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# The effects of recombinant human growth hormone (rHGH) on survival of slender narrow pedicle flap and expressions of vascular endothelial growth factor (VEGF) and classification determinant 34 (CD34)

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**Abstract. – OBJECTIVE: To explore the effects of recombinant human growth hormone (rHGH) on the survival of the mouse slender narrow pedicle flap and the expressions of vascular endothelial growth factor (VEGF) and classification determinant 34 (CD34).**

**MATERIALS AND METHODS: A total of 20 BALB/c mice were randomly divided into the experimental group (n=10) and control group (n=10). The flaps were transplanted for mice in two groups respectively. 6 h after the operation, the mice in the experimental group were administrated with rHGH via local subcutaneous injection, while the mice in the control group were injected with the same amount of normal saline. The laser Doppler was used to measure the subflap blood flow rates before the operation, and 3 days, 7 days and 14 days after the operation, respectively; the flap necrosis and survival areas of mice in two groups were measured respectively, and the survival rate of the flap was calculated 14 days after the operation. Afterwards, the flaps of mice in two groups were exfoliated and the shape and structure of flap tissues were tested by the hematoxylin-eosin (HE) staining. Reverse transcription-polymerase chain reaction (RT-PCR) and Western blot were used to test the levels of mRNA and protein of VEGF and CD34 in the flap tissues.**

**RESULTS: The flaps of mice in the control group mainly exhibited the black or grayish-black and lost the elasticity with the hard texture, while those in the experimental group were ruddy in color with favorable elasticity. The survival rate of flaps of mice in the experimental group was significantly higher than that in the control group (83.61 ± 12.56% *vs*. 46.25 ± 9.70%) and the necrosis area of flaps of mice in the experimental group was significantly smaller than that in the control group (1.32 ± 0.16 *vs*. 4.13 ± 0.35, $p < 0.05$). There were no statistical differences in the blood flow rates of mouse flap both before the operation and three days after the operation between two groups ($p > 0.05$), while the blood flow rates of mouse flap both 7 days and 3 days after the operation in the experimental group were higher than those in the control group ($p > 0.05$). Compared with those in the control group, the levels of VEGF and CD34 were significantly increased, but the levels of the inflammatory factors of the interleukin-6 (IL-6) and tumor necrosis factor-α (TNF-α) were significantly decreased ($p < 0.05$).**

**CONCLUSIONS: rHGH plays an active role in the survival of the flap through promoting the angiogenesis and inhibiting inflammatory reaction.**

*Key Words:*
Recombinant human growth hormone (rHGH), Slender narrow pedicle flap, VEGF, CD34.

## Introduction

Flap transplantation is one of the most important surgical techniques in the reconstruction surgery and widely applied to the wound coverage in the orthopedic surgery, such as the skin repair after the accidental trauma or tumour-reductive surgery[1,2]. However, the local necrosis of flaps is the main challenge for this technique. The survival of flap depends not only by subcutaneous tissues, but also by its blood supply[3]. In particular, the blood hypo perfusion and ischemia reperfusion injury of wound cause some adverse changes in the tissue and vasculature system, leading to flap necrosis[4].

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The mechanism of angiogenesis, as the important foundation of the flap, is very complicated[5]. Angiogenesis starts from the migration of vascular endothelial cells and then new blood vessels are formed to connect to the existing circulation[6]. During this process, the vascular endothelial growth factor (VEGF), as a kind of multi-functional angiogenesis regulator, plays an important role in promoting the epithelial cell proliferation, angiogenesis, and vascular endothelial cell survival[7]. Classification determinant 34 (CD34), as the marker of vascular endothelial cells, is the key factor of the angiogenesis[8] and can participate in the flap survival process.

Recombinant human growth hormone (rHGH) aroused people's interest in increasing the survival rate of flap. As is well-known, rHGH features in the regulation of growth, bone metabolism and wound healing process[9]. However, the mechanism of rHGH facilitating angiogenesis in the flap transplantation is not clear yet. Therefore, the primary purpose of this study was to investigate the effects of recombinant human growth hormone (rHGH) on the survival of the mouse slender narrow pedicle flap and expressions of vascular endothelial growth factor (VEGF) and classification determinant 34 (CD34).

## Materials and Methods

### Building of Animal Model

20 BALB/c mice were randomly divided into the experimental group (n = 10) and the control group (n = 10) and the average weight was 20 ± 1.2 g. All mice received the intraperitoneal anesthesia with 10% chloral hydrate at the dosage of 0.05 mL/20 g. The electric scissors were used to shave off the hair on the back surface of mice. The mice were fixed on the holding plate in the ventral decubitus and were sterilized routinely. Using the Billingham's method, the left hand held the ophthalmic forceps to pick up the integument in a diameter of about 1 cm, while the right hand sheared the integument along the lower edge of ophthalmic forceps. A long and narrow pedicle flap was designed on each side of the dorsal midline of rats, the vertical axis of flap was almost perpendicular to the dorsal midline, and the proximal part of pedicle was about 1 cm away from the midline. The skin was cut along the flap design line till the deep fascia (skin tissue structure of rats: the epidermal layer, corium layer, superficial fascia layer, deep fascia layer and muscle layer from outside to inside), and the flap was carefully lifted. The flap color was observed after hemostasis, and was put back to its original position and sutured in situ (4 cm × 2 cm) (pedicle aspect ratio: 1:1, flap size: 3 cm × 3 cm) (Figure 1). After the fascia and other connective tissues in the implant were removed, the integuments were placed in the culture dish. Afterwards, the implants were transplanted into the backs of the original donor mice and the anastomotic stomas were sutured by needles. Moreover, 0.1% penicillin cotton ball was used to slightly wipe the wound; three layers of fine gauze were used to cover the wound and another one layer of gauze was added outside. At last, the zinc oxide adhesive plaster was used to bind up the wound. 6 h after the operation, the mice in the experimental group were administrated with rHGH (0.05 mL, 10 mg/kg, once per 3 days) via subcutaneous injection at 1 cm place from the skin-grafting anastomotic stoma, while the mice in the control group were injected with the same amount of normal saline. 14 days after the operation, the flap survival of mice in two groups was observed and the total area, necrosis area and survival area of flaps were recorded. The present study was approved by the Ethics Committee on Animal Protection of the Second Affiliated Hospital of Soochow University.

### In Vivo Measurement of Flap Microcirculation and Assessment of Changes in the Flap Blood Flow Volume

Before the operation, and 3 days, 7 days and 14 days after the operation, the laser Doppler hematometry was used to continuously measure the blood vessels at different sites of mouse flaps in two groups with an interval of 1 min; the mean

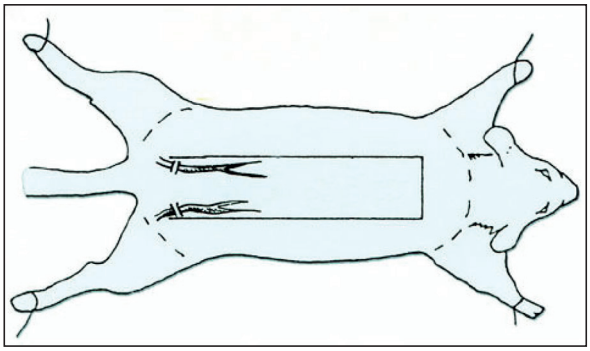

**Figure 1.** Slender narrow pedicle flap model.

value was obtained. Data were collected and Peri-fluxs system 5000 (Perimed AB, Stockholm, Sweden) was used to assess the sub-flap blood flow of mice.

### Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

14 days after the operation, flaps of mice in two groups were exfoliated and the total RNA was extracted with the TRIzol as instructed by the manufacture (Invitrogen, Carlsbad, CA, USA). The extracted total RNA was dissolved into diethylpyrocarbonate (DEPC)-treated water. The spectrophotometry was used to measure the concentration of RNA under 260 nm. The reverse transcription kit (TaKaRa, Otsu, Shiga, Japan) was used to synthesize cDNA with 1 μg mRNA (Article No.: 639505, Tokyo, Japan). ReverTra Ace qPCR RT Kit (Torobo, Article No.: FSQ-101, Tokyo, Japan) was used to determine the mRNA level of each indicator. The reaction conditions are as follows: 50°C for 10 min; 95°C for 15 s; 60°C for 30 s; 40 cycles in total, with the reduced glyceraldehyde-phosphate dehydrogenase (GAPDH) as the internal reference. The calculation formula of the relative expression of each indicator is $2^{-\Delta Ct}$ [$\Delta Ct$ = Ct (target gene) – Ct (GAPDH)]. The primer sequence of each target gene is as follows: GAPDH (forward: GATGCTGGTGCTGAGT-ATGTCG; reverse: TGGTGCAGGATGCATTGCTGA); VEGF (forward: TGCACCCACGACAGAAGG; reverse: GCACACAG-GACGGCTTGA); CD34 (forward: ATCGGAGAGCCACT TATGCT; reverse: ATGCTCGGACATCGAGC); IL-6 (forward: AATCTGCTCTG GTCTTCTGGAG; reverse: GTTGGATGGTCTTGGTCCTTAG); TNF-α (forward: GACTTTAAGGGTTACCTGGGTTG; reverse: TCACATGCGCCTT GATGTCTG).

### Western Blot

Flaps of part of mice were cut into pieces and added with the appropriate amount of mixed solution of RIPA tissue lysate (Beyotime, Shanghai, China) and 1% cocktail protease inhibitor (Proteintech, Chicago, IL, USA) for homogenate. After the resulting solution (at 13,000 g) was centrifuged for 30 min, the supernatant was absorbed to determine the protein concentration. The 40 μg protein sample was separated with sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). The total protein was transferred onto polyvinylidene difluoride (PVDF) membrane and the stripes were incubated with the primary and secondary antibodies of VEGF, CD34, IL-6 and TNF-α. The abundance of the target protein was tested by the hypersensitive chemiluminescence solution (Millipore, Billerica, MA, USA) in enhanced chemiluminescence system (ECL) (Millipore, Billerica, MA, USA).

### Statistical Analysis

All results of the present experiment were analyzed by Graphpad software (La Jolla, CA, USA). The measurement data were expressed in $\bar{x} \pm s$ and the difference among indicators was tested by paired *t*-test method. $p < 0.05$ represented the difference was statistically significant.

## Results

### Comparison of Flap Survival

Most flaps of mice in the control group were black or grayish-black and lost the elasticity with hard texture; hematomas were formed below parts of flaps, even with the fester or exudation in local parts. As for the mice in the experimental group injected with rHGH, the flaps were the same as the surrounding normal tissues and ruddy with favorable elasticity, but a little edema or necrosis was seen. In addition, in the experimental group, the flap survival rate of mice was significantly higher than that in the control group (83.61 ± 12.56% *vs.* 46.25 ± 9.70%, $p < 0.05$) and the flap necrosis area of mice was significantly smaller than that in the control group (1.32 ± 0.16 *vs.* 4.13 ± 0.35, $p < 0.05$) (Figure 2 and Table I).

### Hematoxylin-eosin (HE) Staining Detection of Histological Pattern of Flap

According to Figure 3, a large number of flap tissue cells of mice in the control group necrotized and the cavities were formed. Even inflammatory cell infiltration was seen in local regions. The flaps of mice in the experimental group had the normal histological patterns with few necrosis regions.

### Comparison of Sub-Flap Blood Flow of Mice Between Two Groups

The laser Doppler was used to examine the sub-flap blood flow of mice in two groups before the operation and 3 days and 7 days after the operation respectively. Figure 4 shows that there was no statistical difference ($p > 0.05$) in the sub-flap blood flow rate of mice between

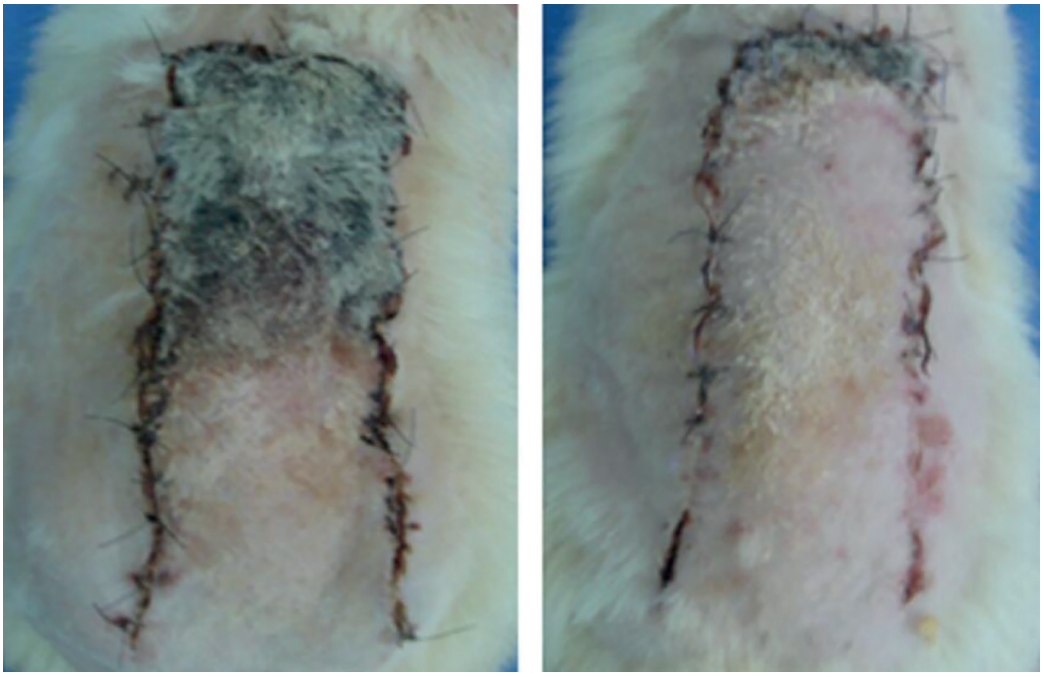

**Figure 2.** Flap survival of mice in two groups.

**Table I.** Comparison of mouse flap survival rate between two groups.

| Group | N | Flap necrosis area ($cm^2$) | Total flap area ($cm^2$) | Flap survival rate (%) |
|---|---|---|---|---|
| Control group | 10 | 4.13 ± 0.35 | 10 | 46.25 ± 9.70 |
| Experimental group | 10 | 1.32 ± 0.16* | 10 | 83.61 ± 12.56** |
| *t*-value | | 1.32 | 10 | 2.31 |
| *p*-value | | 0.027 | | 0.0081 |

Compared with the control group, *$p < 0.05$; **$p < 0.01$.

two groups before the operation and 3 days after the operation, respectively. 7 days and 14 days after the operation, the sub-flap blood flow rate of mice in the experimental group was significantly higher than that in the control group ($p < 0.05$).

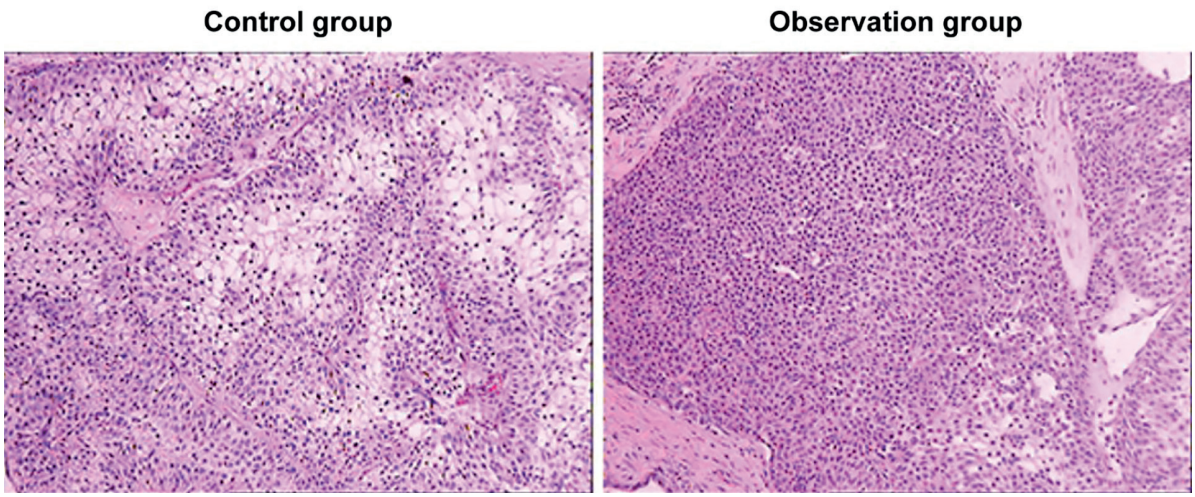


**Figure 3.** Histological patterns of mouse flap in the two groups through HE staining detection (×100).

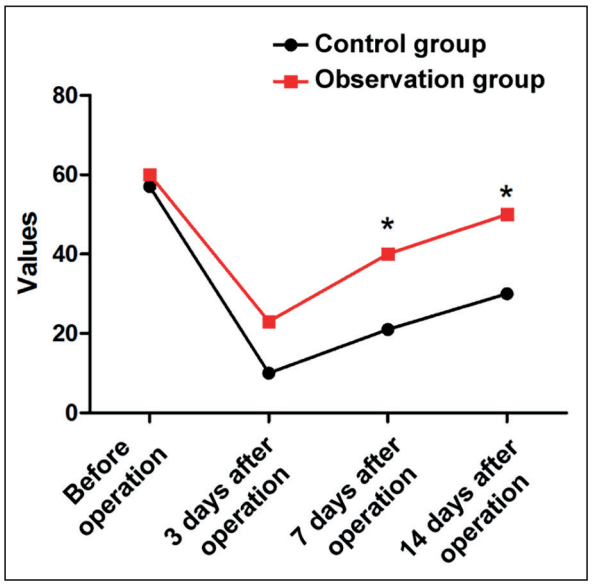


**Figure 4.** Sub-flap blood flow rate detected through laser Doppler. Compared with the control group, *$p < 0.05$.

### *Effects of rHGH on Levels of VEGF and CD34*

14 days after the operation, RT-PCR and Western blot were used to test the mRNA and protein levels of VEGF and CD34 in the mouse flap tissues. According to the results, compared with those in the control group, the levels of VEGF and CD34 in the experimental group were significantly increased ($p < 0.05$) (Figure 5).

### *Levels of Inflammatory Factors in Flap Tissues of Mice in Two Groups*

After the transplantation, the flap inflammation and necrosis appeared; hence, the mRNA and protein levels of the inflammatory factors IL-6 and TNF-α in the flap tissues were further explored. According to Figure 6, the mRNA and protein levels of IL-6 and TNF-α in the experimental group were lower than those in the control group with the statistically significant differences ($p < 0.05$).

## Discussion

As flaps are widely applied to the orthopedics, the researchers have synthesized multiple medicines, such as vasodilator substance, calcium antagonist and prostaglandin inhibitor to alleviate the flap ischemia and increase the survival rate[10,11]. However, these medicines did not radically change the current condition. It is well-known that rHGH belongs to a type of hormone substance, which can irritate the generation of granulation tissues and facilitate the re-epithelialization and angiogenesis[12]. A literature report on the clinical treatment of burned patients showed that the administration of rHGH on burned patients can accelerate the healing of wound, and is conducive to maintaining the ortho-nitrogen balance, reducing the stay time[13]. It has also been proved that rHGH shortens the healing time of the donator site[14]. For large-area burned patients, the systematic administration of rHGH irritates the formation of granulation tissues and increases the collagen deposition, promoting the wound healing[15]. According to the results of another investigation, rHGH accelerates the healing of gastric ulcer through promoting the proliferation of cells at the ulcer margin and growth of new blood vessels[16]. Therefore, it was assumed that rHGH can improve the blood supply

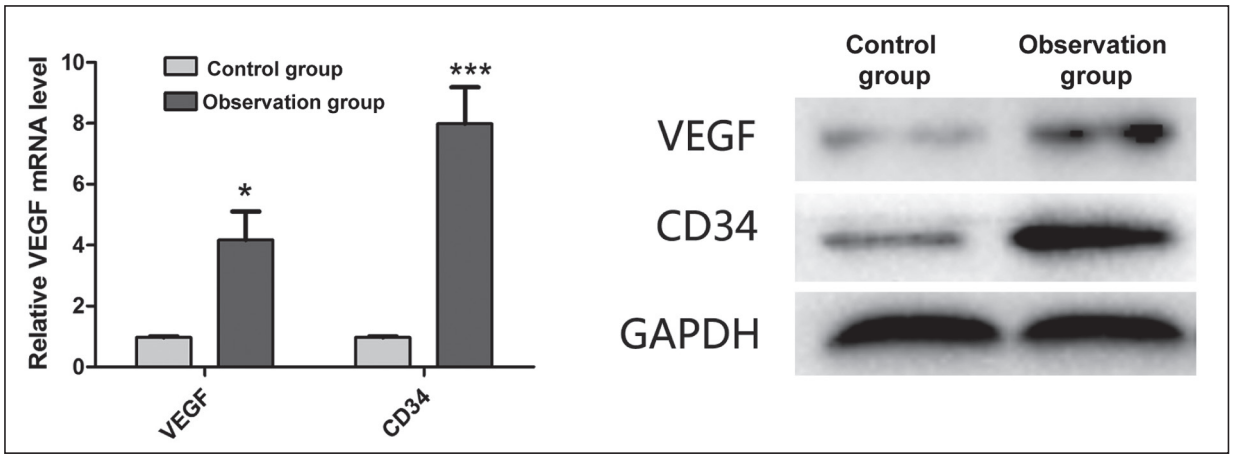


**Figure 5.** Effects of rHGH on mRNA and protein levels of VEGF and CD34. Compared with the control group, *$p < 0.05$; ***$p < 0.001$.

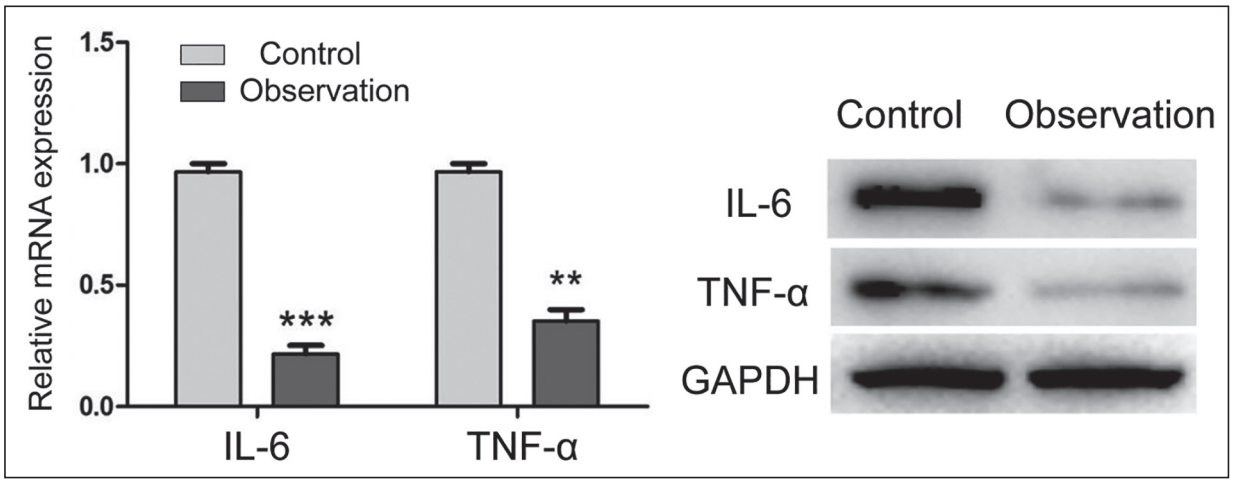


**Figure 6.** Levels of inflammatory factors in flap tissues of mice in two groups. Compared with the control group, $^{**}p < 0.01$; $^{***}p < 0.001$.

in the flap reconstruction process through promoting angiogenesis. The angiogenesis of flap is a complicated process, which is initiated by the activation of vascular endothelial cells, followed by the degradation of the basement membranes. This promotes the formation of vascular buds, cell proliferation and growth of new blood vessels[17] and involves the cooperation between the proliferation of endothelial cells and multiple growth factors. It has been proved that the paracrine mechanism of cell growth factors plays an important role in angiogenesis[18]. According to a previous study[19], the typical signal pathway of VEGF receptor-tyrosine kinase is activated through interactions between VEGF receptors and vascular endothelial cells to play a powerful role in regulating endothelial cell mitosis. Moreover, VEGF can adjust the generation of plasminogen activator and inhibitor in endothelial cells, thus regulating the basement membrane degradation in the angiogenesis[20]. On the other hand, CD34 is highly expressed in the proliferated vascular endothelial cells and the level of CD34 can reflect the vessel density of tissues[21]. Previous studies have shown that the circular HGH has a small effect on the healing of wound, while the injection of rHGH into the local wound tissues is more significant for increasing VEGF mRNA level in the granulation tissues[22]. In the present experiment, the mouse flap was administrated with rHGH via subcutaneous injection after the transplantation and the laser Doppler were used to dynamically test the blood flow. 14 days after the operation, the survival of flaps and VEGF and CD34 expressions in flap tissues were inspected. The results showed that the treatment with rHGH raised the blood flow rate 7 days after the operation, which was consistent with the report by Bry et al[23]. At the same time, according to the results of RT-PCR and Western blot, the mRNA and protein levels of VEGF and CD34 in the experimental group were significantly increased. Compared with the mouse flaps in the control group, the flaps of mice injected with rHGH in the experimental group was ruddy with favorable elasticity and a little edema or necrosis was found. In addition, the levels of inflammatory factors (IL-6 and TNF-α) in the experimental group were lower than those in the control group.

## Conclusions

We showed that rHGH assists the treatment after the flap transplantation and facilitates angiogenesis. Furthermore, it can inhibit the inflammatory reaction, playing an active role in increasing the survival rate of flaps.

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### Conflict of Interest

The Authors declare that they have no conflict of interests.

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