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# Treatment of Ischemia-Reperfusion Injury of the Skin Flap Using Human Umbilical Cord Mesenchymal Stem Cells (hUC-MSCs) Transfected with “F-5” Gene

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**Background:** Recent studies have shown that skin flap transplantation technique plays an important role in surgical procedures. However, there are many problems in the process of skin flap transplantation surgeries, especially ischemia-reperfusion injury, which directly affects the survival rate of the skin flap and patient prognosis after surgeries.

**Material/Methods:** In this study, we used a new method of the “stem cells-gene” combination therapy. The “F-5” gene fragment of heat shock protein 90- $\alpha$  (Hsp90- $\alpha$ ) was transfected into human umbilical cord mesenchymal stem cells (hUC-MSCs) by genetic engineering technique.

**Results:** The synergistic effects of “F-5” gene and hUC-MSCs in the treatment of ischemia-reperfusion injury of the skin flap were confirmed by histochemical and immunohistochemical methods.

**Conclusions:** This study showed that the hUC-MSCs transfected with “F-5” gene can effectively improve the repair of ischemia-reperfusion injury.

**MeSH Keywords:** **Heat-Shock Proteins • Mesenchymal Stromal Cells • Transfection**

**Full-text PDF:** <http://www.medscimonit.com/abstract/index/idArt/905216>

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## Background

As a means of wound repair, skin flap transplantation technique has been widely used in surgeries of many fields, such as tissue repair and organ reconstruction. In the past century, we classified the types of skin flaps into (1) random flaps and (2) axial flaps, in terms of the form of blood circulation in skin flaps. In the late 1970s and early 1980s, the free fibrovascular transfer technology was under rapid development, and using the pedicled musculocutaneous flaps for repair of large-area soft tissue injury has become common. In the late 1980s, Kroll and Rosenfield first used the dorsal perforator flap in surgeries [1]. The application of the perforator flap ushered in a new era of plastic and reconstructive surgery; it effectively improved the survival rate of skin flaps, but the occurrence of partial or complete necrosis of skin flaps were often observed during that period and even in today's surgeries. Actually, this phenomenon is due to the ischemia-reperfusion injury of skin flaps after operations.

The study of ischemia-reperfusion injury has continuously progressed over time. At present, the acknowledged causes of this kind of injury are the production of oxygen-derived free radicals and the infiltration of neutrophils [2]. In 1983, Manson et al., in a study of ischemia-reperfusion injury on rat island-flap models, also proposed that the production of oxygen-derived free radicals is one of the causes of this damage [3]. In a 1992 study of porcine Latissimus dorsi musculocutaneous skin flaps, Lee et al. found that the extent of neutrophil infiltration in the skin flaps with ischemia-reperfusion injury was much higher than that in the common ischemic skin flaps [4,5]. For this disease, the traditional method of treatment is to interfere with or block the process to reduce reperfusion injury of the skin flap. The procedures included: (1) Drug treatment with Xuebijing (XB) [6], tadalafil [7], procyanidin [8], Salvia [9], and Chuanxiong [10]; and (2) Pretreatment with thioredoxin [11], botulinum toxin type A [12], doxorubicin [13], adenosine [14], vitamin A and E [15], heat stress [16], and hyperbaric oxygen [17]. However, there is no effective clinical method for treatment of ischemia-reperfusion injury of the skin flaps due to the imperfect outcomes of all of the above-mentioned methods. Therefore, many researchers have turned their attentions to stem cells. Stem cells are characterized by their potential of proliferation, differentiation, and chemotaxis ability, and they can secrete factors that promote healing. These advantages contribute to the treatment of the ischemia-reperfusion injury of skin flaps. Although there have been some achievements in related research, the applicable categories of stem cells in the treatment of ischemia-reperfusion injury of skin flaps are very limited, which is because of the outside interference from ethical and moral aspects. At present, the categories of stem cells that can be used in research are mainly bone marrow mesenchymal stem cells (BM-MSCs) [18],

adipose-derived mesenchymal stem cells (AD-MSCs) [19], and human umbilical cord mesenchymal stem cells (hUC-MSCs) [20].

In this study, we used hUC-MSCs because the extraction of mesenchymal stem cells from umbilical cord is easy and clinically feasible, and the umbilical cord blood is currently regarded as a type of medical waste which can avoid the ethical problems. Furthermore, there are reports that the stem cells extracted from umbilical cord are superior in number and proliferation ability to BM-MSCs and AD-MSCs due to their characteristic of multilineage differentiation ability. Besides, the immunogenicity and risk of bacterial or viral infections of hUC-MSCs are lower than with BM-MSCs and AD-MSCs [21–23]. The differentiation direction of hUC-MSCs is regulated by the micro-environment; the traumatic stimulation promotes the chemotaxis of hUC-MSCs and induces them to differentiate into the desired cell type, eventually leading to tissue repair [24]. Mackenzie et al. found that the transplanted hUC-MSCs have the ability to migrate to the lesion site and differentiate into the desired type of cells. The combined mesenchymal stem cells can go across the species barrier so as not to induce hemodynamic disorders after the injection [25]. hUC-MSCs have gradually become a promising source of stem cells for repairing damaged tissues.

The wound healing of skin is a complex process, which depends on the cooperation of many categories of cells and biomolecular factors in extracellular matrix. In addition, cell movement also plays a vital role in this process. The lateral migration process at the edge of the wound by epidermal cells (mainly keratinocytes) is known as epithelialization. Human skin cells, including fibroblasts and dermal microvascular endothelial cells, enter the wound to produce and store a large number of matrix proteins, contract and reshape the wound, and also establish new blood vessels [26]. But research shows that not all healing cells are subject to migration to the wound in the early stage of wound healing. Transforming growth factors (TGFs) promote the migration of keratinocytes and inhibit the migration of dermal fibroblasts and microvascular endothelial cells, thus affecting the wound healing [27].

With the emergence of genetic engineering technology in recent years, we are gradually developing a deeper understanding of the 90-kDa heat shock protein (Hsp90). Hsp90s is a heat shock protein (Hsp) family, and are synthesized by organisms during the stress reactions to physical, chemical, biological, and mental stimuli in the environment. It is highly conserved in evolution and widely exists in prokaryotic and eukaryotic organisms. Hsp families are mainly classified into Hsp10, Hsp27, Hsp40, Hsp60, Hsp70, Hsp90, and Hsp110 in terms of their relative molecular weight [28–30]. Chieh-Fang Cheng et al. reported that Hsp90- $\alpha$  can promote wound healing more effectively than platelet-derived growth factor (PDGF), because

a certain concentration (0.1  $\mu\text{mol}$ ) of locally secreted Hsp90- $\alpha$  induced by hypoxia can promote the migration of skin basal cells, fibroblasts, and vascular endothelial cells to the wound, which promotes wound healing [31]. Chieh-Fang Cheng et al. have identified that there is a highly conserved amino acid sequence (aa236-aa350) in the Hsp90 gene sequence, of which the expressed polypeptide can promote wound healing more effectively than can the full-length Hsp90- $\alpha$ . This functional gene sequence (aa236-aa350) is called the “F-5” gene. Based on the mechanism of skin wound healing, we used hUC-MSCs as a carrier, induced “F-5” gene into hUC-MSCs by genetic engineering technique, which compensated the drawback of insufficient migration of basal cells and fibroblasts in wound healing process of the skin flap, and greatly improved the healing effect of ischemia-reperfusion injury of the skin flap (Figure 1).

## Material and Methods

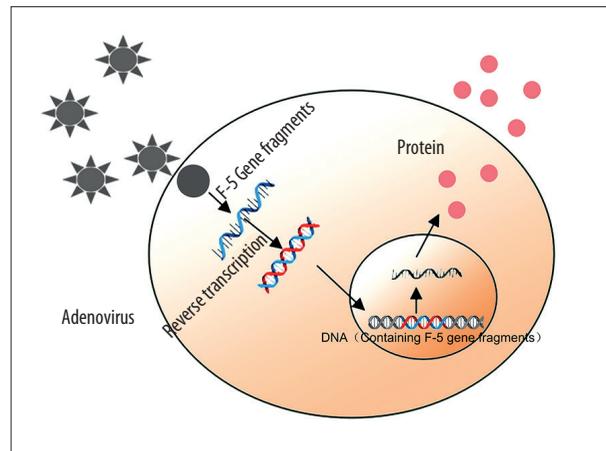
### Materials

#### Reagents

The reagents used in the experiments included: Dulbecco's modified Eagle's medium (DMEM) from Gibco, USA; Fetal bovine serum (FBS) from CellMax, China; Phosphate-buffered saline (PBS) from ZSGB-BIO, China; Adenovirus from Shanghai Genechem Co. Ltd. (China); Primers, RT reagents, Q-PCR reagents and SYBR Green I from Western Biotechnology; DEPC from Sigma, USA; Trypsin and TRIzol from Pik Wan Tin, China; Chlorine Isothiopropanol from Shanghai Chemical Reagent Company, China; Citric acid (PH6.0) antigen repair solution, BSA and hematoxylin from Solarbio; primary antibody (PECAM-1) from Santa Cruz; and secondary antibody (HRP labeled goat anti-rabbit common) from DAKO. The hUC-MSCs were obtained from Qingdao Allcare Biomedical Development Company, China. The SPF Wistar rats were purchased from Jinan City Drug Testing Laboratory Animal Center, China.

#### Instruments

The instruments used in the experiment included: a low-speed desktop centrifuge and high-speed desktop centrifuge from Cheung Instrument Centrifuge Instrument Co., Ltd; a carbon dioxide incubator from Sanyo, Japan; micropipettes and pipettes from Eppendorf, Germany; an inverted microscope from Nikon, Japan; a fluorescent inverted microscope, a camera system and positive microscope from OLYMPUS, Japan; an electronic analytical balance from Precisa12A, Switzerland; various models of cell culture bottles, cell culture plates, centrifuge tubes, pipettes, and cryopreservation tubes from Axygen, USA; real-time fluorescence quantitative PCR from Funplyn Biotech, Canada; electrophoresis from Bio-Rad, USA; Gel Implant Regulator DNA



**Figure 1.** The sketch demonstration of hUC-MSCs transfected by adenovirus carrying “F-5” gene segment.

Electrophoresis from Tianneng, USA; GDS8000 gel scanning system from UVP, USA; and a skeleton machine from Shanghai Leica Instrument Co., Ltd., China.

#### Cells and animals

According to the experimental design, we used hUC-MSCs that were obtained from Qingdao Allcare Biomedical Development Company, China and healthy, adult, male, 200–250 g SPF Wistar rats that were purchased from Jinan City Drug Testing Laboratory Animal Center, China.

#### Methods of preparation for virus and cells

##### Viruses preparation

We used adherent dependent epithelial cells 293T as the packaging cells of Adenovirus and Escherichia coli strain DH5- $\alpha$  to amplify the adenoviral vector while helping to package the vector plasmid, constructed the adenoviral vector that carries the “F-5” gene segment according to the requirements of the experiment, extracted DNA from the adenoviral packaging system, and determined the concentration and purity of the plasmid using UV-light absorption method. Finally, we obtained the recombinant adenovirus through plasmid transfection technique. Then, the adenovirus was stored at  $-80^{\circ}\text{C}$  after concentration and purification for experimental use. At the same time, we prepared the “non-carrying” “F-5” gene adenovirus using the same method, in order to improve the accuracy of the experiment.

##### Cell culture and passage

The culture environment of hUC-MSCs was as follows. Culture medium: low glucose DMEM containing 10% fetal bovine serum. Incubator: 5%  $\text{CO}_2$  incubator at  $37^{\circ}\text{C}$ . Passage cells: until

the cell number reaches the maximum in culture bottles while cells are in good conditions, then we removed the culture medium and added the mixture medium of 1: 1 0.25% trypsin and 0.02% EDTA to detach cells, then added culture medium to inactivate trypsin and centrifuged the cells (1000rad/5min). Then, we removed the supernatant, added new culture medium and split cells into different culture bottles to make sure the cell number in each bottle is appropriate, and we put them back into the incubator and continued culturing.

#### *In vitro transfection of hUC-MSCs*

At 24 h before the transfection, we selected the third generation of hUC-MSCs of good condition, prepared cell suspensions using the method of cell passage, and separated them into a 6-well plate. After the cells had adhered to the surface, we substituted the original culture medium with the compound lentiviral culture medium of gradient concentrations, incubated the cells at room temperature for 15 min, then transferred them into the incubator at 37°C. After 6 h of incubation, we changed the compound lentiviral culture medium to fresh culture medium. After another 72 h, we identified the transfection efficiency by the expression of fluorescein, and determined the value of multiplicity of infection (MOI). We used the same method for the transfection of “non-carrying” adenovirus to improve the accuracy of the experiment. Finally, we selected the stable cell lines by screening them through the medium containing puromycin.

#### *MTT assay*

We prepared a single-cell suspension using culture medium containing fetal calf serum from cells after 3-day passage, inoculated the cells onto a 96-hole plate with the cell concentration of 1000 cell/hole, and added 10 ul of MTT solution per hole (with MTT concentration of 5mg/ml). The volume of each hole was 200 ul. We incubated cells for another 4 h, then stopped culturing and discarded the supernatant by careful suction. (For cell suspension, we centrifuged cells with culture medium and then discarded the supernatant.) We then added 100 ul of DMSO in each hole and shook the plate for 10 min until the crystals were fully dissolved. Afterwards, we selected the 490 nm wavelength of the filter, determined the optical density (OD) value of each hole in ELISA monitor, recorded the results, and used time as the abscissa and OD value as the ordinate to make a curve of cell growth.

#### *qPCR assay*

We used random primers to transcribe RNA into cDNA, then we designed the specific primers, and extracted RNA by referencing to the instructions of the RNA extraction agents kit. After the completion of RNA extraction, we randomly took

several samples for the pre-experiment to determine the time and temperature of reverse-transcription and qPCR amplification. After the determination and confirmation of the above conditions, we drew the standard curve of qPCR and then did the fluorescent quantitative PCR detection using SYBR Green I fluorescent dye.

#### *hUC-MSCs scratch test*

We divided the hUC-MSCs into 3 groups: (a) hUC-MSCs that are transfected with adenovirus carrying “F-5” gene; (b) hUC-MSCs that are transfected with adenovirus but without “F-5” gene; and (c) pure hUC-MSCs without transfection. We added approximately  $5 \times 10^5$  cells in each well of the 6-well plate. After 24 h, until the cells fully covered the culture plate, we drew some perpendicular lines on the plate compared with a ruler using a 200- $\mu$ l gun head. We then washed the plate with PBS 3 times to remove the cells that had been scratched off, and added the serum-free medium, incubated the cells in the incubator under conditions of 5% CO<sub>2</sub> at 37°C. During this period, we observed the cells under a bright-field microscope by 100x amplification at time-points of 0 h, 6 h, 12 h, and 24 h, and took pictures of the results. The relative migration distance was calculated by the formula: relative migration distance =  $[(A-B)/A] \times 100\%$ , where A represents the initial scratch distance and B represents the scratch width of the cell at each observation time-point.

### **Methods of preparation for animal models**

#### *Animal models grouping*

The animal models used for our experiment was rat models with abdominal perforator flaps. The selected rats were healthy adult male SPF Wistar rats, every rat weighs 220–250 g. The 96 Wistar rats were divided into 4 groups: A) rats to be injected with hUC-MSCs transfected by adenovirus carrying “F-5” gene; B) rats to be injected with hUC-MSCs transfected by “non-carrying” adenovirus; C) rats to be injected with pure hUC-MSCs without transfection; and D) control group (rats without injection). There were 24 rats in each group. We randomly selected 10 points on every abdominal skin flap with the distance of 1 cm between each point for point-injection, and randomly selected 6 rats in each group to observe the recovery of the abdominal skin flaps on time-points of day 1, day 3, day 5, and day 7 after the point-injection. During the experiment, 3 rats (2 in Group B and 1 in Group D) died due to unknown causes.

#### *Preparation of animal models*

According to animal care principles of related international legislation, all animals were treated humanely in this experiment. We referred to the methods of Petry and Worthamn to

prepare the abdominal perforator skin flaps with each area of 3×6 cm in a sterile environment. The depth of the flaps was as deep as into the layer of deep fascia. We dissected the flaps bluntly, found the abdominal artery and the abdominal vein (the branches of femoral artery and femoral vein), used a glass needle to carefully separate the abdominal artery and the abdominal vein from the tissue, making a perforator skin flap with the only blood vessels of the abdominal artery and the abdominal vein, clipped the vessels with a small vascular clamp at the same time, and observed the blood supply situation to make sure that the blood supply of all skin flaps had been completely blocked. Then we sutured the skin flaps *in situ*, leaving a 1-cm-wide circle wound without suturing. After occlusion of the blood supply for 6 h, we opened and removed the clamps from the reserved wound, observed whether the blood supply was recovered, then sutured the reserved wound (Figure 2).

#### Cell injection

We made cell suspensions with the cell concentration of  $4 \times 10^5$  cell/ml of all groups of hUC-MSCs using the cell culture methods described in step 2.2.2. When the animal models were successfully prepared, we injected 0.1 ml of cell suspension in each selected injection point on the skin flaps, as described in step 2.3.1. To reduce the variables of this experiment as much as possible, we injected the same amount of saline as cell suspensions in the control group (D).

#### Evaluation of area, color, and hair growth of the skin flaps

We randomly selected 6 rats in each group at every time-point (day 1, day 3, day 5, day 7), observed the color, recovery and hair growth situations of the skin flaps, recorded the results by capturing pictures of the operated areas, and then valued the situations of necrosis by the software "Image Pro Plus", and measured the survival rate (Survival rate=survival area/skin flap area×100%) of each skin flap.

#### Evaluation by HE staining

We cut down the skin flaps from the 24 randomly selected rats (6 rats in each group×4 groups) after general anesthesia at day 7, fixed the skin flaps in 4% paraformaldehyde for 24 h, and then used low-concentration alcohol and high-concentration alcohol to gradually dehydrate the tissues, and put the tissues in xylene to make them transparent by replacing alcohol from the tissues by xylene. We used paraffin to embed the tissues. Afterwards, we cut tissues into slices of 6-μm thickness, washed them in distilled water, put them into hematoxylin for staining for a couple of minutes, then put the slices into acid and ammonia water for separation of colors for several seconds each. After 1 h of washing in flowing water, we

put the slices into the distilled water for a moment, then dehydrated them in alcohol of gradient concentrations, and put them into alcohol-eosin for staining for 2–3 min. The stained sections were then dehydrated in pure alcohol and made transparent again by xylene.

#### Evaluation of platelet endothelial cell adhesion molecule (cluster of differentiation 31, CD31) by immunohistochemistry (IHC) technique

We used the same method of HE staining to prepare for the slices, baked them in the oven at a constant temperature of 60°C, then removed the slices from the oven and put them into xylene for dewaxing and then in ethanol of gradient concentrations for hydration, digestion, and antigen repair. After natural cooling at room temperature, we added antibodies onto the slices according to the instructions of the immunohistochemistry kit. Finally, we used tap water to wash the slices, and repeated the procedures of dying, dehydration, and making transparent. When the tissue samples were completely dry, we used the mountant "buffered glycerol" to mount the slices, then observed the slices under the microscope.

## Result

### Evaluation of *in vitro* transfection

#### Assessment of transfection efficiency

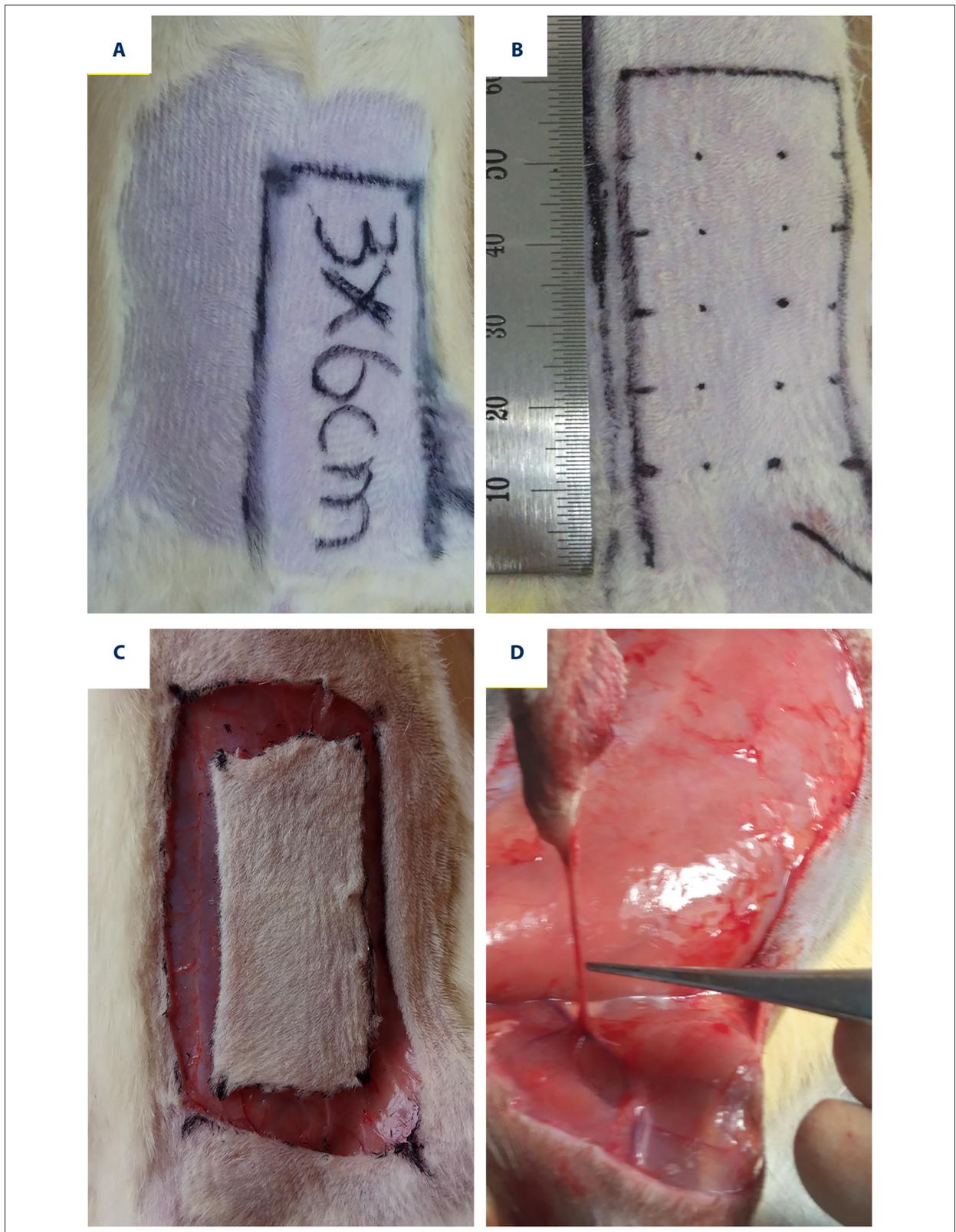
In this study, we used adenovirus to transfect hUC-MSCs. We observed that when MOI=100, the cells with green fluorescence reached the maximum number, accounting for about 93% of the total cell population (Figure 3A–3D).

#### Evaluation of MTT test results

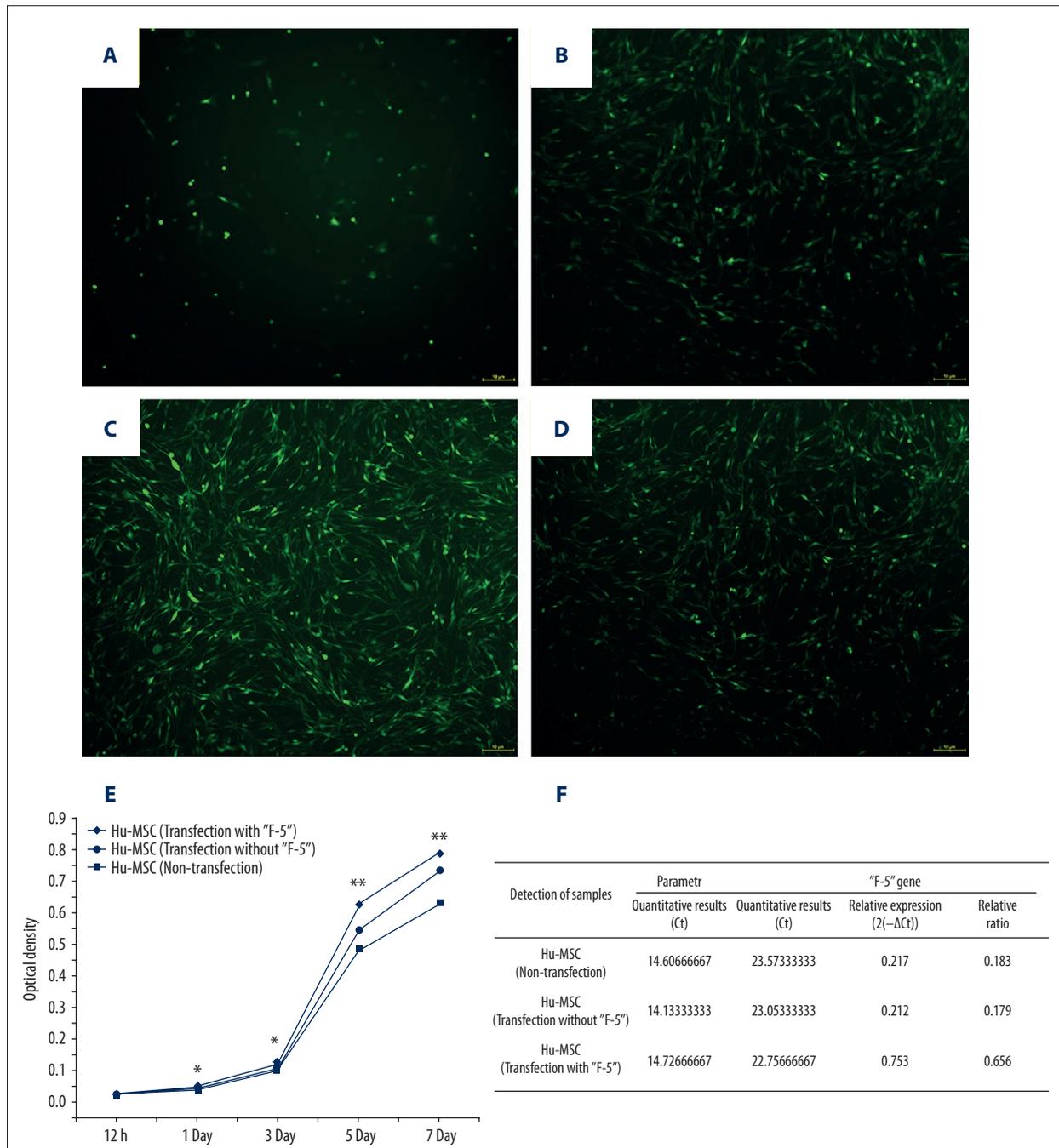
We performed a toxicity test on the transfected hUC-MSCs, and compared the results from the 3 groups of hUC-MSCs – (a) hUC-MSCs transfected with adenovirus that carries "F-5" gene; (b) hUC-MSCs transfected with adenovirus that did not carry the "F-5" gene; and (c) pure hUC-MSCs without transfection – at 5 different time-points (12 h, day 1, day 3, day 5, and day 7). The results showed that the toxicity of adenovirus carrying "F-5" gene segment was very tiny to hUC-MSCs and the effect can be neglected (Figure 3E).

#### Evaluation of qPCR test results

To test the expression efficiency of "F-5" gene in hUC-MSCs, we performed the qPCR test on the 3 groups of hUC-MSCs: hUC-MSCs transfected with adenovirus that carries "F-5" gene; hUC-MSCs transfected with adenovirus that did not carry the



**Figure 2.** (A) The size of the abdominal skin flap on each experimental animal was an area of 3×6 cm. (B) 10 injection points selected on the abdominal skin flap with the spacing of 1 cm. (C) The edge of the flap wound was left open. (D) The blood supply of the flap, only the abdominal artery and the abdominal vein were reserved.



**Figure 3.** The fluorescence expression of 72 h after transfection with different MOI values, using the transfected materials and methods mentioned above. (A) MOI=10, (B) MOI=50, (C) MOI=100, (D) MOI=200. (E) OD values determined by 3 groups of hUC-MSCs: Hu-MSC (Transfection with "F-5"), Hu-MSC (Transfection without "F-5"), and Hu-MSC (Non-transfection) at time-points of 12 h, day 1, day 3, day 5, and day 7, using the materials and methods for MTT assay mentioned above. Data are expressed as the mean  $\pm$ SD of the experiments performed in triplicate. \*  $P < 0.05$ , \*\*  $P < 0.01$ . (F) The expression of "F-5" gene was detected by qPCR. The Ct value represents the number of cycles experienced when the fluorescence signal in each reaction tube reaches the set threshold value.  $\Delta Ct = Ct$  (target gene)  $- Ct$  (reference gene). Relative expression =  $2^{(-\Delta Ct)}$ . Relative ratio = relative copy number of target gene/relative copy number of internal reference gene

“F-5” gene; and pure hUC-MSCs without transfection. The results showed that in the case of similar parameter values, the relative expression value of Group A was 0.753, while values of Group B and Group C were 0.212 and 0.217, respectively. We observed that the relative ratio of the “F-5” gene-transfected group (a) was also much higher than that of the other 2 groups (b and c) (Figure 3F).

#### *Evaluation of cell scratch test results*

Cell migration plays a decisive role in the healing process of skin flaps. We observed the effect of cell migration of the 3 groups of skin flaps with injection through the microscope at time-points of 0 h, 6 h, 12 h, and 18 h. The results showed that the cell migration area out of the total skin flap area at 18 h in the 3 groups were: Group A: 97%, Group B: 70%, and Group C: 72%. Apparently, the effect of cell migration in Group A was much higher than that in Group B and Group C, indicating that the “F-5” gene was able to enhance the effect of cell migration in skin flaps ( $P=0.03$ ,  $n=3$ ) (Figure 4).

#### **Evaluation of animal experiment**

##### *Quality assessment of the animal model*

The quality of the animal model is the key to the success of our experiment. We decided to use the abdominal perforator flap to avoid as much as possible the experimental errors that could be due to the supplementary blood supply from adjacent tissue and capillaries in skin flaps. The blood supply of the abdominal flap mainly depends on the branch of the femoral artery, which is more beneficial to the preparation of ischemia-reperfusion injury model. At 6 h after clipping of the blood vessels in skin flaps, the skin flaps showed symptoms of visible purple color and low temperature. During the process of modeling, we used a microvascular clamp to block the blood supply of the perforator skin flaps for 6 h, then we removed the vascular clamp and point-injected the prepared cells and saline into the skin flaps after the blood supply had been recovered. The distance between each injection point was 1 cm.

##### *Quality assessment of the skin flap*

We observed the changing of color and hair growth situation on the skin flaps at time-points of day 1, day 3, day 5, and day 7. The results showed that, in Group A, the skin flaps showed mild skin contracture, the color was ruddy, the hair growth was good, and there was no sign of necrosis. In Group B and Group C, the skin contracture was worse than in Group A, and there were signs of necrosis in distal areas of the skin flaps, while the color of the sites without necrosis was not good, and hair growth was sparse. In group D, the skin contracture was the worst, there were large areas of necrosis, the color of

skin flaps was blackening, skin lines were unclear, and there was no sign of hair growth. We analyzed the necrotic areas of skin flaps using “Image Pro plus” software, which showed that the average necrotic areas in Group A, B, C, and D) were 2%, 39%, 41%, and 100%, respectively, which indicated that the average necrotic area in Group A was much smaller than that of the other 3 groups ( $P<0.05$ , with statistical significance). The average necrotic area significantly decreased in Group B and C compared to in group D) ( $P<0.05$ , with statistical significance). The average necrotic area in Group B and Group C were similar to each other ( $P>0.05$ , without statistical significance) (Figure 5) ( $P=0.05$ ,  $n=6$ ).

##### *Evaluation of HE staining results*

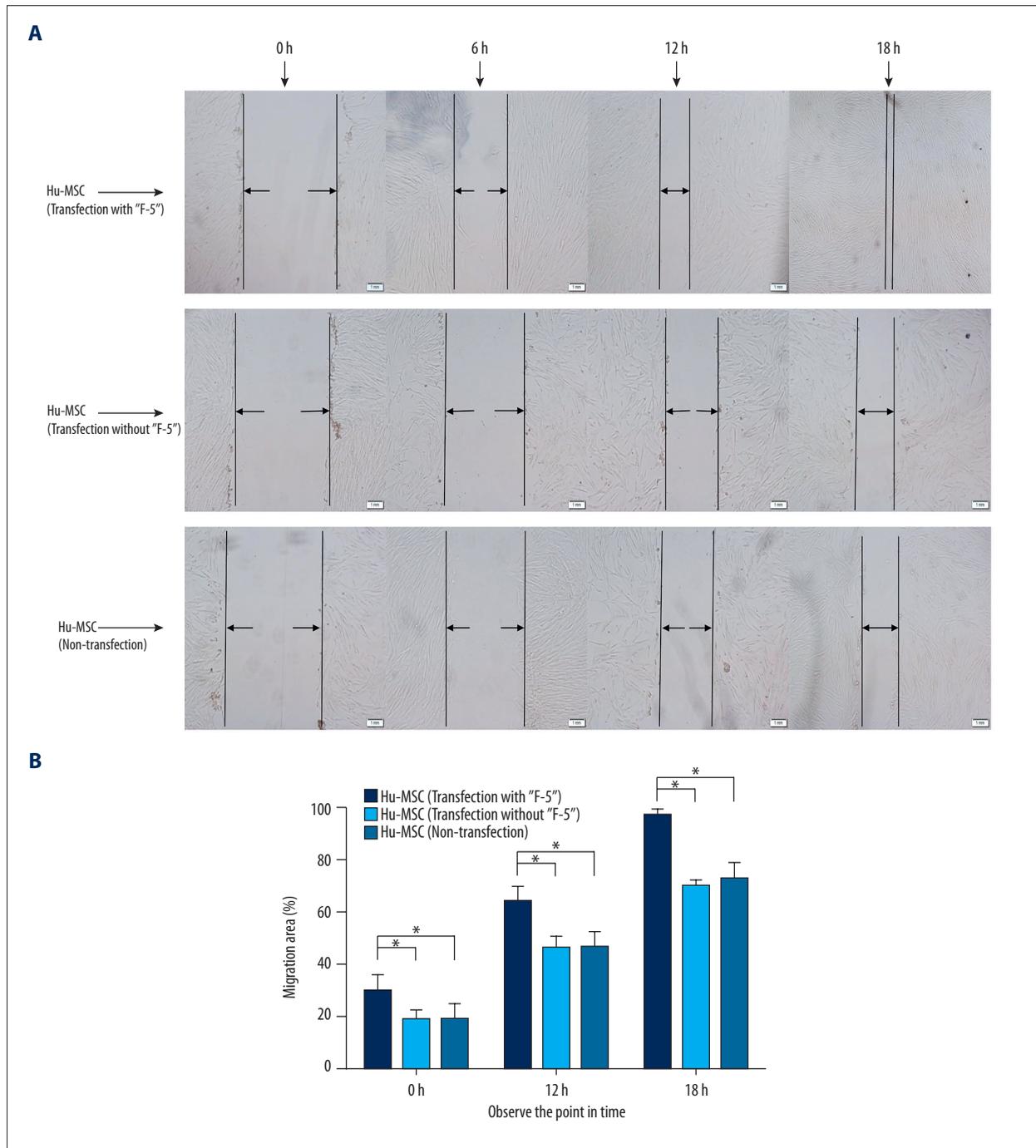
At day 7 observation, we randomly selected 3 slices of the flap samples in each rat group for HE staining, with the staining dimensions including the cross-section and the longitudinal section. The results showed that in Group A the structure of skin was clear on the cross-section, the capillaries were large and uniformly distributed on the longitudinal section. In Groups B and C, the structures of skin were recognizable but the density of capillaries was much lower and unevenly distributed compared to Group A. In group D, the capillaries were few and the structure of skin was undistinguishable (Figure 6).

##### *Evaluation of IHC results*

On day 7 of observation, we randomly selected 6 slices of the flap samples in each rat group for immunohistochemical staining, and measured the content of CD<sub>31</sub> in the peripheral areas of the blood vessels, calculated the integral optical density of each slice, then finally obtained the average values. The results showed that the average integral optical density of group D was higher than that of the other 3 groups (in the comparison of group D with Group A and Group B),  $P<0.01$ , with statistical significance; and in the comparison of group D with Group C,  $P<0.001$ , with higher statistical significance (Figure 7).

## **Discussion**

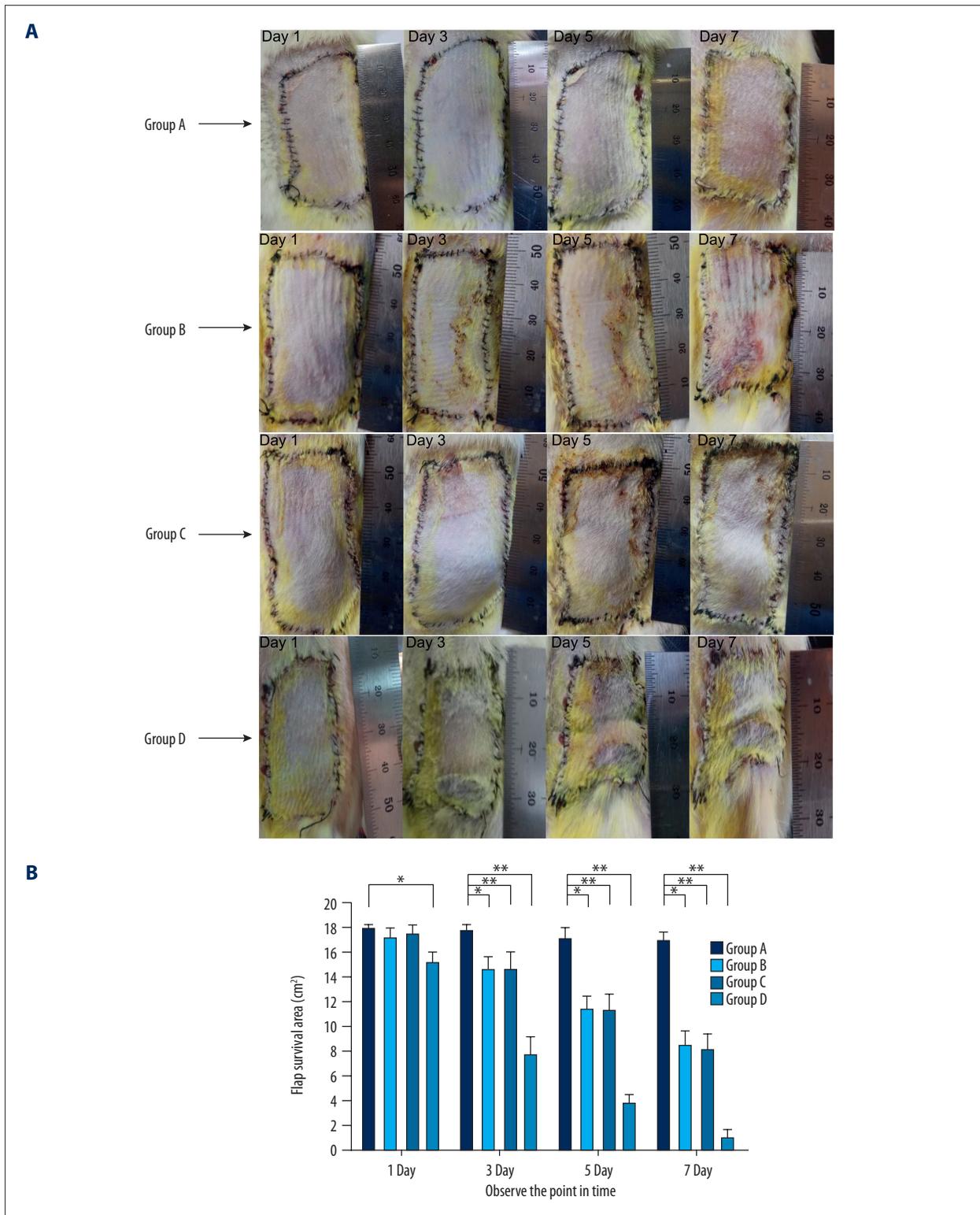
Some studies have shown that estrogen can influence the speed and quality of the wound healing process [32]. In order to reduce this influence, we decided to use only adult male Wistar rats for preparation of animal models. At the beginning of the experiment, we had many considerations on the preparation of animal models. We finally chose rats as our experimental subject mainly because rats are currently the most common subject for animal modeling, with the advantages of fast modeling, high success rate of modeling, easy operation, and low cost. However, there are also some problems in the use of rats for animal modeling; for example, the wound



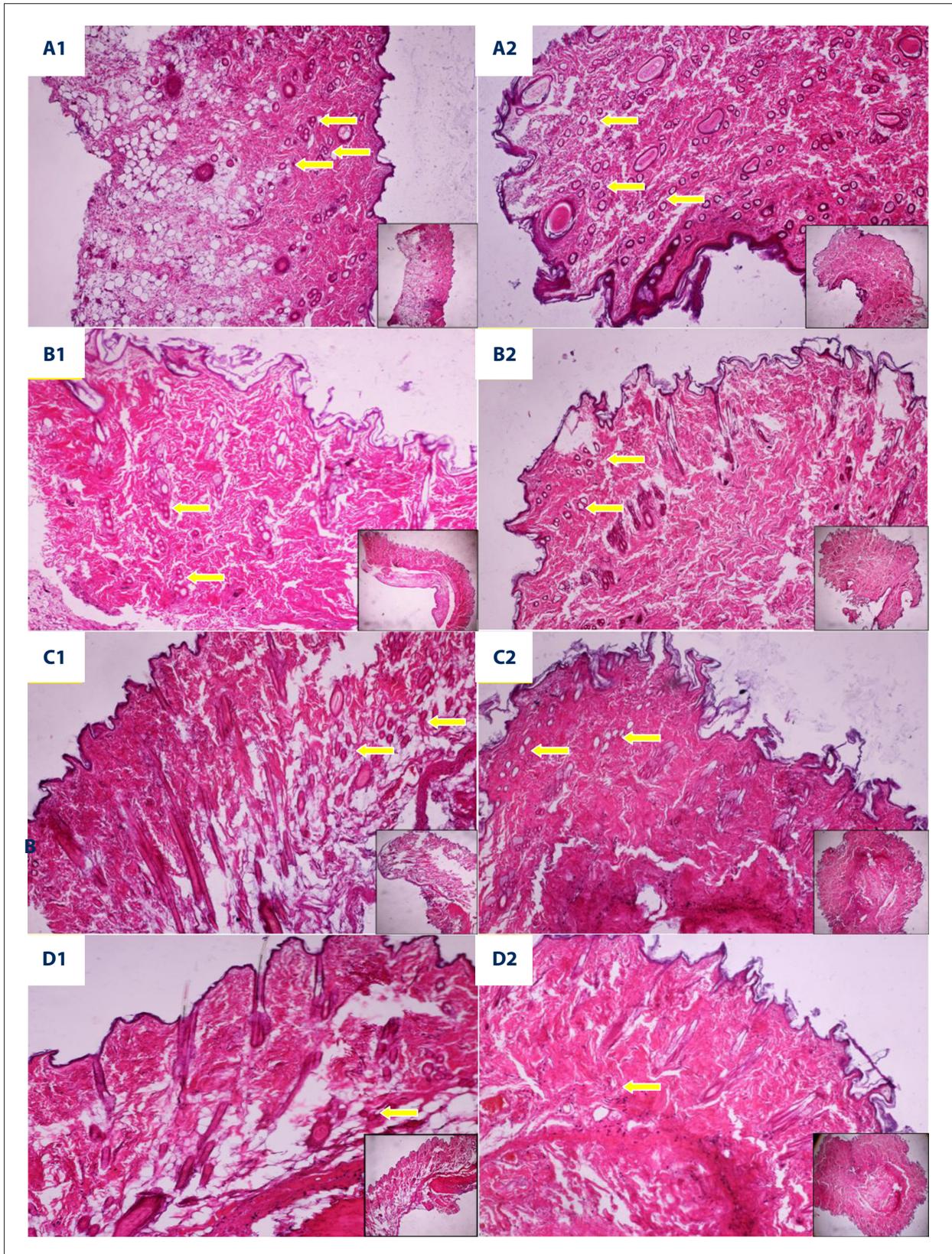
**Figure 4.** (A) The results from 3 groups of Hu-MSC (transfection with "F-5"), Hu-MSC (Transfection without "F-5"), and Hu-MSC (Non-transfection) at 0 h, 6 h, 12 h, and 18 h using the materials and methods of cell scratch test mentioned. (B) Cell migration areas of 3 groups of Hu-MSC (transfection with "F-5"), Hu-MSC (transfection without "F-5") and Hu-MSC (non-transfection) at 6 h, 12 h, and 18 h. Data are expressed as the mean  $\pm$ SD of the experiments performed in triplicate, \*  $P < 0.05$ .

healing process is affected by the elasticity of skin, and the skin of rats heals better than human skin [33]. To reduce the impact of this limitation on the experimental data, we also added a control group in this experiment.

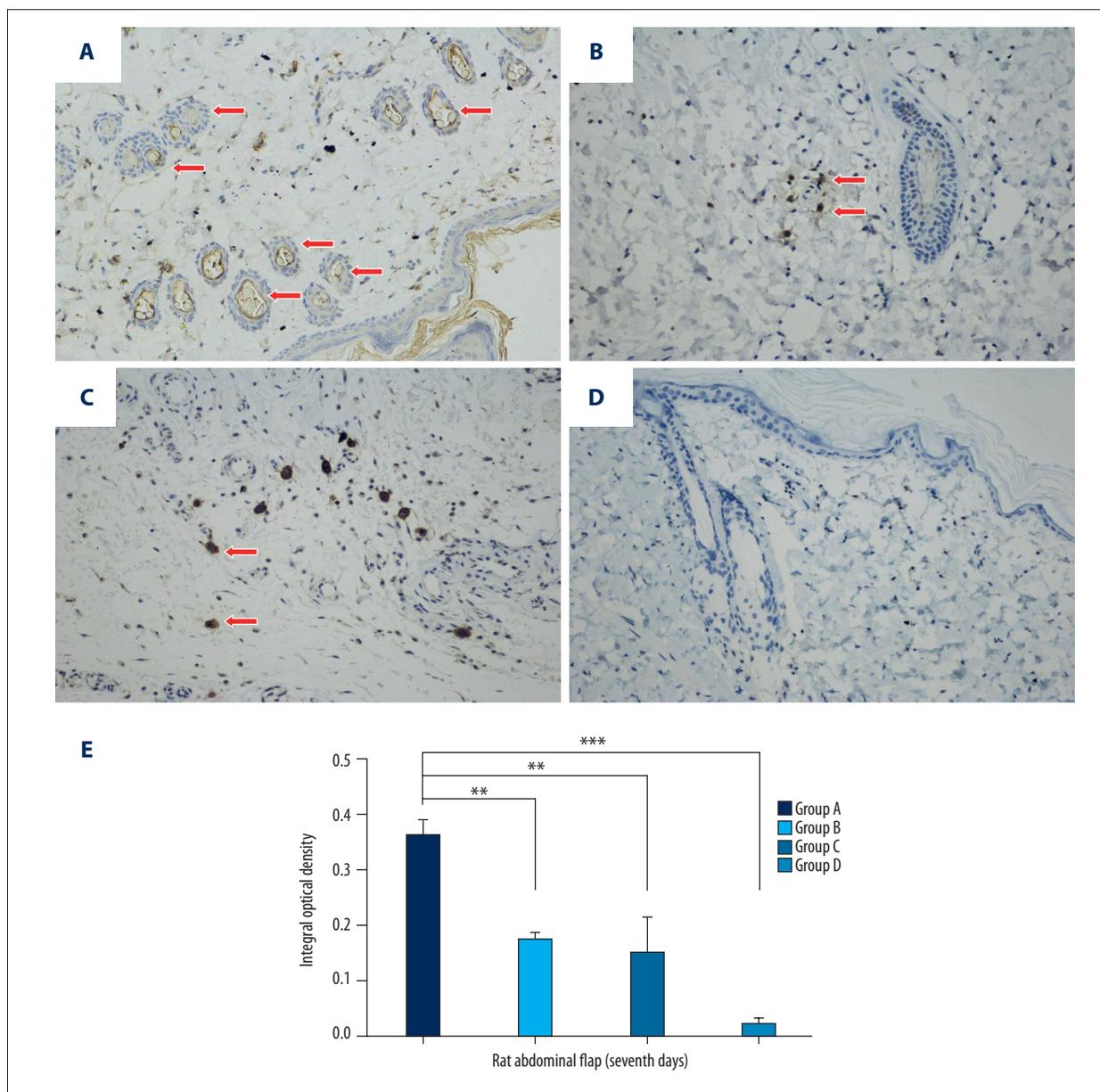
A variety of stem cells have been used in the study of ischemia-reperfusion injury, among which BM-MSCs, AD-MSCs, and hUC-MSCs are the most commonly investigated sources. However, we chose hUC-MSCs combined with "F-5" gene to perform this experiment because of the positive effect of



**Figure 5. (A)** View of the necrosis situation of abdominal perforator flaps in rats from the 4 groups on the day 1, day 3, day 5, and day 7. Rat abdominal perforator flaps were given point-infection with the materials and methods mentioned above. Group A: samples from Hu-MSC (transfection with “F-5”). Group B: samples from Hu-MSC (transfection without “F-5”). Group C: samples from Hu-MSC (non-transfection). Group D: samples from control group. **(B)** The necrotic area of rat flap models from the above 4 groups on day 1, day 3, day 5, and day 7 (n=6, \* P<0.05, \*\* P<0.01).



**Figure 6.** A1, B1, C1, and D1 showed the vascular distribution on the cross-section of the 4 groups of flap tissues. A2, B2, C2, and D2 show the vascular distribution on the longitudinal section of the 4 groups of flap tissues.



**Figure 7.** The results of CD<sub>31</sub> by immunohistochemistry of rat abdominal flaps from the 4 groups on day 7. (A) The morphologic observation of flap tissue injected with Hu-MSC (transfection with "F-5"). (B) The morphologic observation of flap tissue injected with Hu-MSC (transfection without "F-5"). (C) The morphologic observation of flap tissue injected with Hu-MSC (non-transfection). (D) The morphologic observation of flap tissue in the control group. (E) Average integral optical density of the rat abdominal flap slices from the 4 groups on the day 7. n=6, \*\* P<0.01, \*\*\* P<0.001.

stem cells, especially hUC-MSCs, on healing ischemia-reperfusion injury and the promoting effect of "F-5" gene on cell migration in wound healing [34]. The combination of these 2 promoting factors can theoretically improve the effectiveness of either single treatment on ischemia-reperfusion injury of skin flaps, which provides hope for patients to achieve a better prognosis after flap transfer operations.

In this experiment, we used adenovirus to package the "F-5" gene segment and transfect hUC-MSCs. Through the gradient experiments on the effect of viral transfection, we found that when MOI=100, the transfection rate was as high as 93%. Under such circumstance, we used MTT assay to detect cell toxicity and relied on the software "SPSS" for the analysis of variance; the results showed that adenovirus carrying "F-5" gene has very little toxicity on hUC-MSCs, which can be neglected (the results had statistical significance). Afterwards,

we did qPCR test to measure the expression of “F-5” gene in hUC-MSCs, and found that the expression of “F-5” gene in transfected hUC-MSCs was 3.5 times that in pure hUC-MSCs. Finally, we found that the cell migration rate of hUC-MSCs transfected with “F-5” gene was significantly improved in the cell scratch test.

With the assistance of cell transfection technique, we successfully integrated the “F-5” gene into hUC-MSCs. To assess the effect of this new combined treatment, we point-injected the integrated hUC-MSCs on the ischemia-reperfusion injury model of rats’ skin flaps. The results showed that the new treatment can effectively improve the survival rate of skin flaps with much better effect than that of pure hUC-MSCs. According to some study reports, CD<sub>31</sub> can directly improve angiogenesis, while pure hUC-MSCs do not have the ability to secrete this factor, so we could judge the effect of this new treatment by measuring the content of CD<sub>31</sub> and the vascular density in skin flaps [35]. To further confirm this conclusion, on the 7<sup>th</sup> day after we built the animal models, we also used histology methods such as HE staining and immunohistochemistry to measure the results in each group, which were also satisfactory. The content of CD<sub>31</sub> in the group with hUC-MSCs and “F-5” gene was obviously higher than that of the group with hUC-MSCs and “non-carrying” virus and the group with pure hUC-MSCs as well as the control group with no treatment. Moreover, the results from HE staining also showed that the density of capillaries in the group injected with the hUC-MSCs transfected with “F-5” gene was higher and more evenly distributed than that of the other 3 groups.

In summary, the transfection of “F-5” gene provided cells a template with which to synthesize the proteins that can promote

the migration process of epidermal cells. With hUC-MSCs as a promotor for healing process of ischemia-reperfusion injury, both the effectiveness and healing time of ischemia-reperfusion injury of skin flap models were improved. The new “stem cells-gene” combination therapy provides a new approach to the study of treatment for ischemia-reperfusion injury after plastic and reconstructive surgeries.

## Conclusions

The point-injection treatment of hUC-MSCs transfected with “F-5” gene can effectively promote the repair process while shortening the healing time of ischemia-reperfusion injury of skin flaps.

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## Appendix

In this study, we applied picric acid at the edge of the flap wounds to prevent the experimental rats from biting their wounds.

## Conflict of interest

The authors have declared that there is no conflict of interest in this study.

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