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# Allogenic endothelial progenitor cell transplantation increases flap survival through an upregulation of eNOs and VEGF on venous flap survival in rabbits

**Zeyuan Jin, Chenglun Yao, Keshav Poonit, Tao Han, Sunlong Li, Zihuai Huang, Hede Yan***

*Department of Orthopedics (Division of Plastic and Hand Surgery), The Second Affiliated Hospital and Yuying Children's Hospital of Wenzhou Medical University, Key Laboratory of Orthopedics of Zhejiang Province, The Second School of Medicine, Wenzhou Medical University, Wenzhou, Zhejiang, China*

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**KEYWORDS**
Venous flap;
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**Summary** *Background:* Endothelial progenitor cells (EPCs) are one type of bone marrow hematopoietic stromal cells which play a vital role in neovascularization and tissue repair. In this study, we investigated whether EPCs promote flap survival in a rabbit venous model.
*Materials and methods:* EPCs were customized from CHI Scientific, Inc, China. Thirty-six rabbits were randomly assigned to either the sham group ($n=12$), the control group ($n=12$) or the EPC transplantation group ($n=12$). A $10\times6$ cm venous flap was created on the rabbit abdomen. Both the EPC transplantation and control groups had the same volume of EPCs-PBS (phosphate buffered saline) and PBS on postoperative day 1. Flap survival, blood flow, histopathology, expression of endothelial nitric oxide synthase (eNOs) and Vascular Endothelial Growth Factor (VEGF) were detected on postoperative day 10.
*Results:* Cellular immunofluorescence assay positively confirmed that the EPCs were undergoing differentiation. The survival rate of the flap in the EPC transplantation group was $58.4\pm7.1\%$, which was significantly higher than that of the control group ($4.8\pm3.4\%$) ($p<0.01$). Histological examination revealed that the EPC transplantation group had higher microvessel density, fewer inflammatory cells, and a higher expression of eNOs and VEGF. Significantly increased blood flow perfusion was seen in the EPC transplantation group using laser Doppler imaging. The Western Blot technique revealed that the expression of eNOs and VEGF in the EPC transplantation group were both significantly higher than those in the control group.

* Corresponding author.
*E-mail address:* yanhede@wzhealth.com (H. Yan).

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*Conclusion:* This study demonstrated that EPC transplantation improved venous flap survival in rabbits. The present findings may provide insight into the promotion of venous flap survival in clinical practice in the future.


## Introduction

In comparison with traditional flaps, venous flaps are easy to design. Harvesting a venous flap does not require deep dissection, there is no need to sacrifice a major artery at the donor site. There is also less donor site morbidity with optimal postoperative contour compared with other free microvascular tissue transfers.[1–4] Venous flaps are now primarily utilized to cover soft tissue defects of the hands and upper limbs as well as in the reconstruction of the face, head and neck. Furthermore, they are thinner and more pliable than conventional flaps. However, since only veins are present in the flap, ischemia and inadequate nutrient metabolism is inevitable.[5] Moreover, swelling, hemorrhage, shrinkage, and necrosis often occur in the late stage.[6] Many methods have been attempted to improve flap survival, however, few have been consistently effective.[6]

Given the appropriate environmental conditions, endothelial progenitor cells (EPCs), precursors of mature endothelial cells, can exert beneficial angiogenesis and vascular repair functions, including differentiation into endothelial cells and the release of repair-promoting factors.[7] Studies by Ceradini and co-workers have shown that in ischemia-anoxia, tissue is also more conducive to EPC localization, maintenance and regeneration.[8,9] EPC transplantation is a reported therapeutic strategy for promoting blood vessel regeneration.[10] Studies have shown that EPCs have low antigenicity in local allogeneic transplantation.[11] Therefore, EPC transplantation may be an appropriate treatment for the ischemic condition of the venous flap. In this study, we hypothesized that allogenic endothelial progenitor cell transplantation would increase flap survival through an upregulation of eNOs and VEGF on venous flap survival and our goal was to verify the function of allogenic endothelial progenitor cell transplantation in improvement of venous flap survival in rabbits and to develop a feasible strategy to improve the survival of this flap.

## Methods

### Animal

Adult white Japanese rabbits of both sexes were used in the study; each rabbit weighed from 3 to 3.5 kg. All experiments were executed in accordance with ethical guidelines (the Institutional Animal Care and Use Committee of Wenzhou Medical University), and the National Research Council's Guidelines for the Care and Use of Laboratory Animals were followed. The experimental study was based on a superficial epigastric venous flap (10 × 6 cm) (Figure 1(A)). Thirty-six rabbits were randomly divided into three groups with 12 rabbits in each group: the sham group, the EPC transplantation group, and the control group.

### Flap model

We anaesthetized the rabbits with 3% (w/v) pentobarbital (3 ml/kg) using an ear vein injection. Then the abdominal area was carefully shaved with a hair clipper and a 10 × 6 cm (length × width) skin flap was projected along the left thoracoepigastric vein symmetrically (Figure 1(A)). In the sham group, the flaps were dissociated (both ends of the left thoracoepigastric artery and vein were retained as a physiologic flap). In the control and EPC transplantation groups, both ends of the left thoracoepigastric artery were ligated completely, leaving the vein intact. Finally, the flap was returned to the original site and sutured with 4-0 nonabsorbable suture materials. During the operation, the rabbits were anesthetized by multiple injections of pentobarbital. Treatments were given 24 h after the operation. EPCs-PBS (10^5/4 ml) was slowly transplanted into the flap of the EPC transplantation group (hypodermic injection [Hypo]). Rabbits in the sham and control groups received the same volume of PBS (Hypo). All surgical procedures were performed aseptically. When the operation was complete, we used a soft padded dressing to cover the operative site in order to prevent infection.

### Cells and reagents

The primary EPCs of white Japanese rabbits were customized from CHI Scientific, Inc. (Shanghai, China). The anti-VEGF primary antibody and anti-eNOs primary antibody were purchased from Abcam Co, Ltd (Shanghai, China). Dil-ac-LDL was purchased from Yiyuanbiotech (Guangzhou, China). FITC-uea-1 was purchased from Sigma (Missouri, USA).

### Cellular fluorescence assay

The primary EPCs of white Japanese rabbits were expanded to the second generation. Then the cells were cultured for 24 h in media containing 10 ug/ml 1,10-dioctadecyl-3,3,30,30-tetramethylindocarbocyanine-labeled acetylated low-density lipoprotein (Dil-ac-LDL) and mixed with 4% paraformaldehyde for 30 min. Then they were mixed with 10 ug/ml FITC-UEA-1 at 37 °C for 1 h, and observed under a fluorescence microscope (Nikon, Japan).

### Flap viability

On postoperative day 10, the size of surviving and necrotic areas was measured using Image J to assess the survival area of the flap. Areas of darkness and eschar formation were considered to be necrotic. The results are shown as a percentage of the surviving area and were calculated

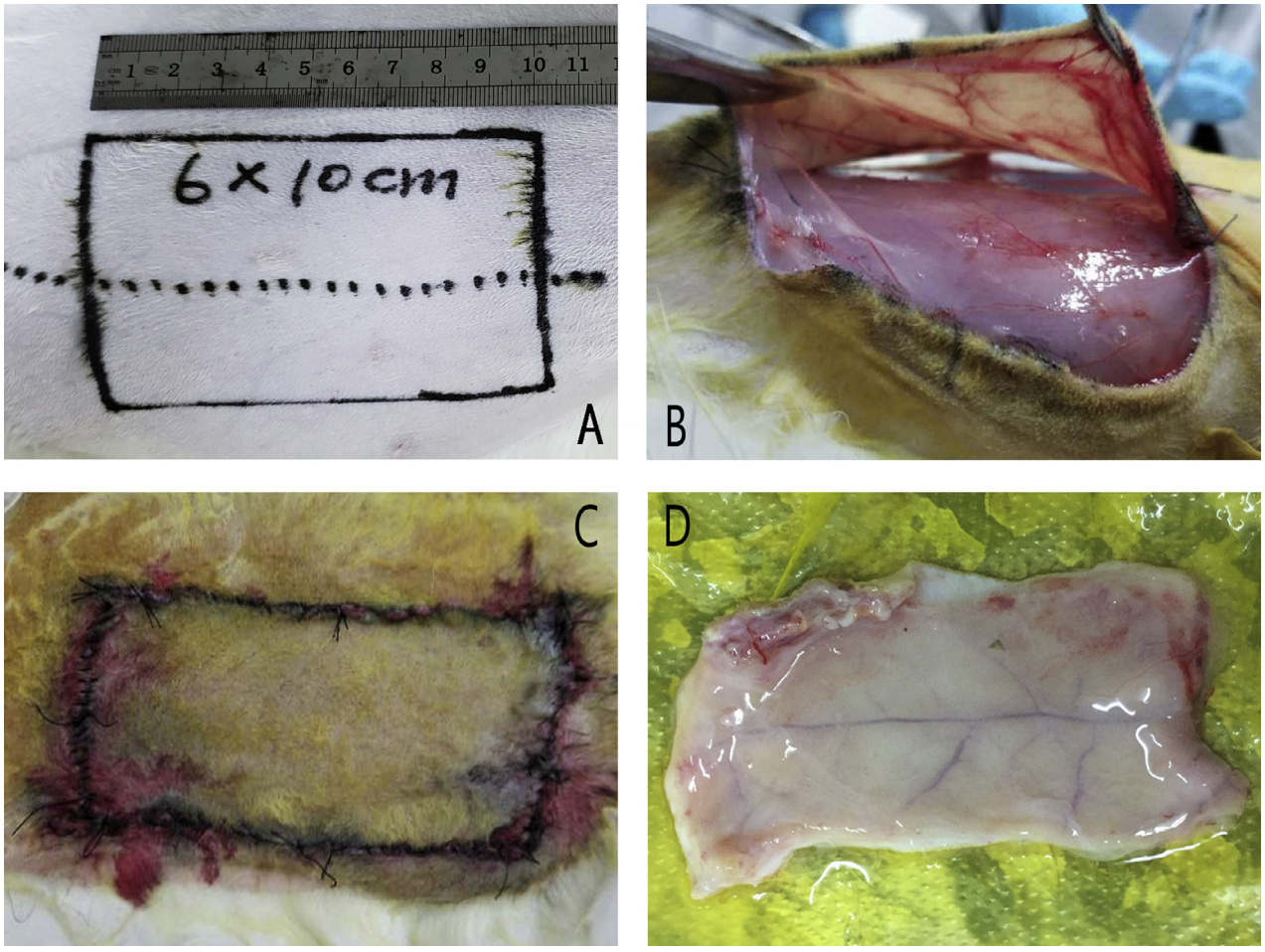


**Figure 1** (A) The venous flap model was designed (6 × 10 cm); (B) The flap was elevated; (C) The flap was sewn in the original position; (D) On the postoperative day 10, the flap was removed and restored for the next test.

according to the formula: Survival rate = survival area/total area × 100%.

## Western blot analysis

On postoperative day 10, after the rabbits were sacrificed, we collected skin samples from the center of the venous flaps. The total protein from rabbit tissues was extracted by a homogenizer. At the same time, a radioimmunoprecipitation assay (RIPA) lysis buffer with 1 mM Phenylmethanesulfonyl fluoride (PMSF) was used to promote better lysis of rabbit tissues. Then the homogenized tissue sample was centrifuged at 12,000 rpm for 30 min at 4 °C. Following this we used a bicinchoninic acid (BCA) protein assay kit (Beyotime Biotechnology, Shanghai, China) to measure the protein concentration. 80ug of protein was separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to a polyvinylidene difluoride (PVDF) membrane. We then blocked the membranes using 5% nonfat milk for 2 h, after which the membranes were incubated with primary antibodies VEGF (1:1000), eNOs (1:500), and beta actin (1:3000) overnight at 4 °C, and then incubated with their respective secondary antibodies for 2 h. Then the stripes were visualized by electrochemiluminescence plus reagent (Invitrogen). Finally, we quantified the intensity of these stripes by using Image Lab 3.0 software (Bio-Rad, Hercules, California).

## Histopathological assessment

After the rabbits were sacrificed, tissues at the center of the venous flaps were removed from all of the animals. Each sample (1 cm × 1 cm) was mixed with 4% paraformaldehyde for 24 h, then embedded using paraffin and cut into 5-μm sections for hematoxylin and eosin (H&E) staining. Finally, the number of microvessels and neutrophil infiltration were recorded.

## Immunohistochemistry staining

On postoperative day 10, skin samples from the rabbits in all three groups were fixed in 4% paraformaldehyde for 24 h, then the tissue was dehydrated with alcohol, embedded in paraffin and cut into 5-μm sections. Paraffin-embedded sections were deparaffinized with xylene and rehydrated. After that, the sections were immersed in 3% $H_2O_2$ to block endogenous peroxidase activities and then incubated with Trypsin-EDTA (Gibco by Life Technologies,

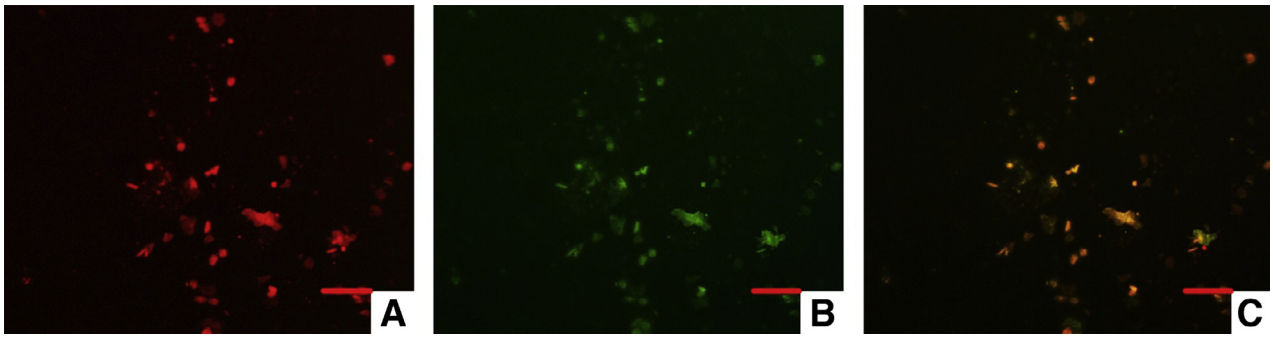


**Figure 2** Inverted fluorescent microscopy images of EPCs (×200). (A) EPC marked by Dil is displayed in red. (B) EPC marked by HITC is displayed in green. (C) Merge. Scale bar = 50 μm.

Canada) at 37 °C for 1 h until antigen retrieval. Non-specific sites were blocked by 10% normal goat serum at 37 °C for 30 min. The sections were then incubated with the primary antibody VEGF (1:200, Abcam) and eNOs (1:200, Abcam) overnight at 4 °C. Horseradish peroxidase labeled goat antibody was used as a secondary antibody. Finally, we counterstained the sections with hematoxylin for 10 min. The sections were dehydrated with alcohol, dealcoholized with xylene, and encapsulated with neutral resin. Sections were imaged using a DP2-BSW image acquisition system (Olympus Corp, Tokyo, Japan) at ×400 margination. Images were assessed using Image Pro Plus software, version 6.0 (Media Cybernetics, Inc, Rockville, Maryland), and the mean of the integrated option density (IOD) was used as an indicator of the VEGF and eNOs expression levels.

### Laser Doppler perfusion image

In a quiet and warm environment, after the rabbits were anesthetized, full-field laser Doppler perfusion images were obtained (Moor Instruments, Axminster, UK). The blood flow of each flap was measured on post operation day 10 and the perfusion images were processed. Each rabbit was measured three times and the mean value was used.

### Statistical analysis

All dates were expressed as a mean with a standard deviation; the results were analyzed using SPSS version 25.0 (IBM, Armonk, New York). The data from the three groups were compared using one-way analysis of variance (ANOVA). A $p$ value of less than 0.05 was statistically significant.

## Results

### Cellular fluorescence assay

EPCs take up DiI-ac-LDL (Figure 2(A)) and FITC-UEA-1 binding (Figure 2(B)) are stained yellow (Figure 2(C)) (overlay of A and B). According to reports in the literature, cells with both markers (DiI-ac-LDL and FITC-UEA-1) were considered to be EPCs which were differentiating.[12,13]

### General observation

From the 1st to the 10th day after surgery, the flaps were observed by the same observer and changes in color, elasticity, and necrosis were recorded. During that period, the flaps of the sham group had no obvious changes. The necrotic area of the other two groups increased from the first day, and the color of the necrotic area gradually became darker. By about the 9th to 10th days, the necrotic area was stable. The extent of necrosis in each flap was variable. All the necrotic sites and the remaining parts had clear boundaries. The necrotic skin color was black or dark brown, and the texture was hard. The remaining part of the skin is relatively soft and the color was the same as normal skin (Figure 3).

### Percentage of survival area

On postoperative day 10, the percentages of survival area were 92.0 ± 6.1% in the sham group, 58.4 ± 7.1% in the EPC transplantation group and 4.8 ± 3.4% in the control group ($p<0.01$) (Figure 3).

### Histopathological evaluation

A hematoxylin and eosin staining of the flaps was prepared to evaluate the effects of EPC transplantation on tissue structure, neutrophil infiltration, and granulation tissue thickness. On the 10th day after surgery, the flap structure in the sham group was well preserved, and intact hair follicles and blood vessels were observed, while the flaps were not significantly thickened. In the control group, the flap structure was broken, and the normal skin structure was not visible. The flap was incrassated and there was a substantial infiltration of neutrophils. The extent of skin flap destruction of the EPC transplantation group was between the sham group and the control group. A small number of hair follicles and blood vessels were observed; the inflammation level was lower in the EPC transplantation group than in the control group. (Figure 4)

### VEGF and eNOs expression

The VEGF level based on the mean of the IOD was 8.2 ± 0.5 in the sham group, 12.0 ± 0.5 in the EPC transplantation

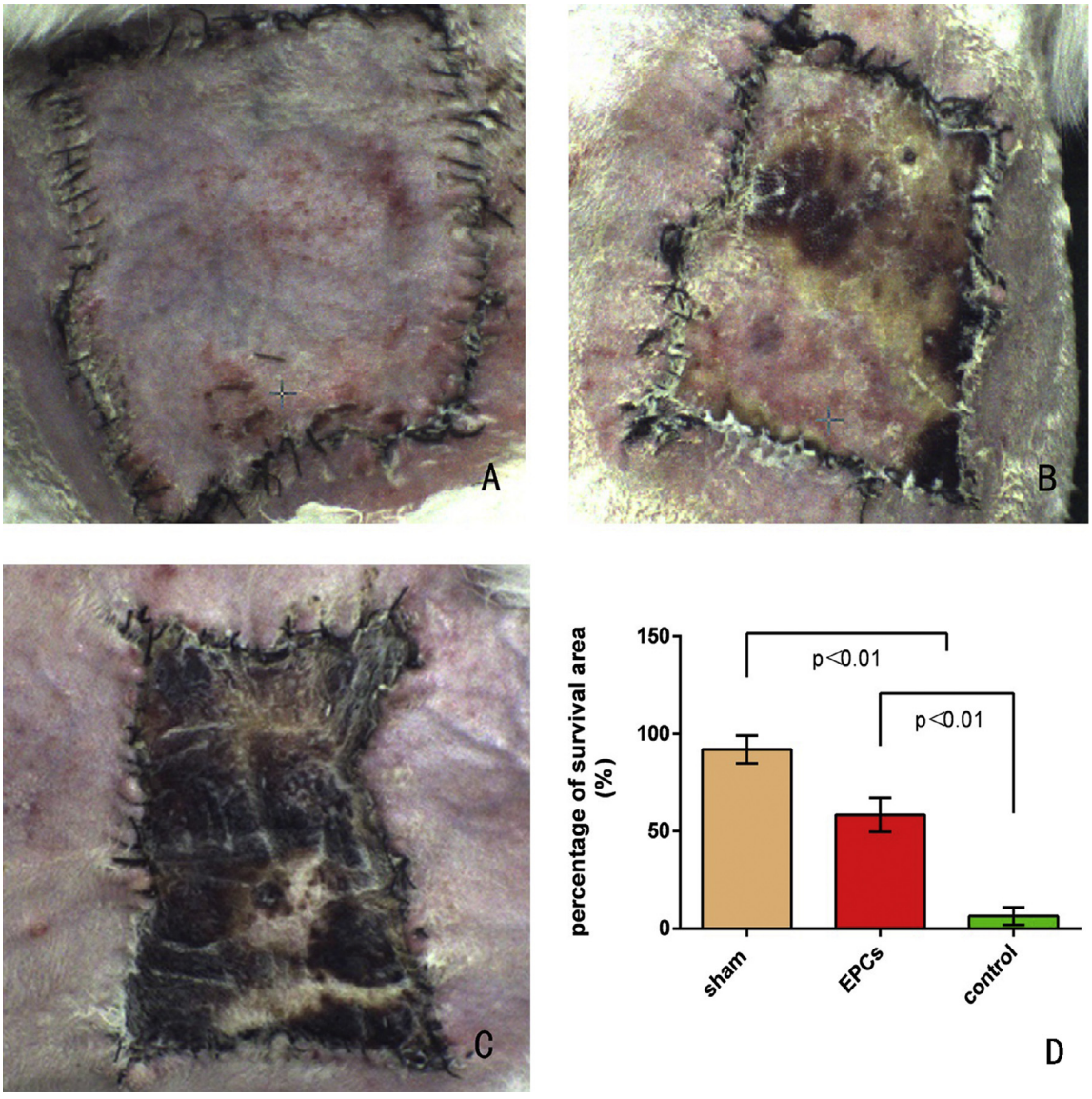


**Figure 3** Digital photographs of venous flaps from three groups (A, sham group; B, EPCs-transplantation group; C, control group). On the postoperative day 10, the surviving area of EPCs-transplantation group was markedly larger than the control group. (D) The survival rate of the three groups.

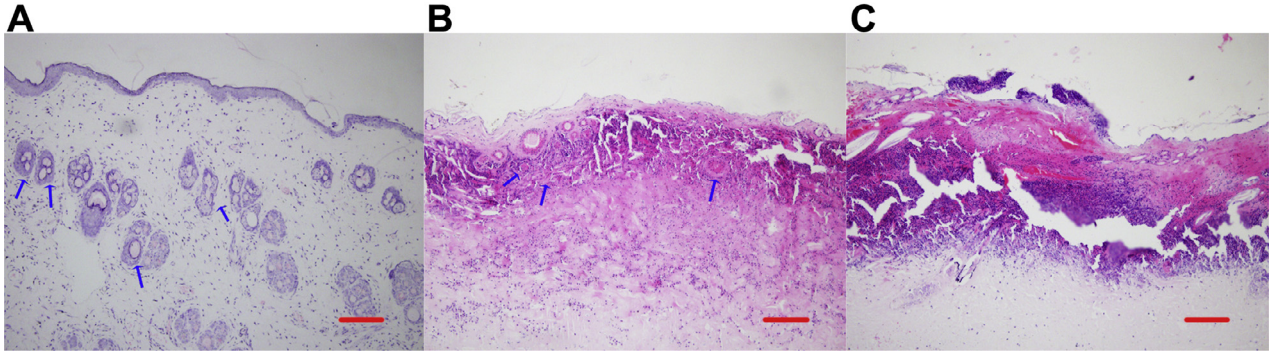


**Figure 4** Histological changes of the flap in the three groups (×100). The blue arrows represent the blood vessels. (A) The sham group: the skin tissue structure was complete, hair follicles and blood vessels could be seen without inflammatory cell infiltration; (B) The EPCs-transplantation group: flap tissue structure was still visible, with some inflammatory cell infiltration; (C) The control group: the structure of the flap was severely broken and there was a great deal of inflammatory cells infiltration. Scale bar=100 μm.

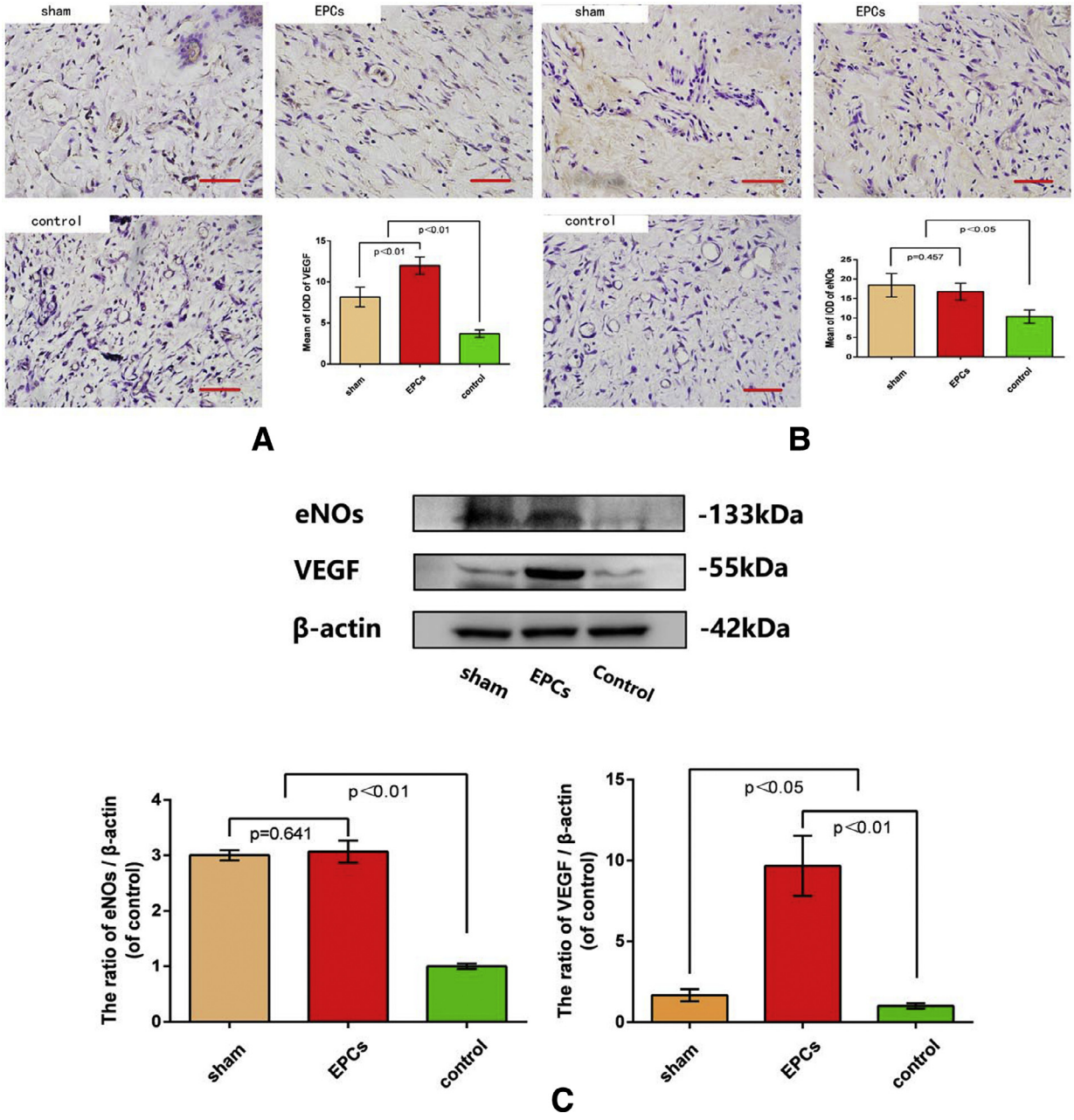


**Figure 5** (A&B) The expression of eNOs and VEGF in venous flap of three groups (×400). Scale bar=25 μm (C) Western blot analysis and quantification of eNOs and VEGF proteins.

group, and 3.7 ± 0.3 in the control group (Figure 5(A)). Immunohistochemical results showed that the mean of the IOD of eNOs in the sham group was 17.7 ± 1.4, 16.7 ± 1.3 in the EPC transplantation group and 10.4 ± 1.0 in the control group (Figure 5(B)). The Western Blot results showed that the VEGF and eNOs protein levels in the EPC transplantation group were markedly upregulated as compared to the control group ($p<0.05$) (Figure 5(C)).

## Blood flow

The percentage of blood flow for the sham group was 368.6 ± 38.4 PU, while that of the EPC transplantation group was 157.4 ± 19.8 PU. Compared with the control group (103.9 ± 9.0 PU), the blood flow of the venous flap in the EPC transplantation group (157.4 ± 19.8 PU) significantly increased ($p<0.05$) (Figure 6).

## Discussion

In comparison with traditional flaps, venous flaps are thin and pliable with a straightforward design and elevation. Most importantly, lower donor site morbidity is guaranteed and there is no sacrifice of the main arteries in the donor sites.[1] Venous flaps can be used as a composite flap to reconstruct the composite defects of vessels and tendons. Given

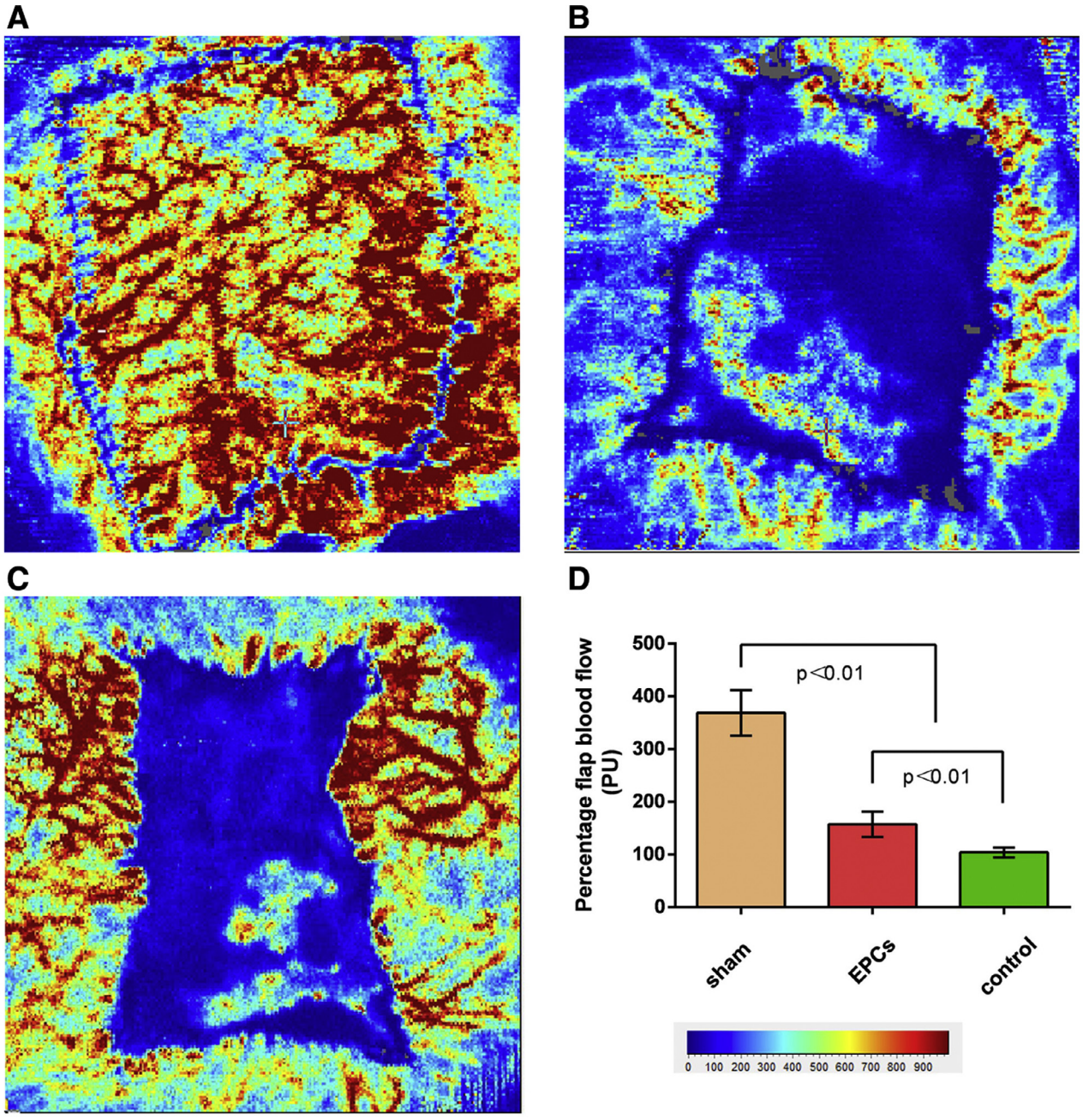


**Figure 6** On the 10th day after operation, venous flaps were performed with full field laser Doppler perfusion imaging. (A, sham group; B, EPCs-transplantation group; C, control group; D, the percentage flap blood flow of three groups). Red showed high perfusion, blue showed low perfusion, and gray showed no perfusion. The scale indicates the color of the perfusion value.

these advantages, venous flaps have been increasingly considered as the optimal choice for reconstruction of soft tissue defects in the hand, especially when conventional flaps are unavailable.[14]

However, because of their unconventional perfusion pattern and inconsistent survival in some cases,[15-18] the clinical application of venous flaps is still under further investigation. To date, many methods for reducing flap ischemia have been reported. They can be generally divided into two categories: preconditioning and postconditioning. Pre-surgical induction of angiogenesis in tissues (preconditioning) can limit postsurgical ischemic complications and improve outcomes. For instances, studies by several research groups have shown an effective, economical, and easy preconditioning to enhance vascularity of soft tissues successfully, safely and promptly.[19-21] As an "after-injury strategy," postconditioning is also a promising approach to reduce I/R injury and improve flap survival after ischemia.[22,23] However, due to the inherent difference in flap perfusion, these interventions for physiological flaps will not all work for the non-physiological venous flaps. In literature, several strategies have been introduced to improve the survival of venous flaps. For instance, a relatively smaller afferent vein and a relatively larger efferent vein were selected[24] in addition to

draining veins. The adaptation of surgical or chemical delay tactics[25-27] were also utilized. The survival of arterial venous flaps was significantly improved. However, very few attempts were made on the pure venous flaps. Approaches for ensuring success in venous flap survival remain elusive. The critical issue related to their survival is venous ischemia—in other words, the unsolved less-perfusion state, which is considered to be the cause of partial or even total flap loss.[28] In our previous study we attempted to overcome the problem of venous ischemia by various techniques, including prefabricating a large skin flap[29] and remodeling the hemodynamics.[30] Nonetheless, the literature on how to improve the survival of pure venous flap is very limited.

Two decades after the first report on EPCs, their key role in angiogenesis and vascular repair has been well documented.[8,9,31] Because of the ability to stimulate the formation of granulation tissue and angiogenesis, as well as reduce inflammation, EPCs became one of the most widely used stem cells.[32-35] Under the appropriate environmental conditions, EPCs can exert favorable angiogenic and vascular repair functions through incorporation into the endothelium and the release of growth factors and cytokines to promote repair.[36-38] EPCs have enormous therapeutic potential and have already been used in various clinical trials for a great number of diseases and disorders.[31] In this study, we found that the survival rate of venous flaps in the EPC transplantation group was significantly higher than that in the control group, indicating that EPCs can also promote tissue perfusion and improve flap survival in venous flaps.

VEGF is a vascular endothelial cell-specific heparin-binding growth factor that induces angiogenesis in vivo.[39] VEGF is reported to promote flap survival by stimulating endothelial cell proliferation and vascular regeneration.[40-42] In this study, we observed that the expression levels of VEGF and the neovascularization status in the flaps after EPC transplantation were significantly higher than those in flaps with saline. The results indicated that an increased expression of VEGF after EPC transplantation contributed to an increase of flap neovascularization, as well as to an upregulation of vascular density. eNOs is a calcium-dependent protease that exists in vascular endothelial cells. Under physiological conditions, it catalyzes the synthesis of trace amounts of NO by arginine and maintains the physiological functions of blood vessels such as vascular tone and sphincter relaxation.[43-45] We postulated that the expression level of eNOs could directly reflect the number of vascular endothelial cells and their function. In this experiment, the level of eNOs in the EPC transplantation group was much higher than that in the control group. This indicates that the EPC transplantation group had more immature endothelial cells than the control group. In other words, the EPC transplantation group has more neovascularization. In literature, eNOs has also been shown to be involved in angiogenesis.[46] It is possible that EPC transplantation has an activation of eNOs to improve the survival rate of pure venous flap.

Inflammation is a very common and fundamental pathological process. The traumatic infection of the body surface and the diseases of various organs all belong in the category of "inflammatory diseases." Inflammation is the defensive response of all living tissues with vascular systems to injury factors.[47] Under hematoxylin and eosin (H&E) staining, we found that the level of neutrophil infiltration in the EPC transplantation group was much lower than that in the control group. Therefore, we speculated that inhibition of the inflammation response after venous flap transfer may also be a potential mechanism for EPC transplantation in the improvement of venous flap survival.

This study revealed that EPC transplantation improved the survival of the venous flap in rabbits. The mechanisms of the favorable effects of EPC transplantation can be generalized as follows: restoring blood perfusion, increasing the microvasculature by the VEGF pathway, and reducing local inflammation of the venous flap.

Nonetheless, there are still many challenges for the allogenic cell translation therapy in clinical settings, including the financial concerns, uncertain regulation of this therapy and the subsequent immune responses. All these issues are worthy of further attention.

## Conclusion

Allogenic EPC transplantation may increase venous flap survival through an upregulation of eNOs and VEGF in rabbits. EPC transplantation offers the potential for the improvement of venous flap survival in clinical practice.

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## Conflicts of interest

No conflicts of interest need to be declared.

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