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Original Article

Improvement of a long random skin flap survival by application of vascular endothelial growth factor in various ways of local administration in a rat model

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ABSTRACT

Background: Vascular endothelial growth factor (VEGF) is a heparin-binding glycoprotein which plays a significant role in angiogenesis and vascular permeability. The effect of various ways of local administration of VEGF on random skin flap survival was studied, using flaps with a relatively high length (*L*) to width (*W*) ratio (5:1). **Materials and Methods:** An 1.5 × 7.5 cm dorsal skin flap with the pedicle orientated, centered, and remaining attached between the lower angles of the scapulae was elevated in 45 Wistar rats in different phases, depending on the group. Rats were divided in five groups of nine. In group A, injections of saline were administered, in equally divided spaces, into flap's fascia and transposed to a created skin defect. In group B, injections of VEGF were applied subdermally, in equally divided spaces, within the limits of a predesigned flap, a week prior to flap dissection and transposition. In group C, injections of VEGF were applied into a recipient bed's fascia just before flap raising and transposition. In group D, injections of VEGF were applied subdermally, only in the distal third of the flap and then the flap was transposed to a recipient area. Finally, in group E, injections of VEGF were applied in the flap intrafascially and in equally divided spaces and then again, the flap was transposed to a recipient area. A week after final flap raising and positioning, rats were euthanatised and flaps were excised. Specimens were photographed, measured, put in formalin 10% and were sent for histological and image analysis. **Results:** Mean flap survival percentage was 35.4% in group A, and 33.7% in group B. In groups C and D, the mean survival area was 56.3% and 80.4%, respectively. In group E, the mean flap survival percentage was 28.3%. Histological analysis demonstrated increased angiogenesis in groups C and D. **Conclusions:** VEGF application improved skin flap survival when injected subdermally in the distal third of a random skin flap or into the fascia of a recipient area even though the length-to-width ratio was high.

KEY WORDS

Angiogenesis; flap survival; neovascularisation; skin necrosis; vascular endothelial growth factor

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INTRODUCTION

The use of viable tissue for the reconstruction of defects often results to a partial failure, due to distal flap necrosis, which is usually the most needed part to cover the defect, when using local or regional flaps. There are a lot of factors that affect the viability of

the flap and wound healing such as ischaemia, hematoma formation, venous congestion and inflammation.°

It has been demonstrated that the processes occurring during wound healing are enhanced by exogenous administration of substances called growth factors, resulting to improved tissue survival. Growth factors are polypeptides that interact with specific cell receptors and control the growth, differentiation and metabolism during the phases of wound healing.[1]

Vascular endothelial growth factor (VEGF), also known as vascular permeability factor (VPF), is a heparin-binding glycoprotein which induces angiogenesis and endothelial cell proliferation and is a potent endogenous stimulator of vascular permeability.[2,3] In addition, it can cause vasodilation by stimulating nitric oxide synthase in endothelial cells.[4,5] The receptors of VEGF are found exclusively on endothelial cells and this mostly explains its actions.[6]

In this study, improvement of skin flap survival with a relatively high length-to-width ratio (5:1), by local administration of 10 μg of rat $VEGF_{164}$ in different modes and in different phases, has been examined. cDNA sequence analysis of human VEGF clones had indicated that VEGF may exist as one of four different molecular isoforms having, respectively, 121, 165, 189, 206 amino acids ($VEGF_{121}$, $VEGF_{165}$, $VEGF_{189}$ and $VEGF_{206}$).[7,8] There is about 88% homology between human and rat VEGF. Rat VEGF is active on human cells and *vice versa*.[9]

MATERIALS AND METHODS

Forty-five adult male Wistar rats, weighing between 250 g and 280 g, were studied. The guidelines from the Prefectural Department of Veterinary Health and Experiments according to National and European Union's regulations were followed, after the authority's license.

The rats were anaesthetised using ketamine (100 mg/kg) and xylazine (10 mg/kg) administered simultaneously by intramuscular injection. Dorsal skin was shaved and rats were put to the prone position with their limbs secured, using adhesive tape. Eyes were protected by administering ophthalmic drops of natural tears.

A standardised random flap measuring 1.5 × 7.5 cm and consisting of epidermis, subcutaneous tissue and panniculus carnosus was raised. Isotonic sodium chloride or recombinant rat $VEGF_{164}$ (Sigma-Aldrich, Inc., St. Louis, Missouri, USA.) was applied, depending on the group examined. Flaps were transposed and sutured, using 4/0 nylon interrupted sutures, to a recipient area. This area was created by making an incision next to the flap, from its base to its edge, in a deviation of 30° and by raising this caudally based flap [Figure 1]. In cases where injections were administered in equally divided spaces, 0.1 ml of either VEGF or natural saline every 0.75 cm was used. Intrafascial injections, in the flaps, were applied between the underlying connective tissue sheath of the panniculus carnosus and the panniculus carnosus itself. Accumulation of the injected fluid was obvious during and shortly after the procedure.

Intraoperatively, all animals received 3.5 mg/kg carprofen (Rimadyl) s.c. for analgesia and 10 mg/kg cefamandole (Acemycin) i.m. for prophylaxis. All rats were housed individually in standard experimental cages, in an environmentally controlled room with regards to temperature and light–dark cycle and were fed standard rat chow and water *ad libitum*. The animals were assigned in five groups as follows:

Group A (n=9): The flap was raised and 1 ml of isotonic sodium chloride was injected into the flap's fascia.

Group B (n=9): The flap's limits were drawn and 1 ml containing 10 μg of recombinant rat $VEGF_{164}$ was injected subdermally, in equally divided spaces, within the limits of the predesigned flap. Seven days later, the flap was raised and transposed to the recipient area.

Group C (n=9): One millilitre containing 10 μg of recombinant rat $VEGF_{164}$ was injected into the fascia of the recipient bed in equally divided spaces. Then, the

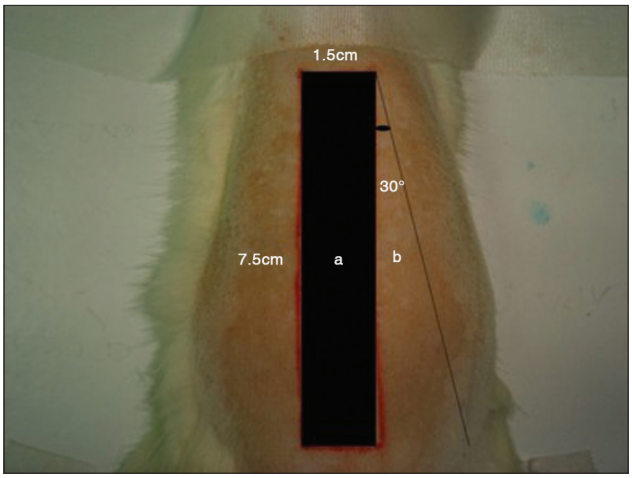

Figure 1: Design of the flaps. Main flap measuring 1.5 × 7.5 cm (a) and secondary flap raised for the recipient area to be created in a deviation of 30° (b)

main flap was raised, transposed, and secured over the injected area using absorbable 5/0 sutures.
Group D ($n=9$): The flap was raised and 1 ml containing 10 μg of $VEGF_{164}$ was injected subdermally, in the distal third of the flap. Five subdermal injections, of 0.2 ml each, were applied, in the middle and in the four corners of the distal third of the flap, in a rectangle measuring 1.5 cm by 2.5 cm.
Group E ($n=9$): The flap was raised and 1 ml of 10 μg/ml $VEGF_{164}$ was injected into flap's fascia, in equally divided spaces.

In all cases, VEGF was freshly prepared. Two rats from group A and one from group C died, but they were replaced by others. Seven days later, all animals were euthanised using an overdose of pentobarbital sodium. Flaps were excised, photographed and measured. All specimens were put into formalin 10% and were sent for histological analysis.

Histological analysis

Following fixation, specimens were transversely cut and embedded in paraffin blocks. Sections (4 mm thick) were cut from formalin-fixed and tissue blocks and placed on poly-l-lysine glass slides for standard eosin–haematoxylin staining and for further immunohistochemical processing.

Masson's trichrome stain was used for the estimation of the necrotizing area. Masson stains blue the viable tissue and red the necrotizing one, so they are distinguishable under a light microscope.

Immunohistochemistry for CD34

Slides were heated overnight at 37°C, deparaffinised in xylene and rehydrated through a graded series of ethanol. For antigen retrieval treatment, slides were boiled in citrate buffer solution 10 mM at pH 9.0 for 15 min. Then, the slides were immersed in 3% hydrogen peroxide for 20 min at room temperature to block endogenous peroxidase activity. Thereafter, the slides were incubated with an anti-CD34 antibody (Clone QBend10, NCL-L-END, Leica Microsystems Gmbh, Germany) at a dilution of 1:50 overnight at room temperature. CD34 protein is being expressed by endothelial cells of blood vessels and is used as a marker of angiogenesis. A two-step technique was used (Envision, K5007, Dako, Glostrup, Denmark). The bound antibodies were visualised using 3,3¢-diaminobenzidine tetrahydrochloride (DAB) as chromogen. Finally, the sections were counterstained with haematoxylin, dehydrated, and mounted. In each batch of staining, positive controls consisted of mouse tonsil with high CD34 expression for the antibody used, whereas substitution of an isotype-matched irrelevant antibody in place of the primary antibody was used as negative control.

Image analysis

Image analysis was used both for the estimation of viable area at Masson's trichrome stain and for the calculation of mean vessel density (MVD) at immunohistochemistry. Digital images were obtained from the stained slides, and the percentage of viable area was calculated semi-automatically.

Also, images of the immunohistochemically stained sections were captured with a Nikon DS-2 MW color CCD digital camera mounted on a Nikon Eclipse 80i microscope (Nikon Co., Tokyo, Japan) under ×400 original magnification and stored as high quality jpg files. Seven to ten images per section were captured. Images were then analyzed with Image-Pro Plus 5.1 software (Media Cybernetics, SilverSpring, MD). In each image, the parameters measured by the image analysis program were the percentage of positively stained area of CD34 in relation to the whole area of the field and MVD, in the meaning of the number of vessels per mm^2 of the examined tissue. Brown diaminobenzidine (DAB) staining, indicative of CD34 expression, was distinguished from the blue haematoxylin counterstain with hue thresholds. Colour threshold settings of DAB-stained pixels were set manually prior to analysis and left unchanged throughout. To determine the hue threshold values for DAB immunostaining, images of the positive and negative control slides were examined for optimal separation between blue-and brown-stained areas. Averaging the quantitative computerised image analysis data from the 7 to 10 images of each tissue section yielded an average percentage of staining area and an average MVD. Single positive cells were not considered as endothelial cells and they were excluded. The physician performing the computerised image analysis was blinded to the experimental data.

Statistics

Data were expressed as mean±standard deviation (SD) or median for quantitative data and as percentages for categorical data. The Kolmogorov–Smirnov test was utilised for normality analysis of the parameters. The comparison of outcome variable among the 5 groups was performed using the one way ANOVA model.

Table 1: Mean vessel density per mm²

	N	*Median ± IQR*	*P value, Kruskal–Wallis*
Group A	9	31.42 ± 9.26*	<0.0005
Group B	9	79.98 ± 107.23*,**	
Group C	9	168.33 ± 24.36*	
Group D	9	139.53 ± 65.39*,**	
Group E	9	49.62 ± 6.43*,**,***	

*P<0.005 *vs*. group A. **P<0.005 *vs*. group C. ***P<0.005 *vs*. group D

Table 2: Mean flap survival area of each group with or without VEGF treatment (total flap area=11.25 cm²)

	N	*Mean ± SD*	*P value, one way ANOVA*
Group A	9	3.99 ± 1.02*	<0.0005
Group B	9	3.80 ± 0.89*	
Group C	9	6.33 ± 0.96**	
Group D	9	9.05 ± 1.10	
Group E	9	3.18 ± 0.50*	

*P<0.0005 *vs*. groups C and D, respectively. **P<0.0005 *vs*. group D

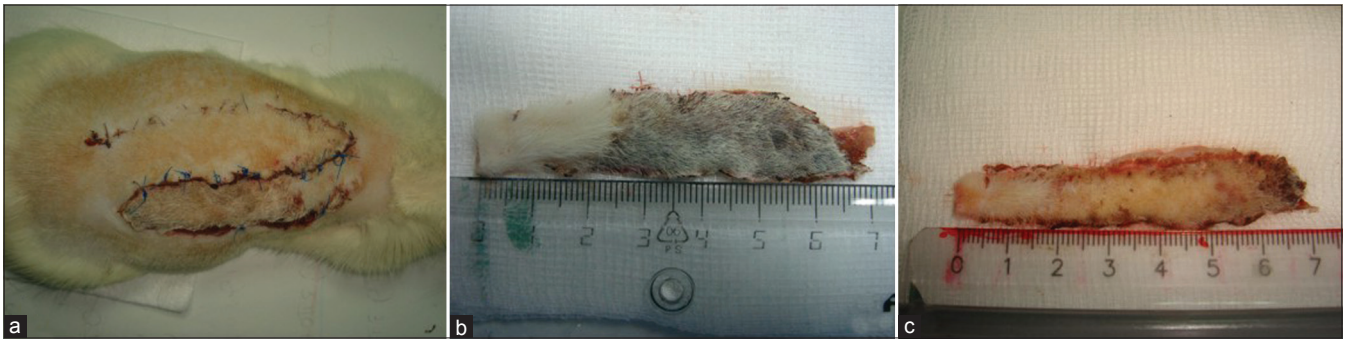

Figure 2: Flaps at the dorsum of the rat a week after raising and transposition. Necrosis is obvious at the middle and distal third of the examined flap (a). Difference between surviving and necrotic skin (b) and flap with high percentage of survival (c)

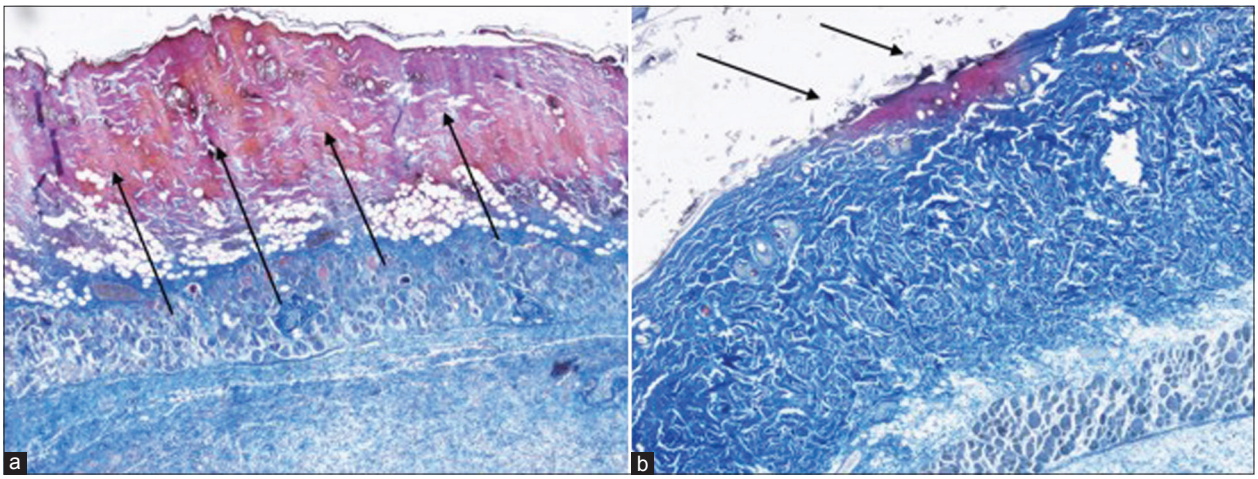

Figure 3: Representative Figures of completely necrotizing skin (a) and partially necrotizing (b). Masson' s trichrome, ×40 original magnification (black arrows)

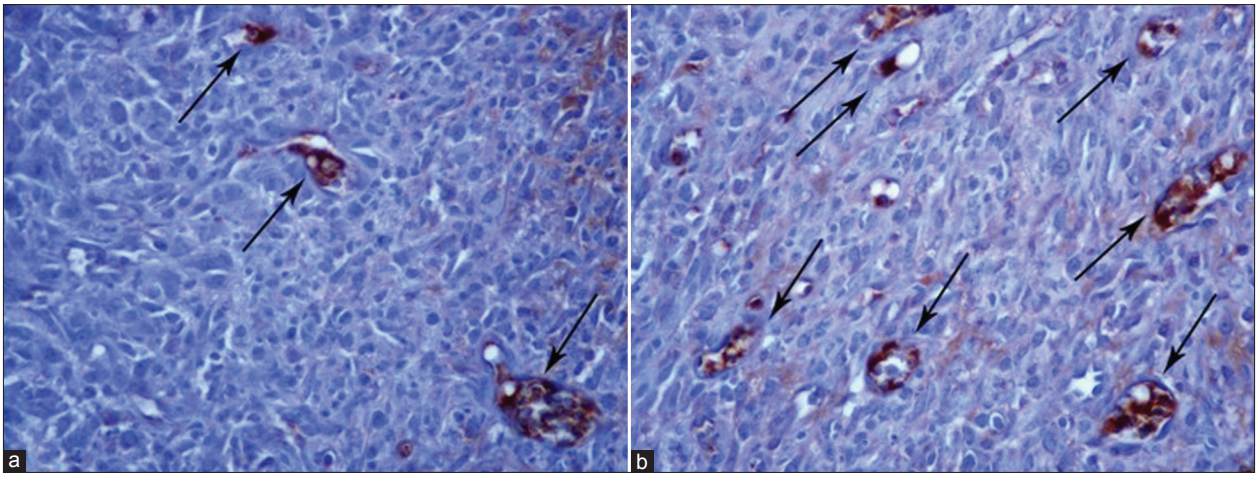

Figure 4: The difference between vessel density (arrows indicating vessels) in the tissues that were not treated (a) or were treated with VEGF (b), anti-CD34 stain, ×400 original magnification.

Pairwise comparisons performed using the Bonferroni test. Kruskal–Wallis test and Mann–Whitney test were used in the case of violation of normality. All tests were two-sided, statistical significance was set at $P<0.05$. All analyses were carried out using the statistical package SPSS v16.00 (Statistical Package for the Social Sciences, SPSS Inc., Chicago, IL, USA).

RESULTS

The survival areas of the flaps were clearly demarcated within seven days time. The surviving skin was pink-white, tender, and normal in its texture [Figure 2]. The necrotic skin was black, rigid, and did not bleed when cut.

Histologically, obvious inflammation was present in flaps' fascia with neutrophil infiltration along with monocytes and sparse macrophages. The necrotizing areas were labelled as red coloured [Masson stain, Figure 3].

Mean vessel density per mm^2 is shown in Table 1 for each group. Increased neovascularisation was demonstrated in every group where VEGF was injected, with the highest values in groups C and D [Table 1, Figure 4]

The mean flap survival percentage in groups A and B was 35.4% and 33.7%, respectively. The mean percentage in group C was 56.3% and in group D 80.4%. In group E, the mean percentage was 28.3% [Table 2 and Figure 5].

DISCUSSION

Extensive research works, regarding the enhancement of skin flap viability, suggest that there are some biologically active substances called growth factors that play a critical role.[10,11] Growth factors are members of a large functional group of polypeptide hormone-like molecules that interact with specific cell surface receptors and regulate the process of tissue repair.[12] VEGF is a potent angiogenic growth factor which increases vascular permeability, enhances endothelial cell growth, and promotes angiogenesis.[13]

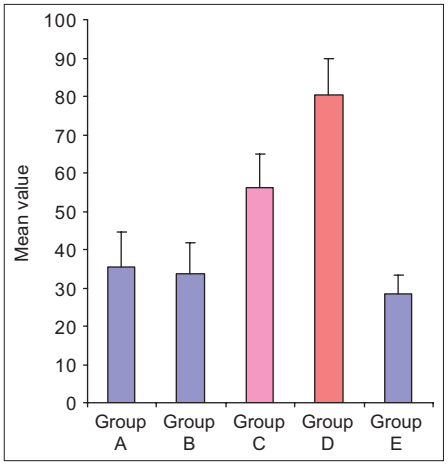

Figure 5: Survival rates of flaps treated with VEGF and control group

In cases where longer flaps are needed to cover large defects, the peripheral flap necrosis rate is high. The length-to-width ratio is known to be one of the most important factors that may affect the survival of random skin flaps. The highest the ratio, the less the survival. Several studies have indicated that skin flap survival has been improved by VEGF administration.[14-22]

In this experimental study, we also investigated the effect of exogenous $VEGF_{r164–h165}$ on flap survival, in a rat model but in flaps with high-length-to-width ratio (5:1), in a dose of 10 μg/ml and in various ways of local administration.

Difference between the rates of flap survival in groups A and B was not statistically significant although a higher percentage of improvement was expected in group B, where signs of neovascularisation were detected. On the other hand, this is consistent to others' suggestion that local application of single-dose VEGF, one week prior to ischaemia, does not have significant clinical angiogenic effects.[23] There are four possible reasons that could explain this result:

First, it is possible that the factor was not present long enough, also considering the fact that its half-life time is approximately 30–45 min under normal conditions.[24]

Second, the dosage might not be enough, resulting to inadequate local concentration. There are studies showing that there was improvement in skin flap viability when one dose of 2 μg of VEGF was injected into the flap immediately after lifting the flap. However, less benefit was observed when either 1 or 20 μg of VEGF was injected.[25]

Third, the fact that the injections were applied in equally divided spaces might not be as effective as if they have been administered in the distal third.

Finally, no other co-factors such as hypoxia or low flow were present. Hypoxia and nitric oxide (NO) seem to

play an important role. NO produced under hypoxic conditions upregulates the expression of VEGF protein[26] and hypoxia induces a fourfold increase in the half-life of VEGF.[27] In this case, the proper environmental conditions that would initiate the secretion and up-regulation of the substances that interact with VEGF, enhancing its angiogenic properties, were not created.

When VEGF was injected into the fascia of the recipient area (group C), the flap survival rate was increased compared to group A. This suggests that angiogenesis was induced by the application of VEGF, as it has also been shown by our results and that the augmented vascular network improved the flaps' viability.

The importance of the recipient bed vascularity, for the survival rates of skin flap transfers in rats, has been well documented. In studies where bed isolation from an overlying flap with artificial sheets was examined, it has been shown that the survival rates of the flaps were significantly reduced compared to the flap survival rates in cases where flaps were transposed directly over the recipient bed.[28,29] The theory of revascularisation of the flap by the tissue of the recipient site either by inosculation or by connection of the vessels may be also supported.

We hypothesise that VEGF improved the flap survival rate in group C either by enhancing neovascularisation at the recipient bed and thus augmenting the density of emerging vessels or by its diffusion through the fascia of the recipient bed and its direct effect on the underlying surface of the flap, *via* the fenestration of the endothelium caused by its vasodilator effect.

In this group, VEGF was injected in equally divided spaces. It is to be examined whether the survival rate would have been even greater if the factor had been injected at the site of the recipient area where the most ischaemic part of the flap would have been put. In addition, site of injection—in equally divided spaces or in the distal part—might also give an explanation why mean flap survival area was higher in group D than in group C although mean vessel density was higher in group C than in group D.

The highest mean survival rate combined with a relatively high percentage of mean vessel density was observed when VEGF was applied subdermally in the distal third of the flap. This is consistent to others' observations that VEGF induces vascular permeability and angiogenesis[25] in two stages, and thus, improves the flap viability significantly when applied in the proper area and in proper dosage although in this study the length-to-width ratio was 5:1.[30] It seems that the exogenous administration of VEGF in combination with the conditions created by hypoxia and production of NO were responsible for the high mean flap survival rate.

However, surprisingly, in the last group the mean survival rate was even lower than the control group and the mean vessel density had no statistical difference compared to group A, although signs of angiogenesis were present. The possible explanation could be that VEGF was rather diffused than having any effect on the skin through the panniculus carnosus because injections were administered subfascially and not subdermally. In addition, it is possible that the division of the total amount of VEGF in consequent sites might not be enough to create a vascular network, capable to support more viable tissue. This might have also impeded blood flow in the vascular network of flap's fascia and in consequent sites and could result in thrombosis. Although proliferation of endothelial cells and vasodilation that was induced by VEGF might have started, they were finally stopped. Application of VEGF, exclusively distally, might have different results, as in group D.

CONCLUSION

Improvement of wound healing by administration of VEGF, in tissues that are ischaemic or in adjacent sites (recipient bed), could allow larger skin flaps with high length-to-width ratio to be created, based on a specific pedicle. Right dosage, accurate site of injection, correct timing and proper dilution are the factors that have to be defined in every case. Further investigation of the cascade of the biological events, after exogenous administration of VEGF and the examination of the interaction with other growth factors, will provide the answers and possibly will expand our knowledge. In the near future, usage of VEGF at the periphery of a perforator flap might expand its limits.

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