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Does VEGF Have an Effect on the Survival of a Long Random Skin Flap by its Application at the Recipient Area?

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Rezumat

Rolul factorului de creștere endotelial, aplicat pe patul receptor, în supraviețuirea lamboului cutanat pediculat

Introducere: Factorul de creștere endotelial (VEGF) este o moleculă *hormone-like*, care s-a demonstrat că acționează asupra unui sistem receptor specific, constituind astfel un mecanism de bază în reglarea angiogenezei. Articolul de față are ca scop evaluarea efectului acestei citokine adminstrate local, asupra supraviețuirii unui lambou cutanat pediculat la șobolanul de laborator.
Material și Metode: Un lambou dorsal standard măsurând 1.5 x 7.5 cm a fost preparat la 18 șobolani Wistar, având pedicului centrat pe unghiurile inferioare ale scapulelor. Raportul lungime/lățime a fost mare (5:1). Șobolanii au fost împărțiți în 2 grupe de câte 9 animale. În grupul A, lamboul a fost preparat și imediat alături a fost creat un defect cutanat de dimensiuni identice. În fascia defectului creat s-a injectat soluție salină, iar lamboul a fost transpozat și fixat pe acest defect. În grupul B lamboul a fost recoltat și transpus în mod similar peste defectul învecinat creat în prealabil, dar, de această dată, în fascia defectului receptor s-a injectat VEGF. Șapte zile mai târziu șobolanii au fost eutanasiați și lambourile excizate. Fragmentele de fascie corespunzătoare paturilor receptoare au fost de asemenea recoltate. Specimenele au fost măsurate, fotografiate și fixate în formaldehidă 10%. Ulterior au fost colorate și examinate histologic și imunohistochimic.
Rezultate: Linia de demarcație între zonele de tegument care au supraviețuit și cele ce s-au necrozat a fost clară macroscopic la sfârșitul celor șapte zile. În grupul A, lambourile au supraviețuit în medie în proporție de 36.8%. În grupul B proporția de supraviețuire a fost de 56.3%. Neovascularizația indusă în urma injectării de VEGF în fascia patului receptor a fost demonstrată histologic.
Concluzii: Adminstrarea exogenă de VEGF în patul receptor al unui lambou cutanat pediculat a ameliorat rata de supraviețuire, chiar în condițiile în care lungimea lamboului a fost mare în comparație cu lățimea sa.

Cuvinte cheie: lambou de supraviețuire, receptor, angiogeneză, factorul de creștere endotelial (VEGF), neovascularizație

Abstract

Background: Vascular endothelial growth factor (VEGF) is a hormone-like molecule which has been shown to act on a specific receptor system and in this way to be the basic regulator of angiogenesis. The effect on the survival of a long random skin flap was examined by exogenous administration of this cytokine, at flap's recipient site.
Materials & Methods: A standard dorsal skin flap measuring 1.5 x 7.5cm was elevated in eighteen wistar rats with the pedicle centered and attached between the lower angles of the scapulae. The length to width ratio was relatively high (5:1). The rats were divided in two groups of nine. In group A, flap was elevated and a skin defect was created next to it.

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Normal saline was injected into the fascia of the defect and the flap was transposed and secured over the previously created recipient site. In group B, flap was elevated and transposed to a previous created defect, as before, where, this time, injections of VEGF were applied into the fascia of the recipient bed. Seven days later the rats were euthanized and the flaps were excised. The underlying fascias of the recipient beds were also excised in the same dimensions. The specimens were measured, photographed and put into formalin 10%. Immunohistochemical staining and histological analysis followed.

Results: The differentiation between the surviving and the necrotic skin was macroscopically clear within seven days time. In group A, the mean flap survival percentage was 36.8%. In group B the percentage was 56.3%, respectively. Neovascularization of the fascia of the recipient bed was also demonstrated when VEGF had been injected.

Conclusions: Exogenous administration of VEGF into the recipient bed of a skin flap improved the survival rate even though the flap's length was relatively high compared to its width.

Key words: flap survival, recipient site, angiogenesis, VEGF, neovascularization

Introduction

Coverage of tissue defects is one of the most important issues that the reconstructive surgeon is being called to resolve. A skin graft is not always the proper solution, especially in complex defects and the need for well-perfused skin flaps often arises. Peripheral necrosis of this living tissue is not an unusual phenomenon and it compromises the final result when occurring. (1) Inadequate arterial inflow and/or insufficient venous outflow are the primary causes of the necrosis. (2)

Inflammatory response, epithelization, connective tissue formation, and finally remodelling are the processes that succeed one another in the wound healing cascade. (3) The growth, differentiation and metabolism of the cells that are involved in this procedure are controlled by specific glycoproteins called growth factors which regulate the tissue repair process by interacting with cell surface receptors. (4,5)

VEGF is a factor which promotes angiogenesis by enhancing the endothelial cell growth and increases the vascular permeability. (6) Angiogenesis is a biological mechanism which is fundamental for the maintenance of the processes of wound healing. (7)

Experimental research has revealed that areas with capillaries lacking blood flow will eventually become necrotic. (8,9) Skin flap viability has been improved after the exogenous administration of VEGF as many studies suggest. (10-18)

In this study, the improvement of viability of a skin flap with high length to width ratio, after subfascial injections of VEGF at its recipient bed, has been examined.

Materials and Methods

Eighteen adult male Wistar rats weighing between 250g and 280g were used in our study. The rats were supplied by Pasteur laboratories. The experiments were performed under the license and by following the guidelines of the Prefectural department of Veterinary Health and Experiments according to the National and European Union's laws.

Ketamine (100 mg/Kg) and xylazine (10 mg/Kg) were used for the anaesthesia by simultaneous intramuscular injection. Shaving of the dorsal skin followed and the rats were put to prone position. Their limbs were secured using adhesive tape. Eyes were protected by application of ophthalmic ointment containing tobramycin.

A standardized skin flap measuring 1.5(w) x 7.5(l) cm was raised at the dorsum of each rat. Flap's pedicle was designed and based centrally between the lower angles of the scapulae. A recipient area was created by raising a caudally based random flap with an incision at the right side of the previous created flap, starting from its edge to its base, in a deviation of 30^{0}.

Recombinant rat $VEGF_{164}$ suspended in phosphate-buffered saline or isotonic sodium chloride was injected at the recipient area depending on the group examined. 88% homology is present between rat and human VEGF. (19) The flaps were transposed and secured into their new positions using 4/0 nylon interrupted sutures. All animals received 3.5 mg/Kg carprofen s.c. for analgesia and 10 mg/Kg cefamandole i.m. for prophylaxis during the operation.

All rats were fed standard rat chow and water ad libitum. They were housed separately in standard experimental cages and in a room that the environmental conditions were controlled as far as it concerns the light-dark cycle and the temperature.

Group A (n=9)

The standard skin flap and the caudally based random skin flap were raised. One ml of isotonic sodium chloride was injected, in equally divided spaces, at the recipient bed which was created after the elevation of the caudally based flap. Flaps were transposed and were stitched at their new positions. The flap that was put over the injected area was secured using 5/0 absorbable sutures so as any possible dislocation to be prevented.

Group B (n=9)

Flaps were elevated in the same way as before. In this group, 1ml containing $10\mu g$ of recombinant rat $VEGF_{164}$ suspended in phosphate-buffered saline was injected, in equally divided spaces, at the recipient bed. Flaps were transposed and secured at their new positions in the same way as in group A.

All rats recovered from the anaesthesia well and were put back into their cages and were treated as it was previously described. Seven days later, rats were anesthetized again and were euthanized using a high dose of pentobarbital. Flaps were excised measured and photographed. The same procedure was

followed for the underlying fascias within the dimensions of the overlying flaps. All specimens were put into formalin 10% and were sent for histological analysis.

Histological analysis

Following fixation, the skin flap 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 – hematoxylin 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, deparaffinized 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 antibody against CD34 (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 stands for a marker of angiogenesis. A two step technique was used (Envision, K5007, Dako, Glostrup, Denmark). The bound antibodies were visualized using 3,3' diaminobenzidine tetrahydrochloride (DAB) as chromogen. Finally, the sections were counterstained with hematoxylin, 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 semiautomatically.

Also, images of the immunohistochemically stained sections were captured with a Nikon DS-2MW color CCD digital camera mounted on a Nikon Eclipse 80i microscope (Nikon Co., Tokyo, Japan) under 400x 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 the 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 hematoxylin counterstain with hue thresholds. Color 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 computerized 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 taken into account as endothelial cells and they were excluded. The physician performing the computerized image analysis was blinded to the experimental data.

Statistics

Data was expressed as mean ± standard deviation (S.D.). The Kolmogorov-Smirnov test was utilized for normality analysis of the parameters. The comparison of outcome variable between the 2 groups was performed using the Independent samples t-test. All tests are two-sided, statistical significance was set at $p < 0,05$. All analyses were carried out using the statistical package SPSS vr 16.00 (Statistical Package for the Social Sciences, SPSS Inc., Chicago,Ill.,USA).

Results

The flaps' survival areas were macroscopically clear within a week's time. The part of the skin that survived had a pink-white colour and a soft texture compared to the necrotic one which was rigid and black *(Fig. 1)*. Inflammation was present with neutrophil infiltration along with monocytes and sparse macrophages. The necrotizing areas were stained red (Masson). The mean survival percentage of group A was 36.8% and the mean survival percentage of group B was 56.3% respectively *(Fig. 2, Table 1)*. The fascia specimens that were excised from group A showed no clear signs of angiogenesis. On the other hand the examination of the fascia specimens that were excised from group B revealed clearly that angiogenesis did occur *(Fig .3)*. Mean vessel density per mm^2 was significantly increased in group B compared to group A *(Table 2, Fig. 4)*.

Discussion

Despite the recent improvement in operative techniques (angiosome concept) and the use of well established axial or perforator arteries supplied flaps, peripheral tissue necrosis still remains a problem, especially in cases where complex defects need to be covered. Although such defects are usually being covered using pedicled, free or perforator flaps, however, there are still some special situations where random flaps are being used.

Tissue repair process involves a series of overlapping biologic events as a response to injury. (20) The exogenous administration of some substances, called growth factors, has been shown to improve the viability of tissues in flap surgery as many studies suggest. (21-26)

These hormone-like polypeptides have been demonstrated

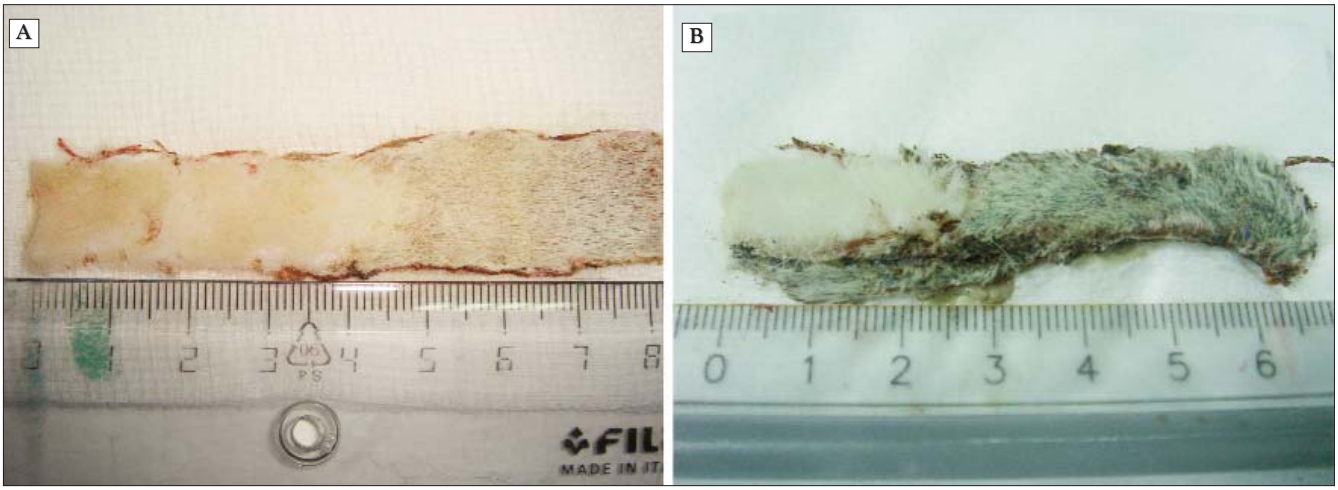

Figure 1. *Increased flap survival percentage in the group treated with VEGF* ***(A)*** *and extended area of flap necrosis in the group where sodium chloride was injected at the recipient site* ***(B)***

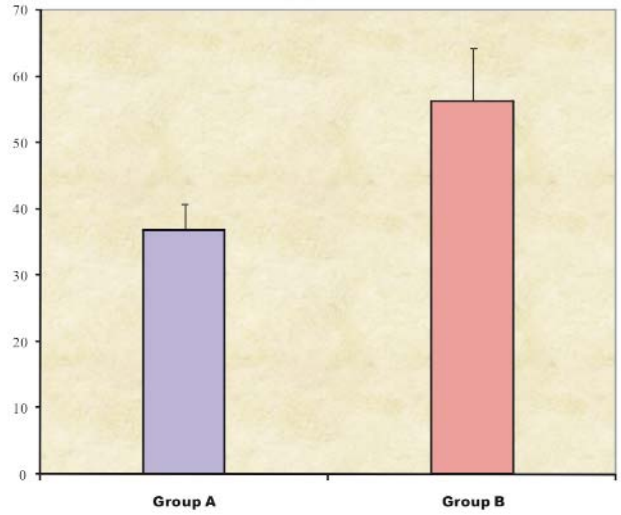

Figure 2. *Comparison of flap survival percentage between the two groups*

Table 1. *Mean survival percentage of groups A and B*

		N	Mean	SD	p-value
Flap survival percentage	Injection of sodium chloride 0.9% (A)	9	36,89	4,00	<0.0005
	Injection of VEGF (B)	9	56,30	8,51	

to stimulate extracellular matrix and collagen synthesis, activate fibroblast and keratinocytes proliferation and migration and induce angiogenesis. (27,28)

In this experimental study the effect of exogenous administration of VEGF on the survival of a long random skin flap by its application at the recipient area has been investigated in rats.

VEGF is one, if not the most, potent endogenous stimulator of angiogenesis and vascular permeability. (29,30) In addition, vasodilation is another biological process that VEGF can initiate, partly by stimulating nitric oxide synthase in

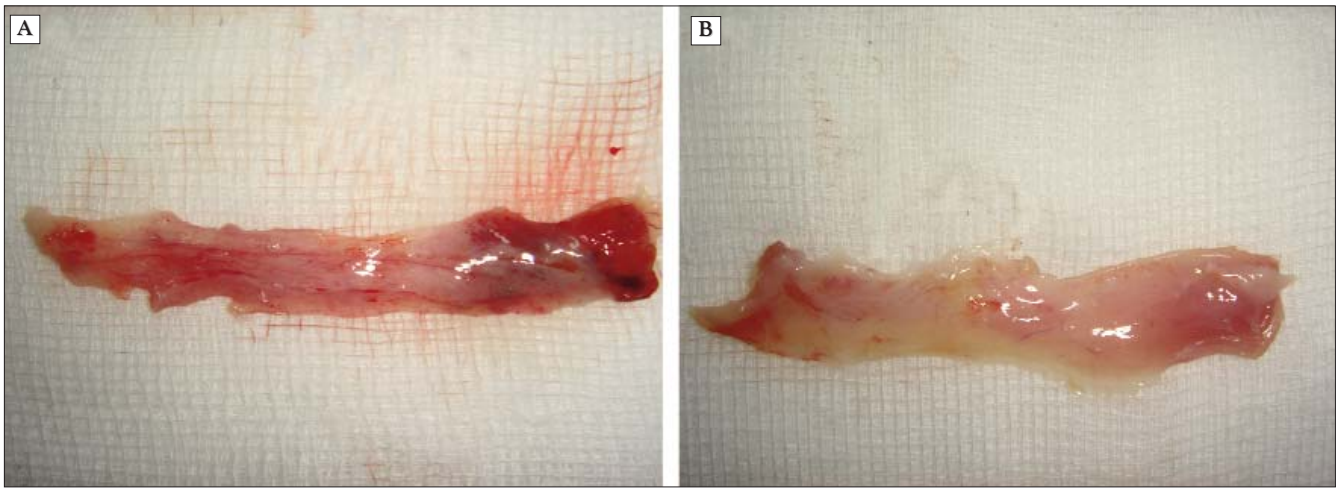

Figure 3. *Macroscopical view of the vascularity of the fascia treated with VEGF* ***(A)*** *and natural saline* ***(B)***

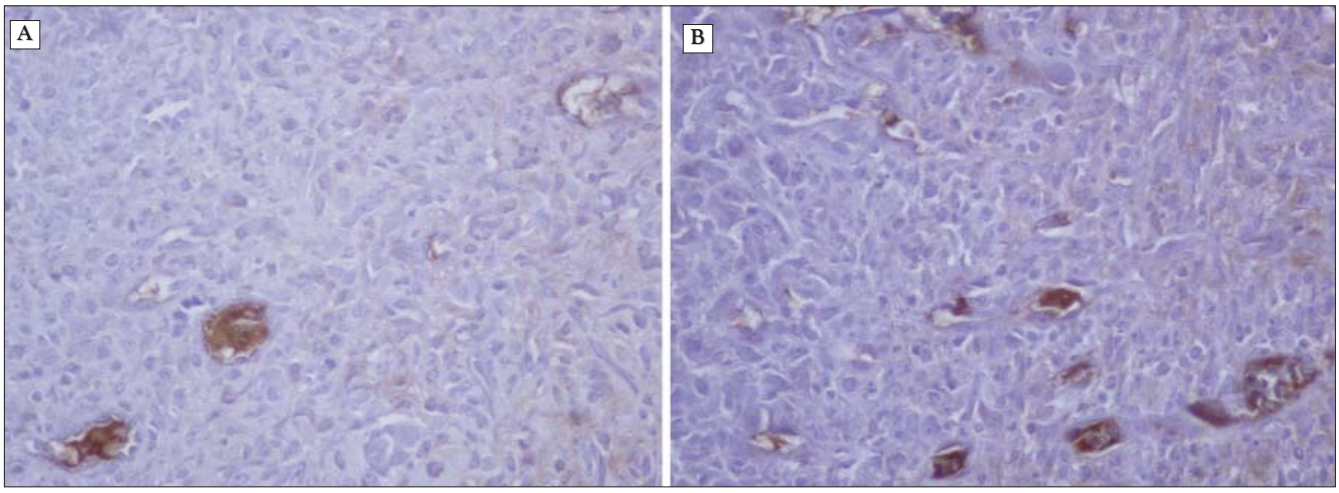

Figure 4. *Vessel density in group treated with sodium chloride* ***(A)*** *and in group treated with VEGF* ***(B)***

Table 2. *Comparison of mean vessel density per mm² between the two groups*

		N	Mean ± SD	p-value
Mean vessel density/mm²	Group A	9	41,87 ± 15,99	< 0.0005
	Group B	9	165,78 ± 17,77	

endothelial cells. (31) Hypoxia or endothelial damage has been found to regulate the expression of VEGF. (32-35) All of these actions are connected to the fact that the receptors of VEGF are situated solely on endothelial cells. (36)

In this study, comparison between the mean survival rate of group A and group B clearly indicates that VEGF application at the recipient site of the flap significantly improves flap's viability. Besides the fact that the angiogenic properties of VEGF are clearly showed, the significance of the role of the recipient bed on the survival of the flap is also emphasized.

It has been demonstrated that the distal segment of a rat skin flap could be partially salvaged by basing its nutrition and oxygenation on the recipient bed. A resemblance between the physiological mechanisms of skin graft's "take" and the mechanisms of the survival of this distal segment of the flap is obvious. (37,38)

Serum imbibition, inosculation of cut vessels of the host bed and the cut ends of the graft, original skin graft vasculature degeneration and vessel invasion from the recipient bed and finally, preservation of the acellular basal lamina of the degenerated vasculature of the skin graft and the host bed's vessel ingrowth into it, are the theories for skin graft survival and revascularization. (39-48)

Studies have supported that serum imbibition and ingrowth of new vessels from the recipient bed could be an explanation for the survival of a random portion of a rat skin flap. It has been found that this partial salvage would have been prevented by isolation of the recipient bed using an artificial barrier. (49) Another study supports that by placing a gelatin sponge soaked with EGF under a skin flap, in a rabbit model, flap's survival could be increased. (50)

Finally it was reported that a pedicled skin flap, subjected to up to 6 hours of warm ischaemia, in a rat model, survived after reperfusion if it was placed on a vascularized bed. On the other hand if the flap was placed on a non-vascularized bed the extent of flap survival in the 6-hour ischaemic flaps was significantly decreased. (51)

In our study, histological analysis demonstrated neovascularization of the recipient area's fascia. Measurements showed that the survival rate in the group, where injections of VEGF had been administrated, was significantly higher compared to the other group.

We hypothesize that VEGF improved the survival rate in group B by enhancing neovascularization at the recipient bed and thus augmenting the density of emerging vessels. In addition, VEGF might have been diffused through the fascia and acted directly on the underlying surface of the flap, via the fenestration of the endothelium caused by its vasodilator effect.

Conclusion

Vascularity of flap's recipient bed plays an important role on the survival of the overlying flap. Well-vascularized bed increases the possibility of augmenting flap's survival rate. VEGF might further improve the viability of the flap when injected at the recipient bed, by both enhancing angiogenesis and its vasodilator effect, even when the length to width ratio is relatively high. Proper dosage and exact site of administration need to be further examined.

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