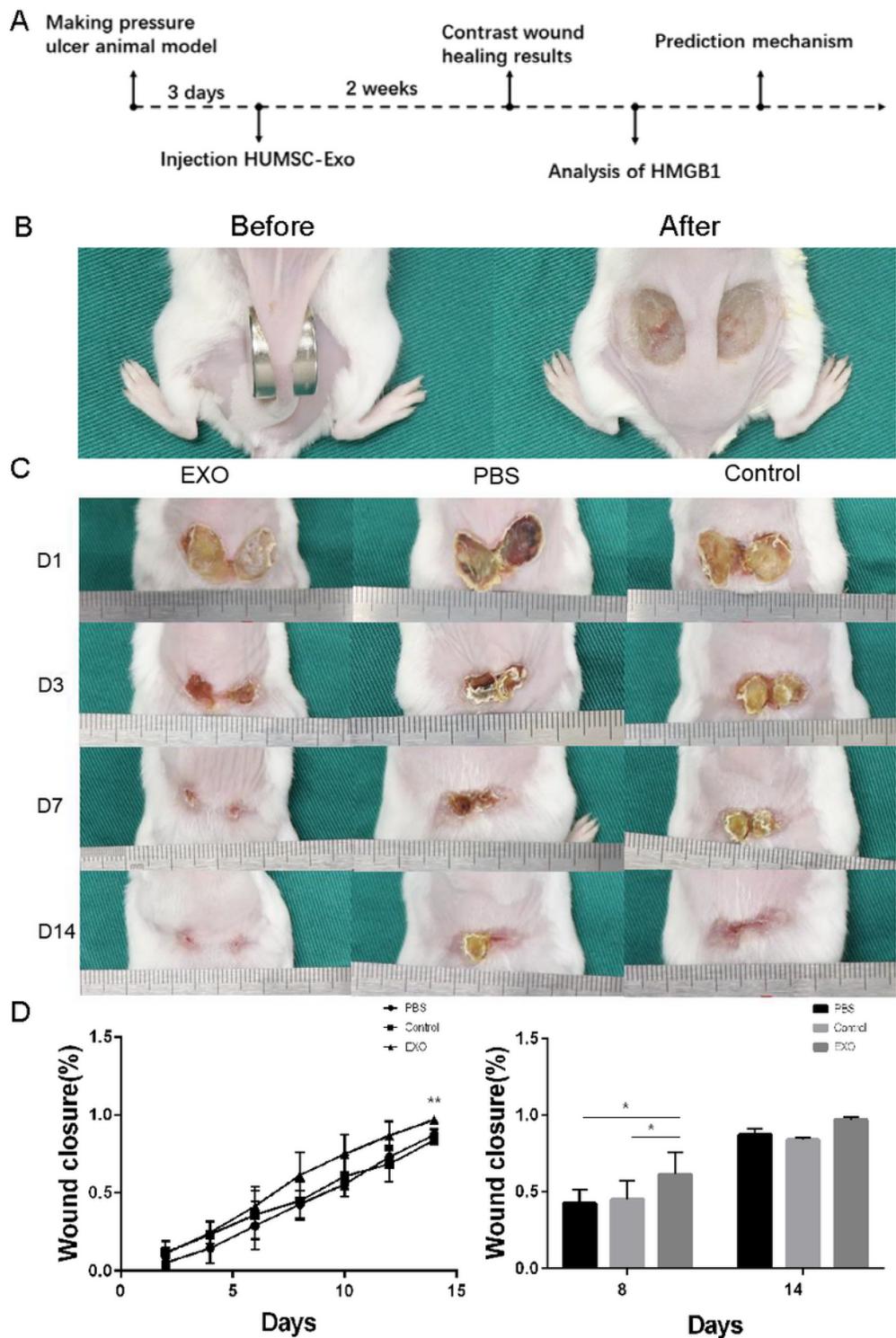
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## Figures



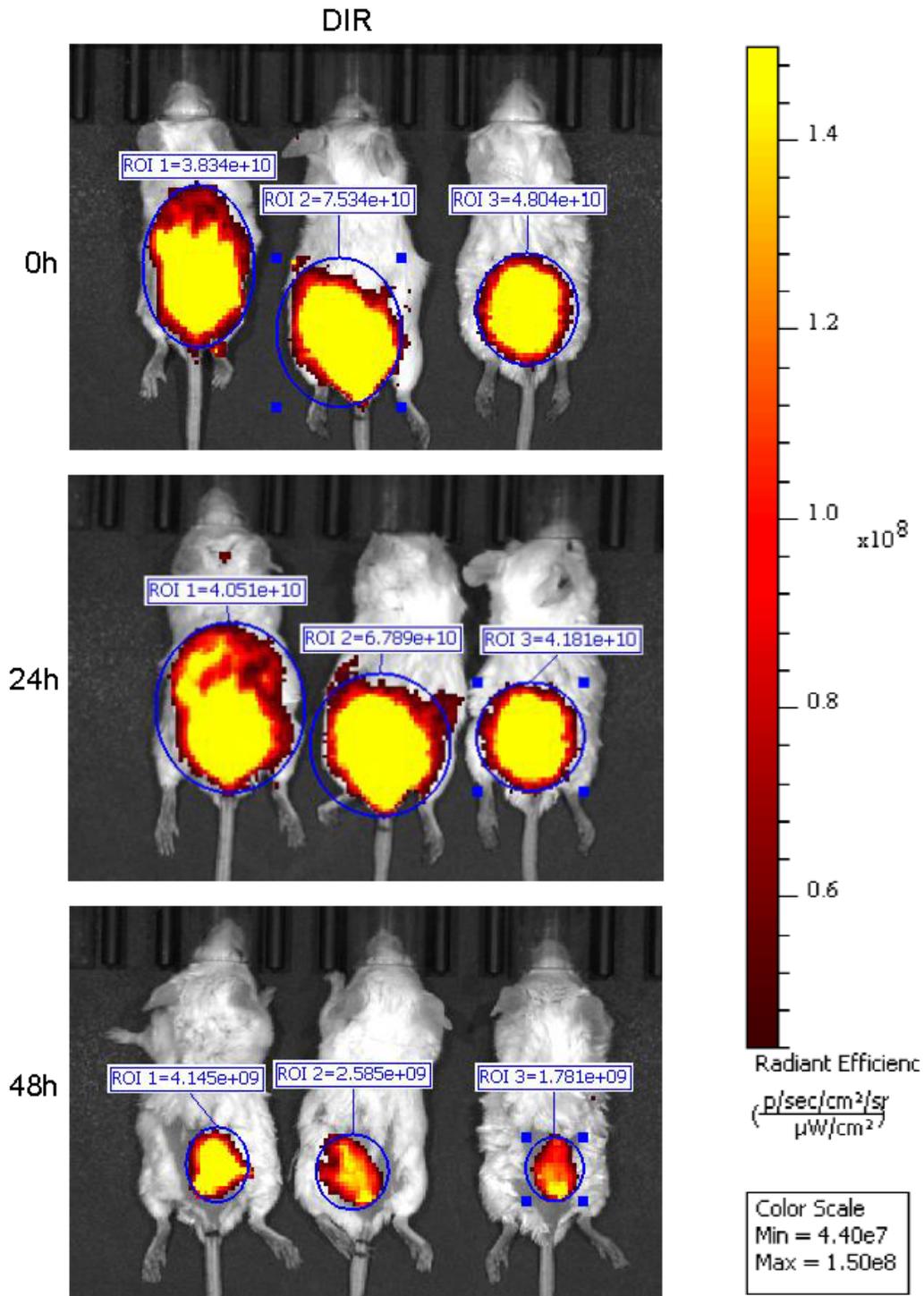
**Figure 1**

Exosome identification (A) Transmission electron microscopy of exosomes isolated from hucMSC-conditioned medium. Arrowheads indicate exosomes. Scale bar: 500 nm. (B) Western blotting was performed with hucMSC-Exo and hucMSC. CD9, CD63, CD81 expression in exosomes was detected. (C) NTA of exosomes isolated from MSC-conditioned medium. It shows the particle size and concentration distribution map: the MSC exosome particle size range is 30-150nm. (D) The exosome image tracked by NTA in real time is a screenshot of the measurement film.



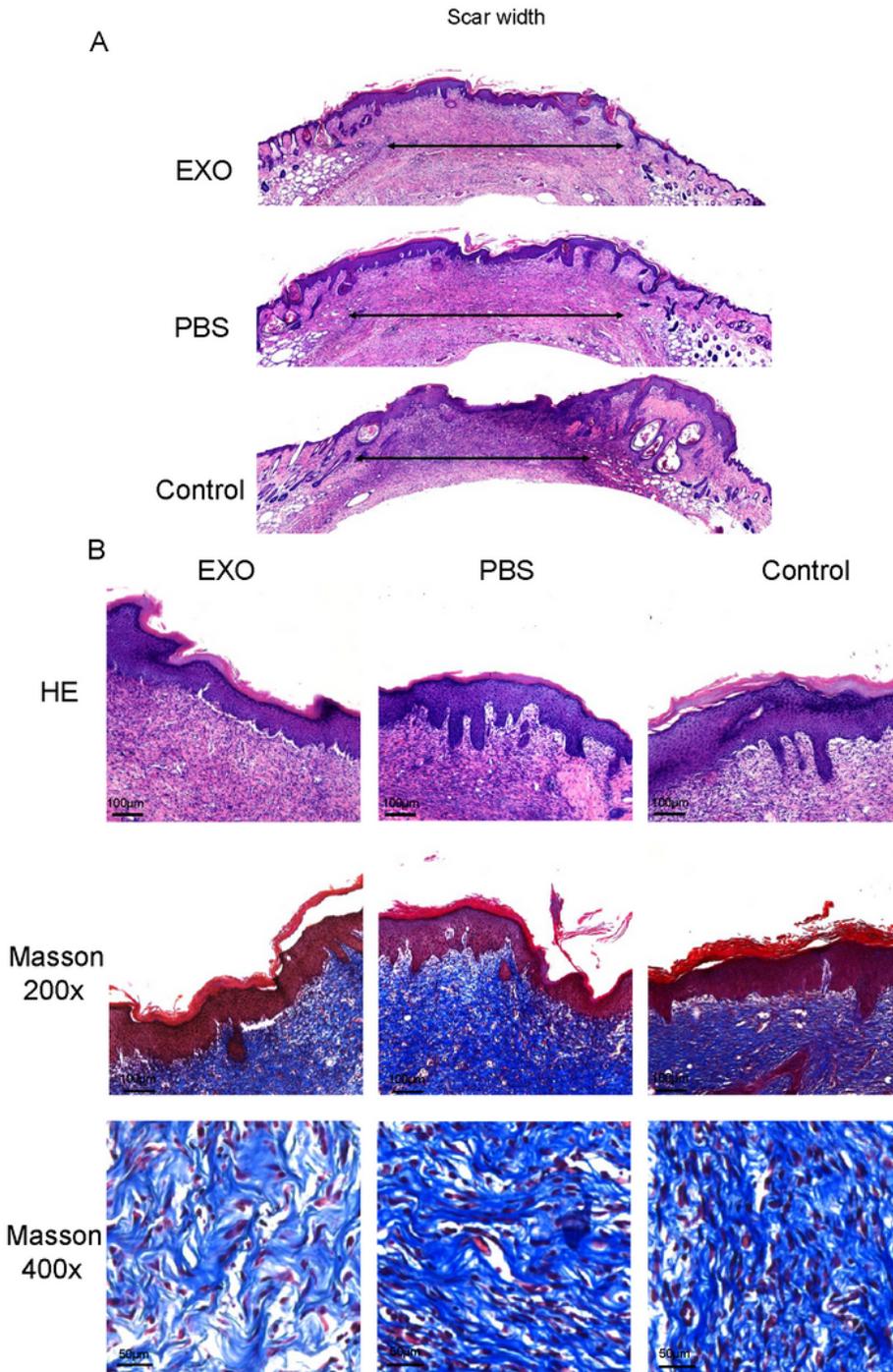
**Figure 2**

Mice macroscopic appearances of cutaneous wounds treated with hucMSC-Exo. (A) The experimental flow chart. (B) The model of pressure ulcer was completed by a pair of magnet disks. (C) Gross view of wounds treated with PBS, hucMSC-Exo at 1, 3, 7, and 14 days. (D) The effects of treatment with PBS, hucMSC-Exo on wound closure at 4, 7, and 14 days. \* $P < 0.05$ .



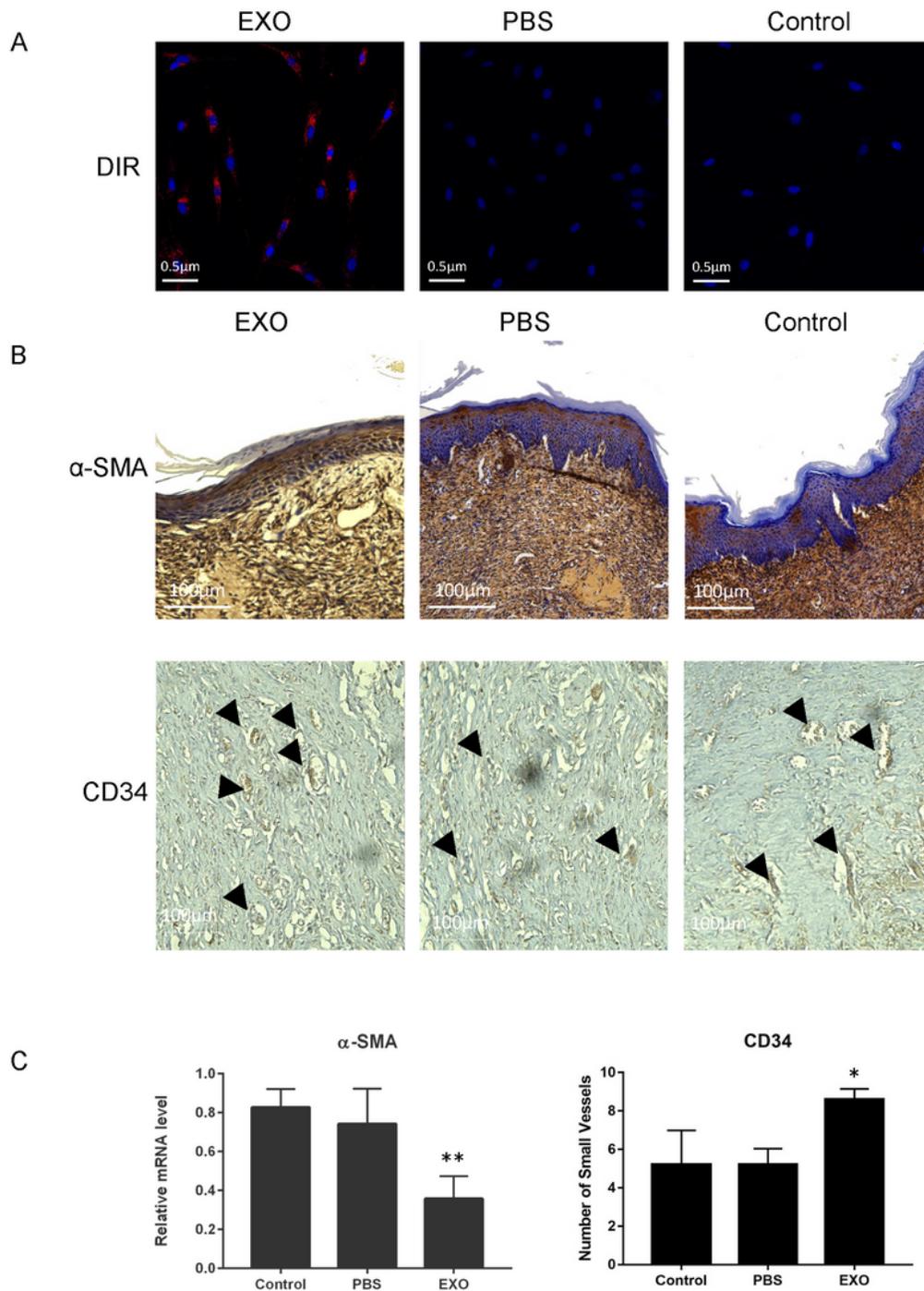
**Figure 3**

In vivo imaging of exosomes. Intradermal metabolism rate of exosomes detected by live imaging of the representative mice on every 24 hours.



**Figure 4**

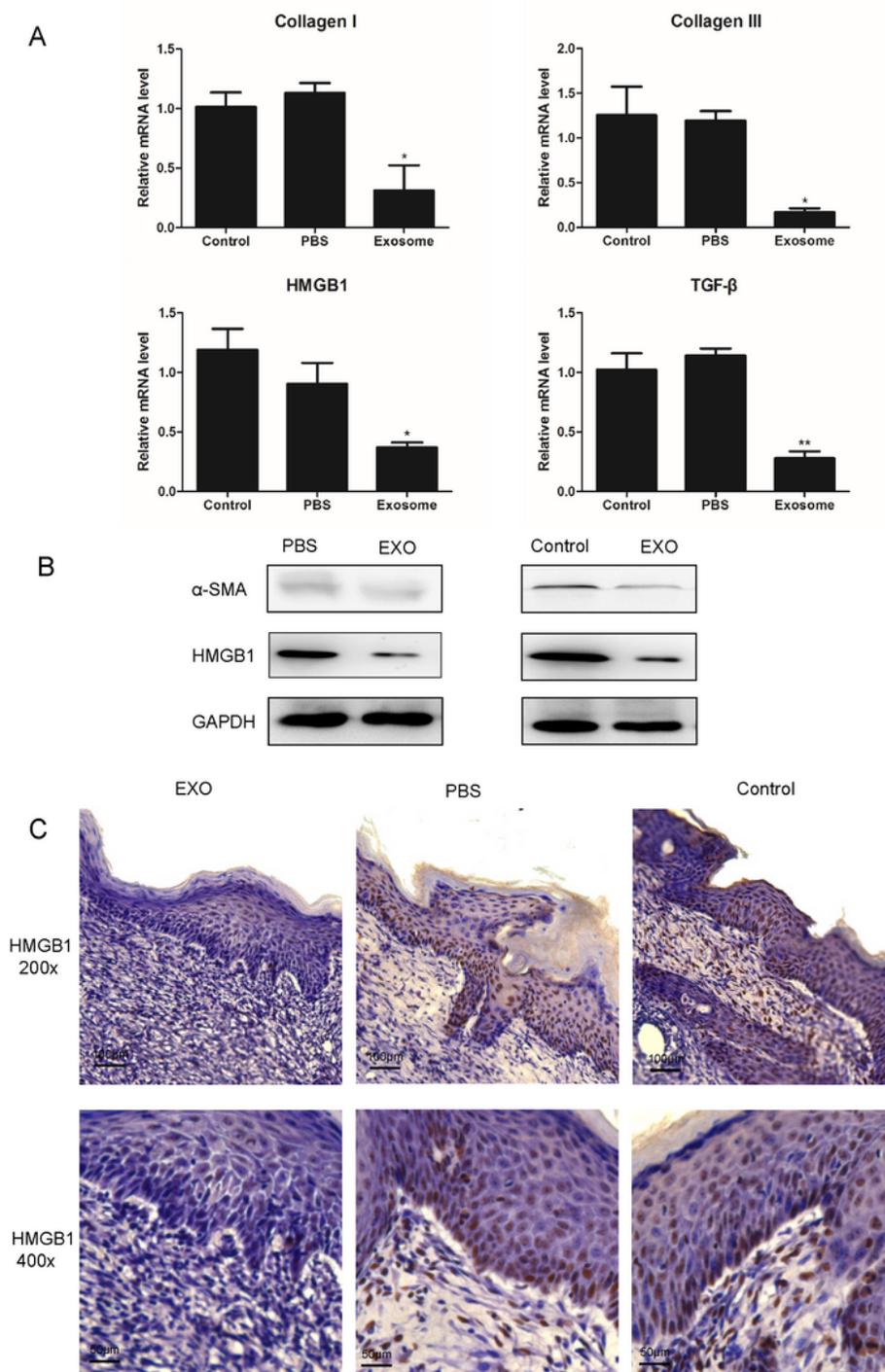
Mice histological analyses of cutaneous wounds treated with hucMSC-Exo, PBS, and untreated at 14 days post-wounding. (A) The double-headed arrows indicate the edges of the scar on H&E staining of wound sections which shows the effects of PBS, HUCMSC-Exo on wound re-epithelialization. (B) Evaluation of Evaluation of collagen maturity by Masson's trichrome staining of wounds following treatment with PBS, HUCMSC-Exo. Scale bar =100 µm.



**Figure 5**

HUCMSC-Exo treatment effect on wounds. (A) In-vitro wound healing properties of exosomes and cellular uptake found that internalized exosomes were visualized by fluorescence microscopy (red) and they mainly localized to the perinuclear zone. Scale bar = 100 $\mu$ m. (B) Immunohistochemical analysis of  $\alpha$ -SMA and content in wounds at 14 days post wounding. Immunohistochemical analysis of CD34 content in wounds at 14days post wounding. Scale bar = 100 $\mu$ m. (C) mRNA expression of  $\alpha$ -SMA in hucMan

fibroblasts treated with HUCMSC-Exo, PBS, and control group for 24 hours. Average number of small vessels in four quadrants. \* $p < 0.05$ , \*\* $p < 0.01$



**Figure 6**

HMGB-1 localization in wounds (A) mRNA expression of HMGB1, COLI, COLIII, TGF-β in wounds treated with 1mg/mL HUCMSC-Exo and PBS at 14days post-wounding. (B) Western blot analysis of the expression of HMGB1 and α-SMA in wounds treated with 1mg/mL hucMSC-Exo and PBS at 14days post-

wounding. \*P < 0.05, \*\*P < 0.01. (C) Immunohistochemical analysis of HMGB1 content in wounds at 14 days post wounding. Scale bar = 100µm.