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RESEARCH ARTICLE

Karakoç et al: Topical nitroglycerin in flap viability

Effect of topical nitroglycerin on neoangiogenesis and pedicle-independent viability in a rat dorsal skin flap model

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ABSTRACT

Interpolated flaps are frequently used in reconstructive surgery when free tissue transfer is not feasible, but they require staged procedures due to pedicle dependence. Flap autonomization, the process by which transferred tissue develops new vascular connections and survives independently of its pedicle, is essential before division. Although methods such as delay techniques, hyperbaric oxygen (HBO), vascular endothelial growth factor (VEGF), and stem cell therapies have been tested to enhance angiogenesis, the effect of topical nitroglycerin (NTG), a nitric oxide (NO) donor with vasodilatory, anti-inflammatory, and angiogenic properties, has not been investigated. This study aimed to evaluate the effect of topical NTG on neoangiogenesis and flap autonomization in a rat dorsal skin flap model. Sixty Wistar-Albino rats were divided into five groups ($n = 12$). A 3×3 cm dorsal flap with a caudal pedicle was elevated in all animals. In groups 1–3, pedicles were transected on day 5: Group 1 received vaseline, group 2 received NTG for 5 days then vaseline, and group 3 received NTG continuously. Groups 4 and 5 were sacrificed on day 5 to assess early angiogenesis after vaseline or NTG. Flap survival was analyzed with ImageJ, angiogenesis with VEGF, CD34, and CD105 staining, and histology with Hematoxylin and Eosin (H&E) and Masson's Trichrome. Flap survival was significantly greater in groups 2 (485.5 mm²) and 3 (757.3 mm²) than in group 1 (273.5 mm²), with group 3 highest ($p < 0.01$). NTG-treated groups showed increased VEGF, CD34, and CD105 expression, with the strongest angiogenesis in group 3. Group 5 also had higher vascular proliferation than group 4 ($p < 0.001$). Histology showed that NTG reduced epithelial disruption, hemorrhage, collagen degradation, and leukocytic infiltration while enhancing vascular proliferation. In conclusion, continuous topical NTG enhanced angiogenesis and accelerated flap autonomization, leading to greater viability after pedicle division. NTG may help shorten pedicle division intervals and improve outcomes in reconstructive surgery, but further molecular and clinical studies are needed.

Keywords: Flap autonomization, topical nitroglycerin, interpolation flaps, angiogenesis, rat flap model.

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INTRODUCTION

Although reconstructive surgery is sometimes the first choice, the patient's condition may occasionally necessitate reconstruction using interpolated flaps. In interpolated flaps—one type of local flap—the donor site is not contiguous with the recipient site; instead, normal healthy tissue exists between them. Consequently, the pedicle must remain either beneath, or more commonly on top of, the intervening tissue. Therefore, most interpolated flaps are performed in at least two stages, including necessary tissue revisions. The forehead flap, Abbe flap, Cutler-Beard flap, cross-finger flap, and pedicled groin flap are among the most common examples. The main disadvantage of these flaps is the requirement for multiple stages; however, they are primarily preferred when repair with similar tissue is desired, when sufficient adjacent skin is unavailable at the defect, or when free flap repair is not feasible. For the closure of an open wound, a pedicle is not required; it merely supplies blood circulation to the tissue that will cover the defect, and its presence can be problematic for the patient. The flap tissue, through the angiogenesis developed in the recipient area, becomes capable of surviving independently of the pedicle. At this stage, the pedicle is no longer necessary and can be excised for the outcome. The process by which the transferred tissue eventually no longer depends on its original vascular supply is defined as flap autonomy (pedicle independent viability). In one study, after ischemic preconditioning and transection of the pedicle of a superficial inferior epigastric artery-based skin flap on day 5, it was observed that only 10% of the distal skin of the flap remained viable.[1] It is known that free flaps can survive despite issues with their vascular pedicles; however, very little is known about the precise impact of various factors—such as anatomical localization and internal or external conditions—or about the duration of the flap autonomization process.[2], [3]Furthermore, there is no evidence as to whether neoangiogenesis occurs in all flaps or to what extent flaps remain dependent on their main vascular pedicles over time.[4] According to the literature, the influencing factors can generally be categorized as wound bed characteristics, flap location, and flap type. Various strategies have been investigated to enhance neovascularization, including physical methods such as the delay technique and intermittent pedicle compression, as well as experimental approaches like L-theanine, hyperbaric oxygen therapy, epinephrine injection into the pedicle, adipose-derived stem cell application, epidermal growth factor (EGF) treatment, exogenous vascular endothelial growth factor (VEGF) administration.[5] [6], [7], [8]

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Wounds compromised by infection, tumor, radiation, and vascular insufficiency (such as in atherosclerosis, smoking, and diabetes) may delay the neoangiogenesis process.[9], [10] Additionally, research on the therapeutic use of nitric oxide (NO) continues due to its ability to activate multiple intracellular mechanisms. Numerous clinical studies have examined the wide-ranging efficacy of nitroglycerin (NTG) in conditions such as anal fissures, tendinopathies, and the prevention of mastectomy skin flap necrosis. A rapid autonomization process—where the flap develops a vascular network independent of its pedicle—can prevent the need for revision surgeries caused by inadequate circulation at the pedicle or free tissue transfer anastomoses. This leads to reduced flap loss, fewer complications from prolonged hospital stays, expedited patient recovery, and lower healthcare costs. The primary aim of this study is to investigate the effect of topical nitroglycerin on both the duration a transferred skin flap can survive without pedicle dependency (flap autonomization) and the process of neoangiogenesis.

MATERIALS AND METHODS

Study design

In this study, a priori power analysis was performed using G*Power 3.1 to estimate the minimum required sample size for one-way ANOVA. Assuming a large effect size (f = 0.4), an alpha level of 0.05, and a power of 0.80, the total sample size required for five groups was calculated to be 60 animals (n = 12 per group). 60 female Wistar-Albino rats weighing 250–300 grams, provided by the Süleyman Demirel University Animal Production and Experimental Research Laboratory, were used. Ethical approval for this experimental study was obtained from the Süleyman Demirel University Local Ethics Committee for Animal Experiments. (Approval Date: 08/06/2023; Approval No: 06-178) During the experiment, the rats were individually housed in separate cages within the same room under controlled environmental conditions (24 °C, 12-hour light/dark cycle). They were provided with standard chow and water ad libitum.

To evaluate the microscopic and macroscopic effects of topical nitroglycerin on the flap, the 60 rats were randomly divided into five equal groups (n=12 per group) using a simple manual randomisation process to avoid selection bias.

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Prior to surgery, anesthesia was induced by intraperitoneal injection of 10 mg/kg xylazine and 30 mg/kg ketamine. In all groups, 3×3 cm skin flaps were designed with a caudal pedicle such that the pedicle remained between the caudal and iliac crests. The flap tissue was elevated, then repositioned, and secured with 5.0 polypropylene sutures (Figure 1). Immediately following surgery, the animals were placed under an infrared heating lamp for 30 minutes to maintain postoperative warmth. Subsequently, they were returned to their cages and allowed ad libitum access to food and water. Behavioral monitoring was performed daily, and no signs of distress, reduced activity, or feeding abnormalities were observed. All histological evaluations and ImageJ-based analyses were performed by investigators blinded to the treatment groups.

Experimental groups

The first 3 groups were evaluated both clinically and microscopically. On the 5th day after flap elevation, the pedicles of the flaps were transected to allow the flaps to develop a new capillary network for circulation. One week after pedicle transection, biopsies were taken for microscopic evaluation and photographs were obtained for macroscopic assessment.

Group 1: Vaseline was applied for 5 days (until pedicle transection) and for an additional 7 days thereafter.

Group 2: Topical nitroglycerin was applied for 5 days (until pedicle transection), followed by Vaseline for the subsequent 7 days.

Group 3: Topical nitroglycerin was applied continuously for 5 days (until pedicle transection) and for 7 days after transection.

In addition to the clinical evaluation groups, two extra groups were included to assess tissue vascularization on the 5th day—prior to pedicle transection. In these groups, the vascular network of the flaps on day 5 was evaluated:

Group 4: Vaseline was applied for 5 days following flap elevation.

Group 5: Topical nitroglycerin was applied for 5 days following flap elevation.

In groups 4 and 5, the animals were sacrificed on day 5 and tissue vascularization was evaluated microscopically.

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Topical nitroglycerin application

The topical nitroglycerin used in the study was a 5% ointment obtained from the Turkish Magistral Pharmacists Association. Topical nitroglycerin was applied using the Finger Tip Unit (FTU) method, where 1 FTU corresponds to approximately 0.5 grams of ointment. For each application, 1/3 FTU (~0.17 grams) of 5% nitroglycerin ointment was applied every 12 hours. This dosage corresponds to approximately 8.5 mg of nitroglycerin per application. The ointment was evenly spread over the entire flap surface, which measured 3 cm × 3 cm (9 cm^2), resulting in an estimated dose of ~0.94 mg/cm^2 per application.

Clinical evaluation

On the 7th day after pedicle transection, the necrotic areas were accepted as final. For the macroscopic evaluation, the ratio of the viable flap area (excluding the necrotic area) to the total flap area was calculated. Measurements were performed in square millimeters using the ImageJ program (an open-source Java image processing software, USA), based on a ruler included in the photographs taken with the rats (Figure 2).

Histological analysis

For microscopic evaluation, a single-piece biopsy including the middle portion of the flap edge and the adjacent normal skin was taken from each rat. The excised tissues were left under running water overnight to remove the fixative, then dehydrated by passing through 6 different alcohol solutions of increasing concentration (from 50% to absolute) for 1 hour each. The tissues were subsequently cleared in xylene for 10–15 minutes, infiltrated with paraffin in an oven at 60°C for 3 hours, and embedded in paraffin blocks. From these blocks, 4 µm thick sections including all skin layers parallel to the long axis of the tissue sample were obtained using a rotary microtome (Leica RM2155 RT, Nussloch, Germany).

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For immunohistochemical analysis of endothelial proliferation, three primary antibodies—VEGF, CD34, and CD105 (dilutions: 1:100, 1:100, and 1:50, respectively; Elabscience, Texas, USA)—were used. Sections (4 μm thick) obtained from the deparaffinized tissue blocks were mounted on polylysine-coated slides. The sections were deparaffinized by overnight drying at 60°C and by two 20-minute xylene treatments. They were then rehydrated through a series of decreasing alcohol concentrations using PBS. Antigen retrieval was performed by microwaving the sections in 10% citrate buffer for 2 minutes, followed by cooling for 20 minutes. The section peripheries were marked with a pappen pen, and endogenous peroxidase activity was blocked with 3% H_2O_2. After protein blocking, the sections were incubated with the VEGF, CD34, and CD105 primary antibodies for 1 hour at room temperature and then overnight at +4°C in a humid chamber. After incubation with a secondary antibody, the sections were treated with streptavidin-HRP, developed with DAB chromogen, counterstained with Mayer's Hematoxylin, mounted with entellan, and evaluated using a digital microscope. In two randomly selected sections per animal, five random fields at ×400 magnification were evaluated, and all vessels showing immunoreactivity were counted regardless of their size; the averages were then calculated.

Additionally, using Hematoxylin and Eosin and Masson's Trichrome staining, five parameters were evaluated: disruption in epithelialization, hemorrhage, collagen degradation, vascular proliferation, and leukocytic infiltration. Five histopathological parameters were evaluated for each subject: disruption in epithelialization, hemorrhage, collagen degradation, vascular proliferation, and leukocytic infiltration. Each parameter was scored on a semi-quantitative scale as follows: 0 = absent, 1 = mild, 2 = moderate, 3 = severe, and 4 = very severe. For each rat, two randomly selected tissue sections were examined, and five randomly chosen fields at ×200 magnification were evaluated per section. The mean score of these five fields was calculated for each parameter.

Statistical analysis

Data were analyzed using SPSS Version 26.0 (IBM Corp., Armonk, NY, USA). Normality was assessed using the Shapiro–Wilk test. For normally distributed variables, descriptive statistics are presented as mean ± standard deviation and analyzed using one-way ANOVA with Bonferroni post-hoc corrections. For non-

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normally distributed data, the Kruskal–Wallis test was applied, followed by Dunn's post-hoc test with Bonferroni correction. A type I error rate of 5% was applied for all analyses, and adjusted p-values <0.05 were considered statistically significant.

RESULTS

Clinical findings

The average surviving flap areas were as follows: Group 1 (5 days TV + 7 days TV) had 273.5, Group 2 (5 days TN + 7 days TV) had 485.5, and Group 3 (5 days TN + 7 days TN) had 757.25. Statistical analysis revealed that Group 3 had a significantly larger surviving area compared to Groups 1 (p <0.001) and 2 (p <0.001), and significant difference was found between Groups 1 and 2 (p = 0.002). (Table 1) (Figure 3, 4, 5, 6)

Immunohistochemical findings

The highest reactivity for the vessel proliferation markers VEGF, CD34, and CD105 was observed in Group 3, whereas the lowest reactivity was seen in Group 1. On day 12, Group 3 demonstrated significantly greater angiogenesis compared to both Group 1 ($p < 0.001$) and Group 2 ($p < 0.001$) following vascularization assessment. Comparison between Group 4 (surgery only) and Group 5 (surgery with topical nitroglycerin) revealed a statistically significant increase in vascularization in the nitroglycerin-treated group ($p < 0.001$). (Table2) (Figure 7, 8, 9, 10)

Histochemical analyses were performed using both Hematoxylin and Eosin and Masson's Trichrome staining and five histopathological criteria were evaluated: Disruption of epithelialization, hemorrhage, collagen degradation, vascular proliferation, and leukocytic infiltration. (Table 3 and 4) (Figure 11)

All groups showed minimal impact on epithelialization. Histopathological evaluation showed that epithelial disruption was most prominent in the group 1 (G1: 1.0 [1.5–0.0]) and nearly absent in the group 3 receiving prolonged nitroglycerin treatment (G3: 0.0 [0.5–0.0]).

Group 1 exhibited the highest scores (indicating severe hemorrhage), while Groups 3 and 5 had scores near normal levels. Group 1 and group 2 showed statistically

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significantly higher hemorrhage compared to Groups 3. However, no statistical significance was found between groups 4 and 5 (p=0.097).

The highest degree of tissue degradation was observed in Group 1, followed by Group 2. In contrast, Group 3 exhibited the lowest degradation scores, indicating histological features most comparable to normal tissue. Statistically significant less degradation was observed in Group 3 compared to Groups 1, 2. No statistical significance was found between groups 4 and 5.

The highest level of vascular proliferation was observed in Group 3, while Groups 1, 2 had the lowest levels. In Group 3, proliferation was significantly higher than in Groups 1, 2. There was no statistically significant difference between Groups 4 and 5.

The highest leukocytic infiltration was seen in Groups 1, 2, and 4, while Group 3 exhibited the lowest infiltration. Groups 1, 2 showed statistically significantly higher infiltration compared to Group 3 but statistical analysis revealed no significant difference between Groups 4 and 5.

DISCUSSION

In plastic surgery, two-stage skin flaps that require pedicle division are still frequently used for the reconstruction of cutaneous defects. Owing to factors related to both the patient's overall condition and the surgeon's resources, microvascular procedures may sometimes be unsuitable. In such cases, relatively shorter and safer pedicled local or distant flaps are preferred. However, patients are restricted in various ways during the period preceding pedicle division [11], making a shortened pedicle division interval highly desirable. In our study, we hypothesized that topical nitroglycerin applied to a pedicled flap harvested from the rat's back would accelerate the autonomization process—defined as the flap's ability to survive independently of its pedicle—by enhancing angiogenesis along three sides of the flap. Our clinical findings demonstrated that the group receiving nitroglycerin for 5 days before pedicle division (Group 2) and the group receiving nitroglycerin both before and for an additional 7 days after pedicle division (Group 3) exhibited significantly higher flap viability compared to the control group treated with vaseline (Group 1). Most studies in the literature evaluating flap survival with topical nitroglycerin have used the McFarlane model, and no study to date has specifically assessed flap autonomization. In this regard, our experimental model distinguishes itself by focusing on the evaluation of

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flap autonomization. Flap tissue can develop a capillary network with the surrounding recipient tissue, thus ensuring its survival without dependence on the pedicle—a process known as flap autonomization—which is necessary before the pedicle can be safely divided. Clinically, an average interval of about 3 weeks is recommended for pedicle division [12]. To enhance flap autonomization and shorten this interval, temporary pedicle compression via silicone or staplers (i.e., physical ischemic preconditioning) is the most commonly used method [13].

In a 2023 study, Yanis Berkane et al.[1] demonstrated in a rat superficial inferior epigastric artery skin flap model that the distal, most hypoxic portion of the flap exhibited greater viability after pedicle division, attributing this to hypoxia-induced angiogenesis. This finding aligns with our observation of necrosis in areas close to the pedicle in nearly half of our flaps. The literature contains numerous publications investigating the angiogenic effects of growth factors such as transforming growth factor (TGF), epidermal growth factor (EGF), fibroblast growth factor (FGF), and vascular endothelial growth factor (VEGF). For example, Zhang et al. [14] reported that exogenous VEGF improved early pedicle division outcomes and flap survival in a rat tube pedicle flap model; however, due to its potential modulatory role in cancer, clinical application of exogenous VEGF is currently not feasible [15]. Another agent used for enhancing angiogenesis is nitroglycerin. As a prodrug, nitroglycerin is rapidly metabolized in the tissue to release nitric oxide (NO), a mediator with diverse biological effects. NO activates guanylate cyclase in vascular smooth muscle cells, leading to increased cGMP production. cGMP then activates protein kinase G, cGMP-dependent ion channels, and cGMP-sensitive phosphodiesterases, with the best-known effect being smooth muscle relaxation via intracellular calcium redistribution [16]. Moreover, studies have shown that inhibition of NO synthesis prevents angiogenesis in models of portal hypertension [17], [18] and that VEGF's effect via its receptor VEGFR-2 is NO-dependent [19]. Thus, NO-mediated mechanisms have been implicated in VEGF-induced angiogenesis. John Russell et al. [20]used sodium nitroprusside as an NO donor in a McFarlane flap model and reported significant improvements in flap survival with both topical and injection applications. Despite NO's established role as an angiogenic mediator, no previous study has investigated whether nitroglycerin can enhance neoangiogenesis and consequently improve flap

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autonomization. This provided the rationale for our study, in which we evaluated flap survival by transecting the pedicle of a 3×3 cm dorsal skin flap on day 5.

Historically, topical nitroglycerin was first used in an animal study by Rohrich et al. in 1984, where a 30 mg dose applied preoperatively and three days postoperatively resulted in improved axial flap viability in both rats and pigs compared to controls [21] Commercially, nitroglycerin is available in 0.2%, 0.4%, and 2% formulations. The 0.2% and 0.4% forms are prescribed for anal fissures and hemorrhoids, while the 2% form, used for angina, is not available domestically. Although most studies have shown benefits of nitroglycerin, some have failed to demonstrate improvements in tissue viability; these studies often employed a single daily dose [22] or low doses (approximately 5 mg) [23]. Considering the short duration of nitroglycerin's effect, we opted for a higher concentration available on the market. One study even found no benefit from a single postoperative dose on flaps and grafts [22]. In our experiments, Group 3—receiving topical nitroglycerin both before and after pedicle division— and group 2, which received nitroglycerin only for 5 days followed by vaseline exhibited a significantly larger area of viable flap tissue than the group 1. The greater mean surviving flap area observed in Group 3 suggests that continuous topical nitroglycerin application may be more effective in promoting flap viability. In the immunohistochemical evaluation, although both Groups 2 and 5 received 5 days of topical nitroglycerin, Group 5 demonstrated a statistically significantly higher vessel count. Two hypotheses may explain this: first, while vessel density on day 5 in Group 2 might have been comparable to that in Group 5, cessation of nitroglycerin thereafter could have led to incomplete maturation of the nascent angiogenic vessels, causing them to lose their vascular characteristics. Second, in Group 5, the vasodilatory effect of nitroglycerin prior to sacrifice may have rendered the early angiogenic vessels more distinctly visible upon staining. This is supported by the observed clinical reduction in flap viability and decreased vessel counts in Group 2.

To isolate the effect of flap elevation and the angiogenic influence of topical nitroglycerin, we compared Groups 4 and 5. Although immunohistochemical staining showed better vessel counts in Group 5, this difference was not statistically significant. A statistically significant increase in vessel counts was observed in Group 5 based on immunohistochemical staining, suggesting that topical nitroglycerin enhances neovascularization.

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Regarding other histological findings, nitroglycerin significantly affect epithelialization across the groups , statistically significant difference was observed among the repeated measures ($p = 0.048$), although the result was close to the threshold of significance. In line with immunohistochemical results, vascular proliferation was most pronounced in Group 3, followed by Group 5. Furthermore, nitroglycerin's well-known anti-inflammatory and ischemia-reperfusion injury-preventing properties were evidenced by a statistically significant reduction in inflammatory cell infiltration in Groups 3 and 5 compared to the others [24]. Collagen degradation—a marker of ischemia-reperfusion injury—was significantly higher in Groups 1 and 2, where nitroglycerin was not used post-pedicle division.
While immunohistochemistry offered valuable data on angiogenesis, incorporating molecular techniques—such as VEGF gene expression profiling by RT-PCR or the assessment of other angiogenic markers—could enhance the robustness of the results. These methods are recommended for subsequent research.

CONCLUSION

In summary, our findings indicate that topical nitroglycerin enhances flap autonomization by promoting angiogenesis, thereby improving flap viability and potentially reducing the time required before safe pedicle division in reconstructive procedures.

Conflicts of interest: Authors declare no conflicts of interest.

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REFERENCES

[1] Y. Berkane *et al.*, "The Superficial Inferior Epigastric Artery Axial Flap to Study Ischemic Preconditioning Effects in a Rat Model," *Journal of Visualized Experiments*, no. 191, Jan. 2023, doi: 10.3791/64980.

[2] J. Granzow, A. I. Li, A. Caton, and J. B. Boyd, "Free Flap Survival Following Failure of the Vascular Pedicle," *Ann Plast Surg*, vol. 75, no. 1, pp. 44–48, Jul. 2015, doi: 10.1097/SAP.0000000000000136.

[3] C. J. Salgado *et al.*, "Effects of Late Loss of Arterial Inflow on Free Flap Survival," *J Reconstr Microsurg*, vol. 18, no. 7, pp. 579–584, 2002, doi: 10.1055/s-2002-35095.

[4] "Ludolph, Ingo et al. 'Indocyanine Green Angiography and the Old Question of Vascular Autonomy – Long Term Changes of Microcirculation in Microsurgically Transplanted Free Flaps'. 1 Jan. 2019 : 421 – 430.".

[5] J. Wang, Y. Xu, Y. Ruan, and X. Hu, "L-theanine promotes angiogenesis in limb ischemic mice by modulating NRP1/VEGFR2 signaling," *Biomolecules and Biomedicine*, vol. 25, no. 5, pp. 1063–1078, Apr. 2025, doi: 10.17305/bb.2024.11256.

[6] E. Karacaoglu, F. Yuksel, S. O. Turan, and R. J. Zienowicz, "Chemical Delay: An Alternative to Surgical Delay Experimental Study," *Ann Plast Surg*, vol. 49, no. 1, pp. 73–80, Jul. 2002, doi: 10.1097/00000637-200207000-00012.

[7] L. Richards, W. C. Lineaweaver, F. Stile, J. Zhang, and F. Zhang, "Effect of Hyperbaric Oxygen Therapy on the Tubed Pedicle Flap Survival in a Rat Model," *Ann Plast Surg*, vol. 50, no. 1, pp. 51–56, Jan. 2003, doi: 10.1097/00000637-200301000-00009.

[8] S. A. Vourtsis, P. K. Spyriounis, G. D. Agrogiannis, M. Ionac, and A. E. Papalois, "VEGF Application on Rat Skin Flap Survival," *Journal of Investigative Surgery*, vol. 25, no. 1, pp. 14–19, Feb. 2012, doi: 10.3109/08941939.2011.593693.

[9] T. Mücke, K. D. Wolff, A. Rau, V. Kehl, D. A. Mitchell, and T. Steiner, "Autonomization of free flaps in the oral cavity: A prospective clinical study," *Microsurgery*, vol. 32, no. 3, pp. 201–206, Mar. 2012, doi: 10.1002/MICR.20984.

[10] "Weinzweig N, Gonzalez M. Free tissue failure is not an all-or-none phenomenon. Plast Reconstr Surg. 1995; 96: 648".

EARLY ACCESS

[11] S. F. Rudy, M. Abdelwahab, C. K. Kandathil, and S. P. Most, "Paramedian forehead flap pedicle division after 7 days using laser-assisted indocyanine green angiography," *Journal of Plastic, Reconstructive & Aesthetic Surgery*, vol. 74, no. 1, pp. 116–122, Jan. 2021, doi: 10.1016/j.bjps.2020.05.079.

[12] F. J. Menick, "A 10-year experience in nasal reconstruction with the three-stage forehead flap," *Plast Reconstr Surg*, vol. 109, no. 6, pp. 1839–1855, 2002, doi: 10.1097/00006534-200205000-00010.

[13] D. W. Furnas, R. C. Lamb, B. M. Achauer, I. M. Turpin, and K. S. Black, "A pair of five-day flaps: early division of distant pedicles after serial cross-clamping and observation with oximetry and fluorometry.," *Ann Plast Surg*, vol. 15, no. 3, pp. 262–7, Sep. 1985.

[14] F. Zhang *et al.*, "Accelerating flap maturation by vascular endothelium growth factor in a rat tube flap model," *Br J Plast Surg*, vol. 55, no. 1, pp. 59–63, Jan. 2002, doi: 10.1054/bjps.2001.3704.

[15] N. Ferrara and H.-P. Gerber, "The Role of Vascular Endothelial Growth Factor in Angiogenesis," *Acta Haematol*, vol. 106, no. 4, pp. 148–156, 2001, doi: 10.1159/000046610.

[16] R. C. Webb, "SMOOTH MUSCLE CONTRACTION AND RELAXATION," *Adv Physiol Educ*, vol. 27, no. 4, pp. 201–206, Dec. 2003, doi: 10.1152/advan.00025.2003.

[17] D. Fukumura *et al.*, "Predominant role of endothelial nitric oxide synthase in vascular endothelial growth factor-induced angiogenesis and vascular permeability," *Proceedings of the National Academy of Sciences*, vol. 98, no. 5, pp. 2604–2609, Feb. 2001, doi: 10.1073/pnas.041359198.

[18] L. T. Sumanovski, E. Battegay, M. Stumm, M. van der Kooij, and C. C. Sieber, "Increased angiogenesis in portal hypertensive rats: Role of nitric oxide," *Hepatology*, vol. 29, no. 4, pp. 1044–1049, Apr. 1999, doi: 10.1002/hep.510290436.

[19] S. Thibeault *et al.*, "S-Nitrosylation of β-Catenin by eNOS-Derived NO Promotes VEGF-Induced Endothelial Cell Permeability," *Mol Cell*, vol. 39, no. 3, pp. 468–476, Aug. 2010, doi: 10.1016/j.molcel.2010.07.013.

EARLY ACCESS

[20] J. A. Russell, N. P. Connor, and G. K. Hartig, “Iontophoretic delivery of nitric oxide donor improves local skin flap viability.,” *J Rehabil Res Dev*, vol. 47, no. 1, pp. 61–6, 2010, doi: 10.1682/jrrd.2008.10.0144.

[21] R. J. Rohrich, G. W. Cherry, and M. Spira, “Enhancement of Skin-Flap Survival Using Nitroglycerin Ointment,” *Plast Reconstr Surg*, vol. 73, no. 6, pp. 943–948, Jun. 1984, doi: 10.1097/00006534-198406000-00016.

[22] C. L. Dunn, D. G. Brodland, R. D. Griego, M. J. Huether, M. J. Fazio, and J. A. Zitelli, “A Single Postoperative Application of Nitroglycerin Ointment Does Not Increase Survival of Cutaneous Flaps and Grafts,” *Dermatologic Surgery*, vol. 26, no. 5, pp. 425–427, May 2000, doi: 10.1046/j.1524-4725.2000.99307.x.

[23] L. S. Nichter, M. W. Sobieski, and M. T. Edgerton, “Efficacy of Topical Nitroglycerin for Random-Pattern Skin-Flap Salvage,” *Plast Reconstr Surg*, vol. 75, no. 6, pp. 847–852, Jun. 1985, doi: 10.1097/00006534-198506000-00016.

[24] J. R. de Berrazueta, I. Sampedro, M. T. Garcia-Unzueta, J. Llorca, M. Bustamante, and J. A. Amado, “Effect of transdermal nitroglycerin on inflammatory mediators in patients with peripheral atherosclerotic vascular disease,” *Am Heart J*, vol. 146, no. 4, p. 746, Oct. 2003, doi: 10.1016/S0002-8703(03)00391-0.

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TABLES AND FIGURES WITH LEGENDS

Table 1. Mean surviving flap areas (mm²) and their percentages, presented with standard deviations and 95% confidence intervals

	Average surviving areas (mm²)	95% CI		Adjusted *p* values
Group 1	273,5 (%30,40) ±176,74	161,2-385,8	**Group 1-2**	0.002
Group 2	485,5 (%53,94) ±147,67	391,6-579,3	**Group 1-3**	<0.001
Group 3	757,25 (%84,14) ± 61,40	720,5-830,6	**Group 2-3**	<0.001

Note: Adjusted *p* values represent pairwise comparisons between groups following one-way ANOVA with Bonferroni correction. A p value < 0.05 was considered statistically significant. Abbreviation: CI, confidence interval.

Table 2. Mean immunohistochemical vessel counts of the groups, and *p* values of the groups

	VEGF	CD34	CD105
Group 1 (5 day TV+7 day TV)	5,41±2,15 4,04-6,78	5,25±2,17 3,86-6,63	4,91±1,97 3,66-6,17
Group 2 (5 day TN+7 day TV)	6,91±2,42 5,37-8,46	6,75±2,41 5,21-8,28	6,16±2,79 4,39-7,93
Group 3 (5 day TN+7 day TN)	22,66±2,93 20,80-24,53	21,58±2,81 19,79-23,36	20,75±2,66 19,05-22,44
Group 4 (5 day TV)	11,41±3,02 9,49-13,34	10,83±2,79 9,06-12,60	10,41±2,10 9,07-11,75
Group 5 (5 day TN)	20,08±2,35 18,58-21,57	19,41±2,31 17,94-20,88	18,83±2,72 17,10-20,56

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	***p* values**		
Group 1-2	1.0	1.0	1.0
Group 1-3	<0.001	<0,001	<0,001
Group 2-3	<0.001	<0,001	<0,001
Group 4-5	<0.001	<0.001	<0,001

Note: Data are presented as mean ± standard deviation and 95% confidence intervals (95% CI). *p* values obtained from one-way ANOVA and < 0.05 was considered statistically significant. Abbreviations: VEGF, vascular endothelial growth factor; TN, topical nitroglycerin; TV, topical vaseline.

Table 3. Histopathological scoring of all groups

	Disruption in epithelialization	**Hemorrhage**	**Collagen degradation**	**Vascular proliferation**	**Leukocytic infiltration**
Group 1	1,0(1,5-0)	3(4-2)	4(4-3)	1(2-1)	3(3,5-2)
Group 2	1,0(1-0,5)	3(3-1,5)	3(4-3)	2(2-1)	2,5(3-2)
Group 3	0(0,5-0)	0(0-0)	0(1-0)	4(4-3)	0(1-0)
Group 4	0(1-0)	1(1-1)	1.25(2-1)	2(2,5-1)	2,5(3-2)
Group 5	0(1-0)	0(0,5-0)	1(1-1)	3(3-2,5)	1,5(2-1)
	***p* values**				
Group 1-2	0.999	0.786	0.617	0.842	0.962
Group 1-3	0.171	<.001	<.001	<.001	<.001
Group 2-3	0.102	<.001	<.001	<.001	<.001
Group 4-5	1.000	0.097	0.087	0.097	0.062

Note: Histopathological evaluation of flap tissue in groups 1–5 using Hematoxylin & Eosin (H&E) and Masson's Trichrome staining. Five parameters were assessed: disruption of epithelialization, hemorrhage, collagen degradation, vascular proliferation, and leukocytic infiltration. Data are presented as median and interquartile range (IQR).

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Table 4. Comparison of histopathological parameters among study groups using the Kruskal–Wallis test with effect size

	p value	**Effect size (ε^2)**	**Interpretation**
Disruption in epithelialization	0.048	0.163	Medium
Hemorrhage	<.001	0.697	Large
Collagen degradation	<.001	0.777	Large
Vascular proliferation	<.001	0.548	Large
Leukocytic infiltration	<.001	0.564	Large

Note: Comparison of five histopathological parameters (disruption of epithelialization, hemorrhage, collagen degradation, vascular proliferation, and leukocytic infiltration) among groups. Effect size is reported as epsilon squared (ε^2), where 0.01 indicates a small effect, 0.08 a medium effect, and 0.26 a large effect. Abbreviation**:** ε^2, epsilon squared.

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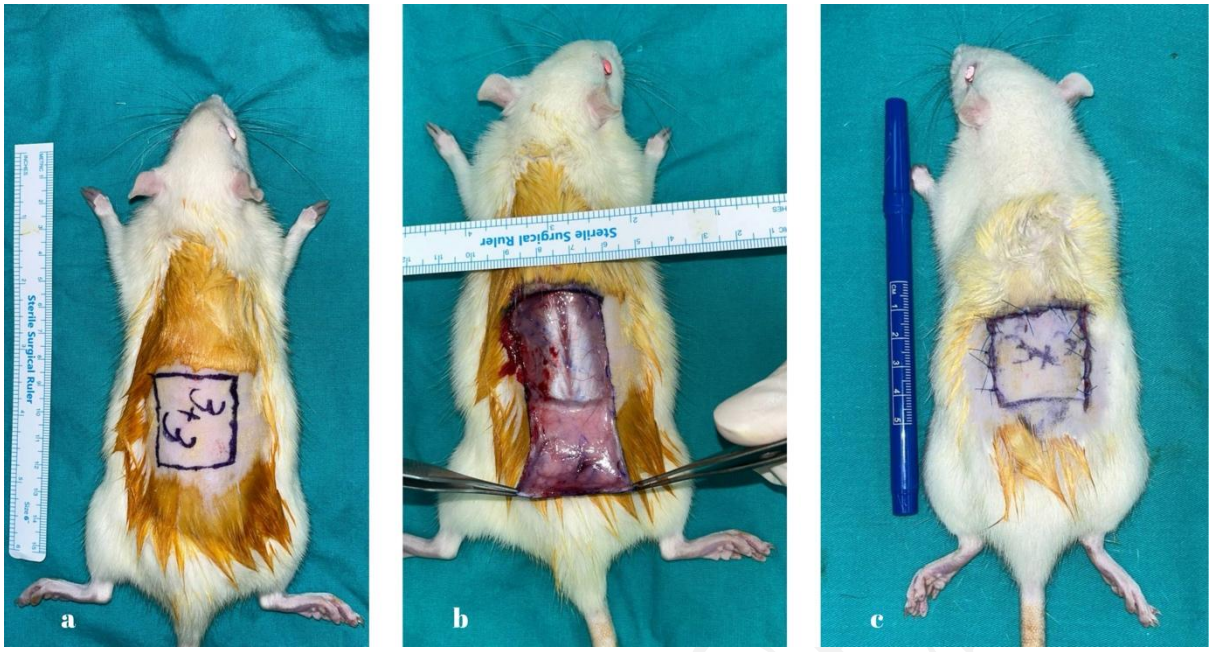

Figure 1. Creation of the dorsal caudally pedicled skin-flap model in Wistar-Albino rats. (a) Preoperative marking of a 3 × 3 cm dorsal skin flap; the pedicle is positioned between the caudal and iliac crests. (b) Elevation of the flap on its caudal pedicle. (c) Flap repositioning into the original bed and primary closure with interrupted 5-0 polypropylene sutures. The same standardized flap design and procedure were applied in all groups. Rulers indicate centimeters.

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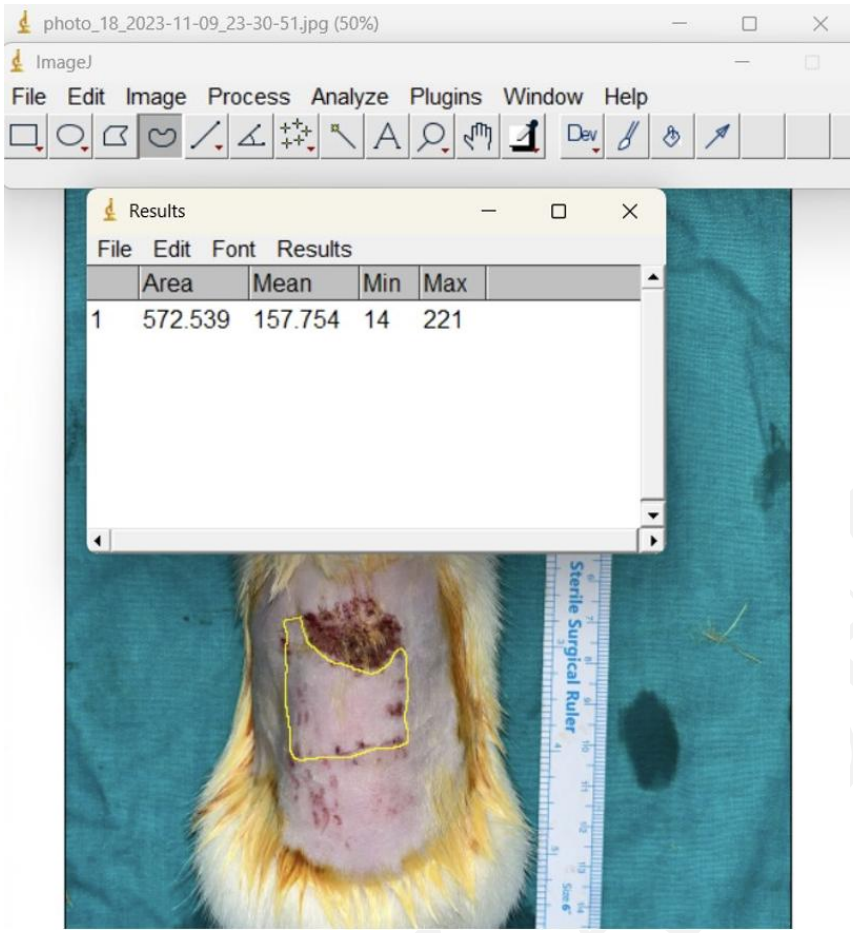

Figure 2. Quantification of viable flap area (mm²) using ImageJ.

Representative planimetric analysis performed on day 7 after pedicle transection, when necrosis was considered final. A metric ruler within the photograph was used to calibrate scale. The viable (non-necrotic) portion of the flap was traced with a polygon ROI (yellow), and ImageJ (open-source Java software, USA) returned the area in square millimeters (Results window). For each animal, percentage viability was calculated as (viable area ÷ total flap area) × 100.

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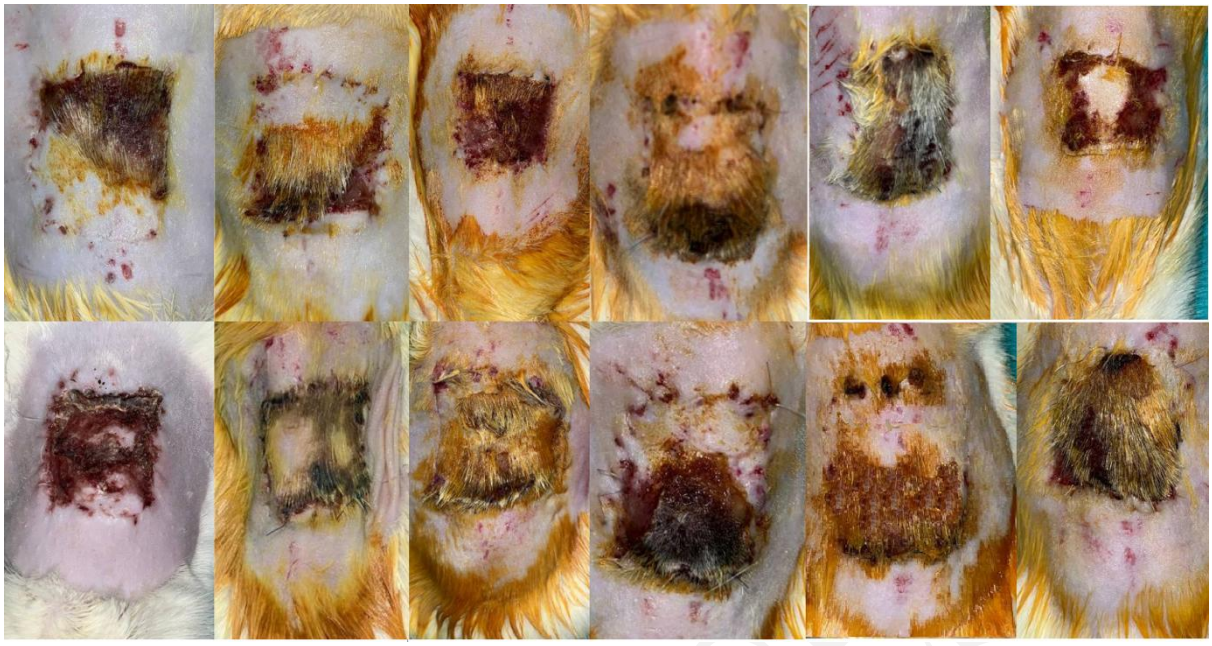

Figure 3. Surviving areas of flaps in group 1 on the 7th postoperative day after pedicle division. The viable regions are markedly smaller compared to the other experimental groups, showing extensive necrosis and limited tissue survival.

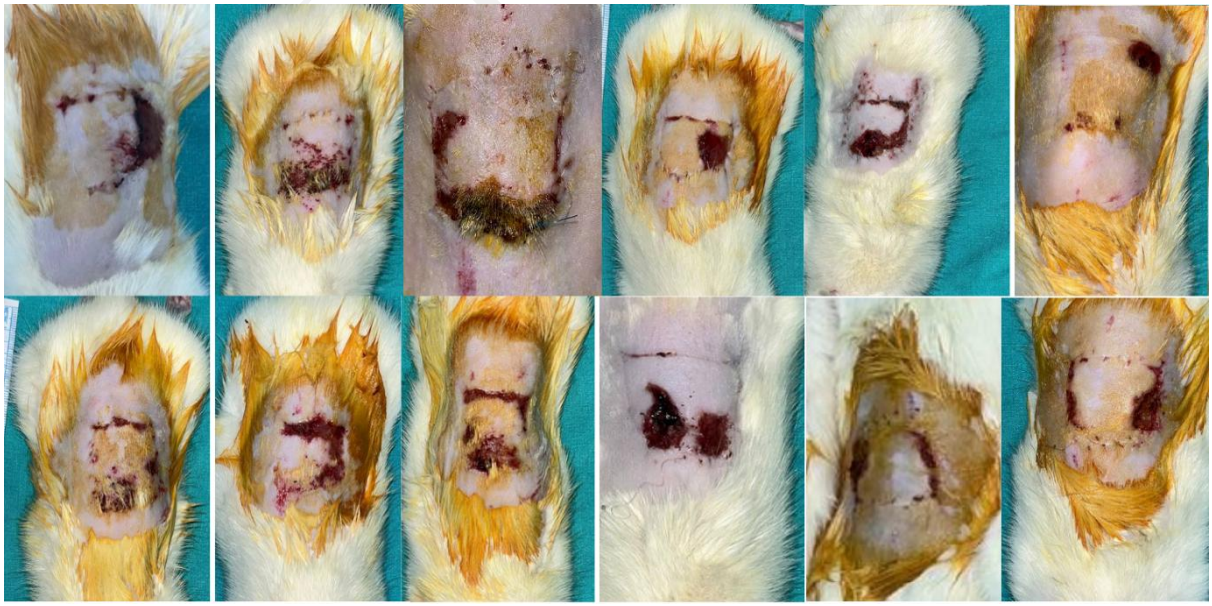

Figure 4. Representative appearance of surviving flap areas in group 2 on the 7th postoperative day after pedicle division. The extent of viable tissue is greater than in group 1, but remains smaller when compared with group 3.

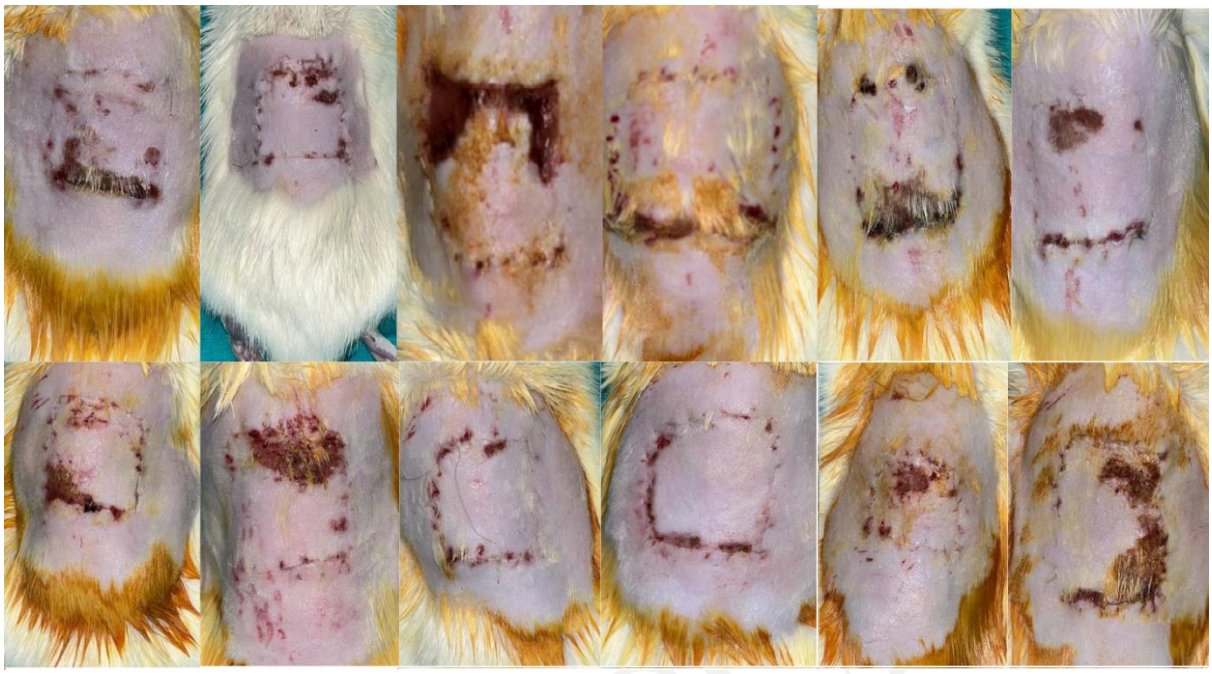

Figure 5. Representative appearance of surviving flap areas in group 3 on the 7th postoperative day after pedicle division. A visibly larger viable region is present compared with both group 1 and group 2.

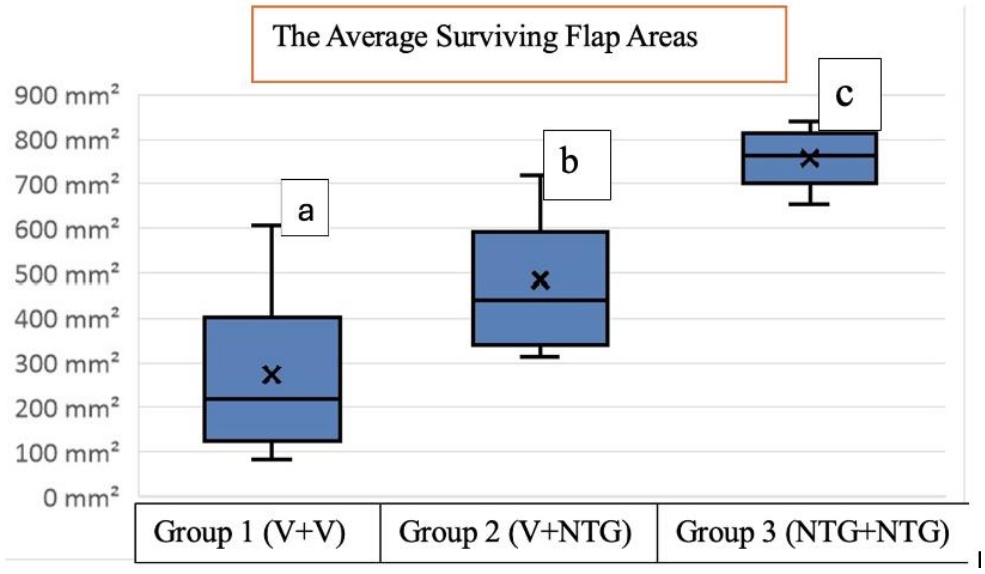

Figure 6. Box-plot representation of average surviving flap areas in the three experimental groups on the 7th postoperative day. Groups marked with different letters differ significantly. Abbreviations: V, vehicle; NTG, nitroglycerin.

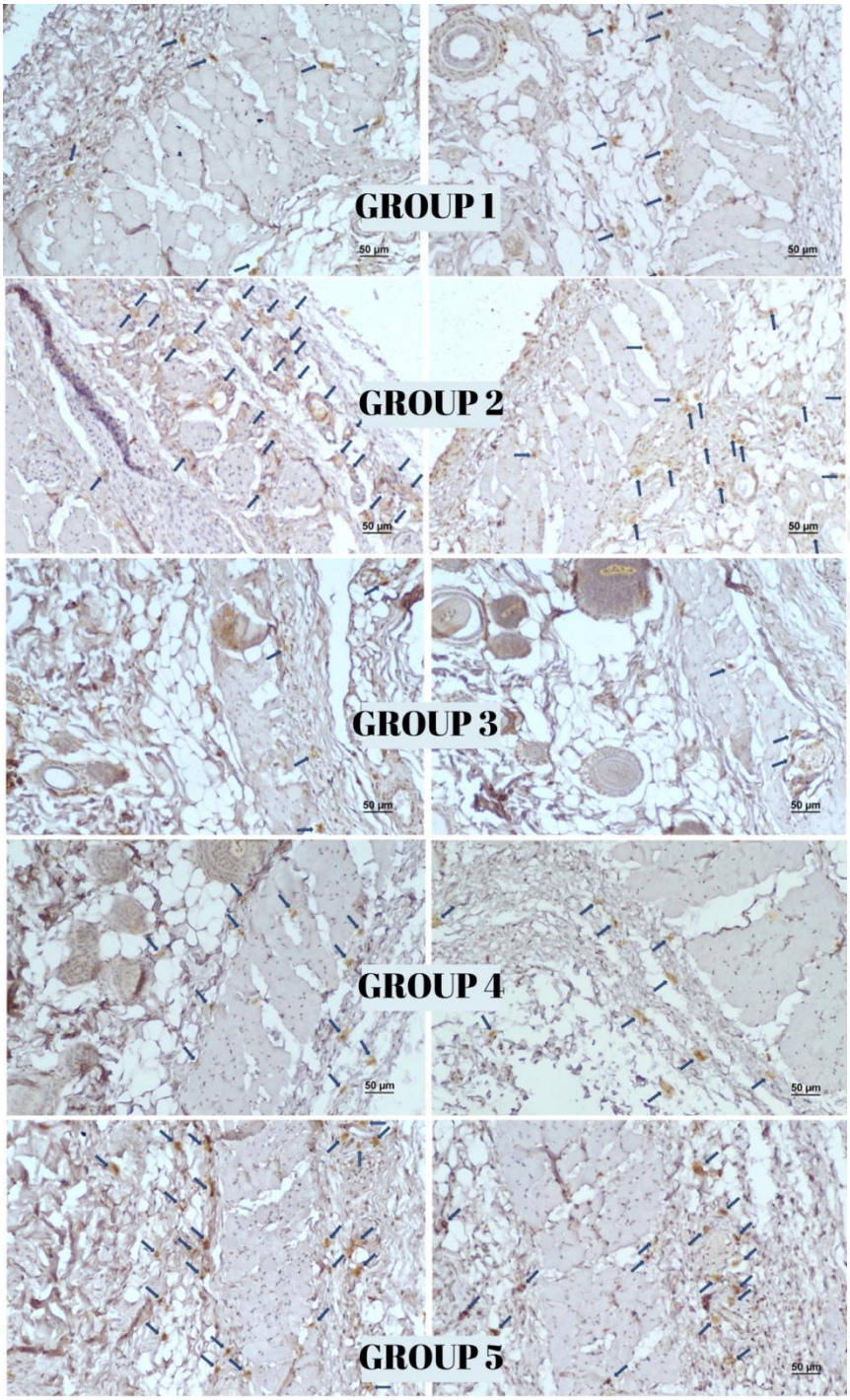

Figure 7. Immunohistochemical staining for VEGF expression in flap tissues across experimental groups. Representative micrographs demonstrate differences in staining patterns among groups. (Scale bar: 50 µm; Streptavidin–biotin peroxidase method, 100× magnification.). Abbreviation: VEGF, vascular endothelial growth factor.

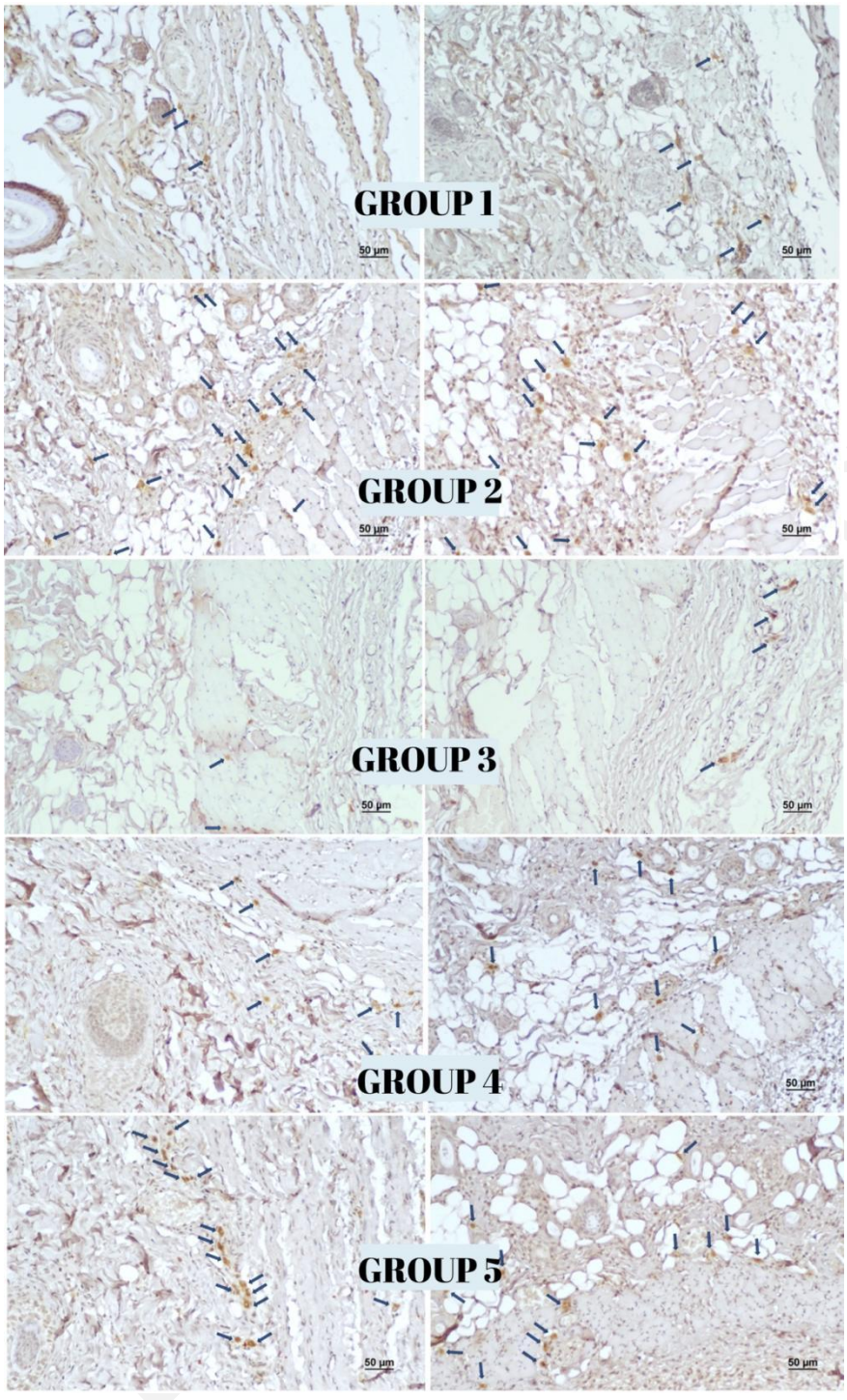

Figure 8. Immunohistochemical staining for CD34 in flap tissues across experimental groups. Strong staining intensity is evident in groups 3 and 5, moderate staining in group 4, and mild staining in groups 1 and 2. (Scale bar: 50 µm; Streptavidin–biotin peroxidase method, 100× magnification.)

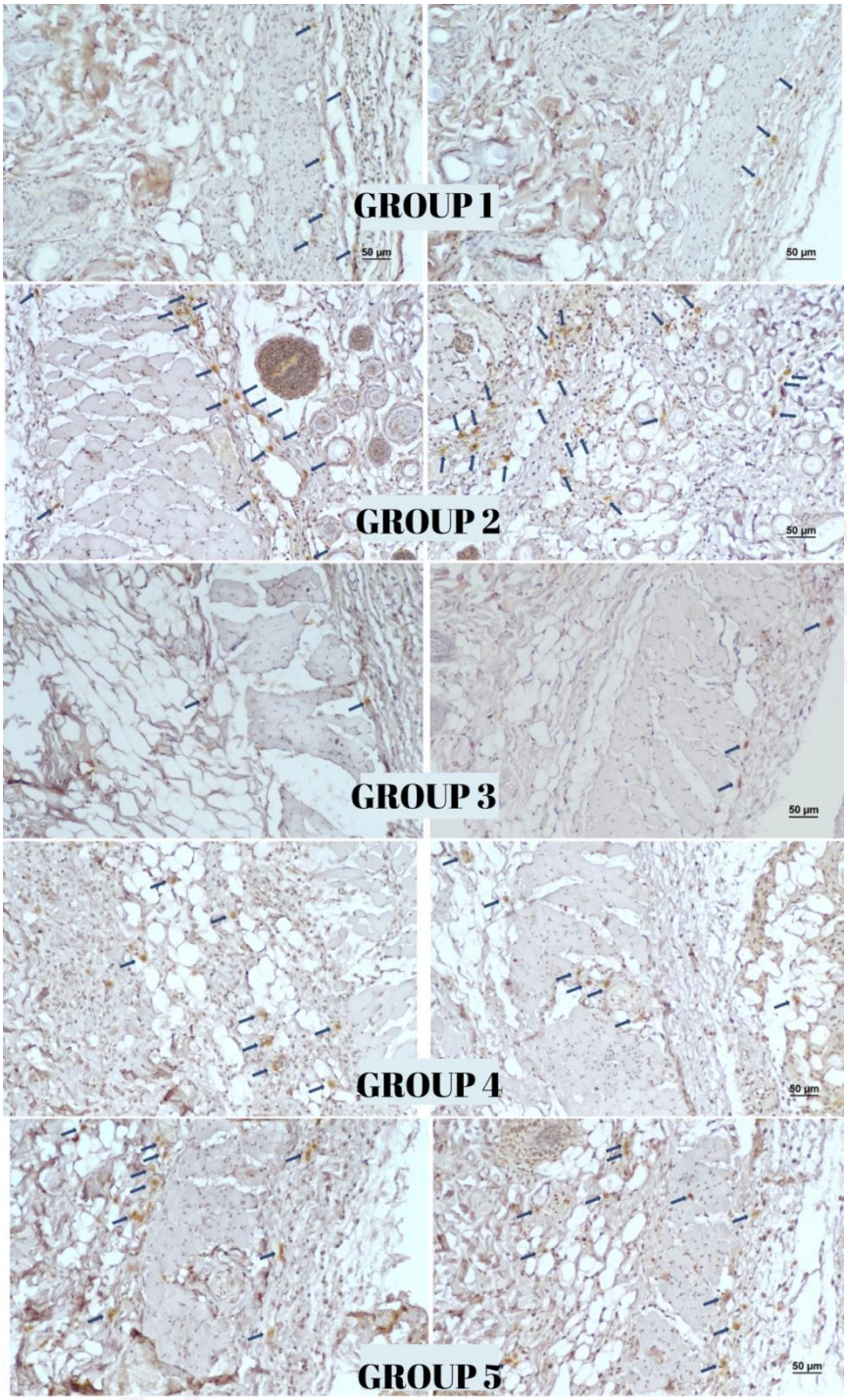

Figure 9. Immunohistochemical staining for CD105 in flap tissues across experimental groups. Strong staining intensity is observed in groups 3 and 5, moderate staining in group 4, and mild staining in groups 1 and 2. (Scale bar: 50 µm; Streptavidin–biotin peroxidase method, 100× magnification.)

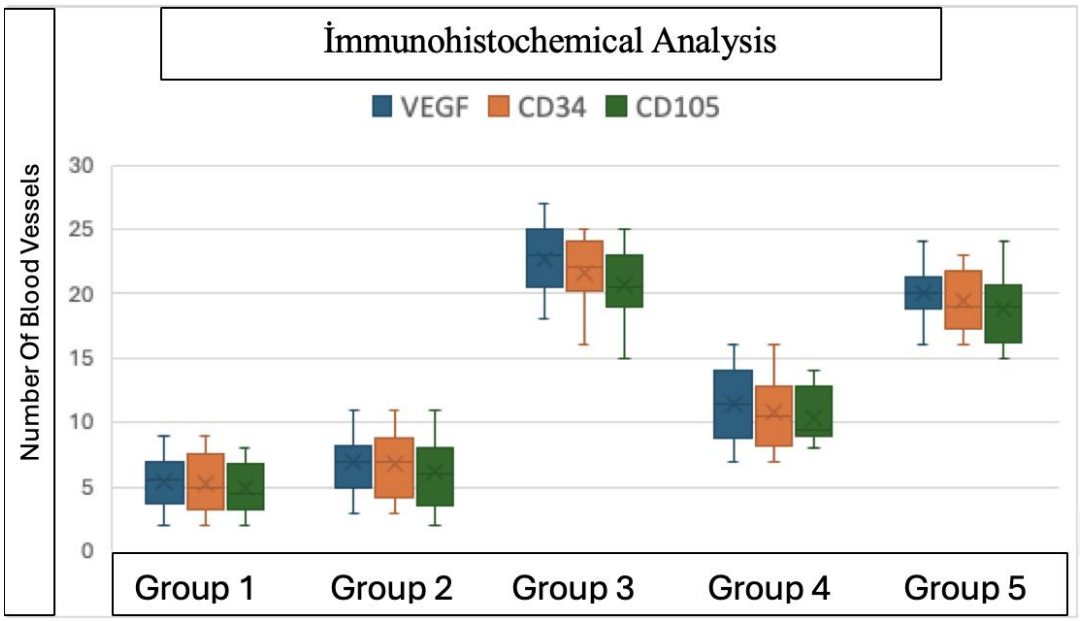

Figure 10. Graph showing immunohistochemical vascular counts across all groups. Group 3 demonstrated significantly higher vessel counts compared with groups 1 and 2, with no significant difference between groups 1 and 2. Group 5 exhibited significantly higher vascular counts compared with group 4.

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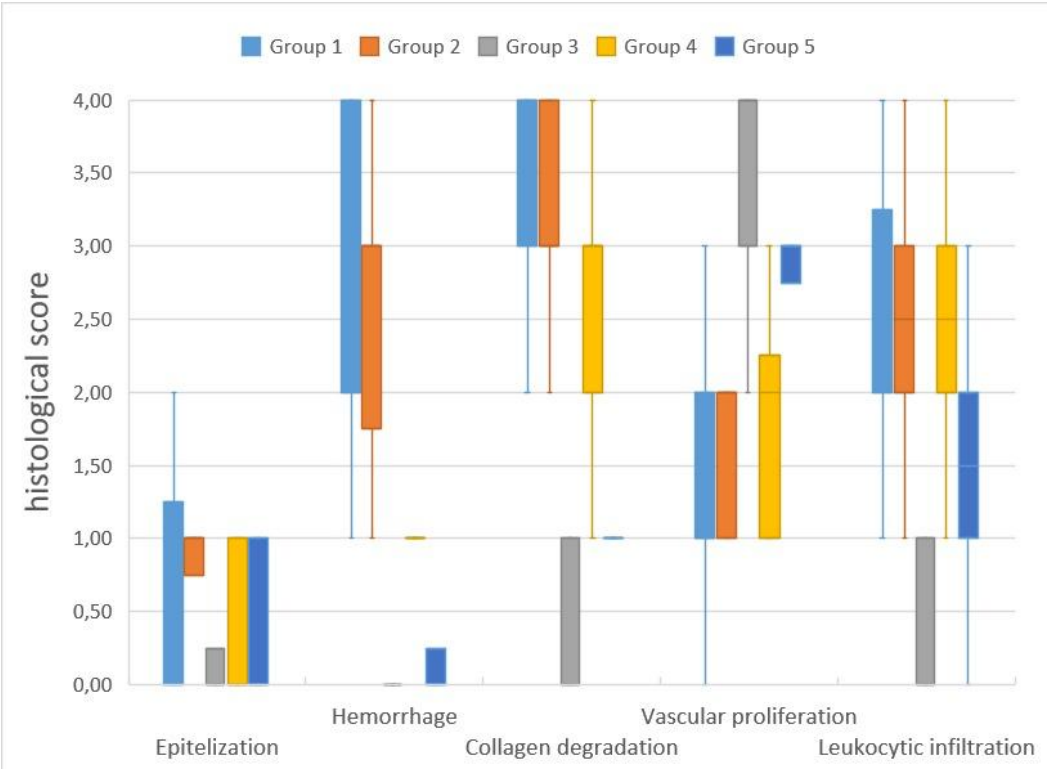

Figure 11. Graph of histological wound healing scores in all groups. The evaluated parameters include epithelialization, hemorrhage, collagen degradation, vascular proliferation, and leukocytic infiltration.

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