International Journal of Stem Cells & Research

Research Article

Nitroglycerin Ointment Enhances the Wound Healing Effect of Adipose-Derived Stem Cells

Sang Wha Kim*, Hyeonwoo Kim and MiRi Hyun

Department of Plastic and Reconstructive Surgery, Seoul National University College of Medicine, Korea

***Address for Correspondence:** Sang Wha Kim, Department of Plastic and Reconstructive Surgery, Seoul National University College of Medicine, ehak-roJongno-gu, Seoul 03080, Republic of Korea, E-mail: sw1215@snu.ac.kr

Submitted: 20 April 2020; Approved: 16 April 2020; Published: 22 June 2020

Cite this article: Kim SW, Kim H, Hyun M. Nitroglycerin Ointment Enhances the Wound Healing Effect of Adipose-Derived Stem Cells. Int J Stem Cells Res. 2020; 4(1): 001-005.

ABSTRACT

Background: Adipose-Derived Stem Cells (ADSCs) have provided new prospects in the treatment of impaired wound healing; however, the therapeutic effect of ADSCs requires improvement. Nitroglycerin Ointment (NTGO) has been shown to enhance the proliferation of mesenchymal stem cells and promote wound healing. The aim of the present study was to evaluate the efficacy of combining NTGO and ADSCs as a superior wound healing treatment, which was assessed in a mouse model.

Methods: Two splinted dorsal full-thickness skin defects (6 mm in diameter) were made in mice: NTGO was applied to one defect and the other was treated with conventional ointment as a control. ADSCs labeled with the fluorescent probe PKH-26were intravenously injected to the mice, and the paired wounds on were evaluated. For comparison, another group of mice with the same treatment of paired wounds received only saline injection without ADSCs.

Results: The number of recruited ADSCs at the NTGO-applied wounds was significantly higher than that in the conventional ointment-applied wounds, and the NTGO-applied wounds were significantly narrower than those of the control at day 4 and day 7 in the groups with ADSC injection.

Conclusion: The wound-healing rate was accelerated in the NTGO-applied wound compared with that in the control.

Keywords: Stem cells; Wound healing; Stem cell transplantation; Nitroglycerin

INTRODUCTION

Adipose-Derived Stem Cells (ADSCs) are known to home to the sites of injury or inflammation and can thus accelerate the wound-healing process [1,2]. This homing behavior of ADSCs to specific sites occurs upon their intravenous [3-5] or intra-arterial [6,7] administration. However, the specific mechanism underlying ADSC homing remains unclear, which is essential to best apply these cells in targeted therapies.

Two possible mechanisms of ADSC homing have been suggested: active and passive homing [8]. Ley et al. [9] suggested that ADSCs actively home to the inflamed endothelium through selectin- and integrin-mediated cell arrest. By contrast, Walczak, et al. [10] demonstrated the passive entrapment of ADSCs in capillaries or microvessels. The pitfall of ADSCs homing is that only 1% of administered ADSCs can successfully home *in vivo* [11], which limits their therapeutic application potential.

To overcome this limitation, various studies have reported the enhanced therapeutic response of ADSCs by additional factors. Brenner, et al. [12] used retroviral vectors encoding homing receptors such as CXCR4 to enhance ADSC homing. Lin, et al. [13] showed that combined treatment with sildenafil and ADSCs more effectively preserved the heart function of rats with dilated cardiomyopathy than treatment with either alone. However, these strategies focused on the active mechanism of ADSC homing, while the passive mechanism remains unclear. We have been focusing more on the passive entrapment of ADSCs because the size of ADSC clusters is known to be enlarged during *in vitro* culture [14].

Nitroglycerin (NTG) is a potent vasodilator that relaxes the vascular smooth muscles [15]. NTG is also known to stimulate the cell proliferation and osteoblastic differentiation of mesenchymal stem cells, and can be administered through intravenous, oral, or cutaneous routes. The cutaneous application of NTG as an ointment is non-invasive, provides sustained hemodynamic effects, and can be conveniently discontinued [16].In particular, Nitroglycerin Ointment (NTGO) has been shown to increase dermal blood flow [17] and improve the rate of wound healing [18]. Therefore, we hypothesized that NTGO may enlarge the terminal vessels near wound beds and that ADSCs would be able to reach these wounds more efficiently before being arrested passively, thus enhancing their homing ability. To test this hypothesis, we evaluated the efficacy of combining NTGO and ADSCs as a superior wound healing treatment in a mouse model.

MATERIALS AND METHODS

ADSC isolation and characterization

We harvested the inguinal fat tissues from two male ICR mice purchased from ORIENT Inc. (Seongnam, Seoul). ADSCs were isolated and cultured according to our previously described method [19]. ADSCs were identified according to positive expression of the markers CD44, CD90, and CD105, and negative for CD31 and CD45 [20]. For cell tracking, ADSCs were labeled with PKH-26 (Mini26-1Kt, Sigma-Aldrich), which was incorporated into the lipid region of the cell membrane [1]. For intravenous injection, 5×10^5 ADSCs were mixed with 100 μl of normal saline.

Wound model

Fifteen 5-week-old male ICR mice were used to establish wound models in this study. The mice were fed a standardized diet for 1 week during the adjustment period prior to the experiment. The mice were epilated using NiClean (Ildong, Seoul, Korea) before the experiment. Under anesthesia, two identical wounds were made on the back of each mouse using a 6-mm punch biopsy device (Kai Medical, Seki City, Japan) to compare the effect of NTGO with that of a control conventional ointment. The wounds were located at a relatively cephalic position to prevent self-harming. Silicone splints (Bioplexus, CA, USA) with a 6-mm-diameter hole were fixed on each wound to prevent contraction and to estimate the change in wound size [2]. The splints were fixed at the wound margin using prolene 5-0 (Ethicon, NJ, USA). The two wounds were separated to the greatest extend from each other as possible to minimize any reciprocal local effects (Figure 1).

Different ointments were applied to both wounds on each mouse after fixing the splint: NTGO (Pasarect; Daehwa, Seoul, Korea) was applied on one wound, while practical ophthalmic ointment (Effexin; Ildone, Seoul, Korea) was applied on the other wound. The wounds were then covered with Tegaderm film (3M, MN, USA). Then the prepared ADSC suspensions were injected into each mouse intravenously through retrobulbar injection.

Pictures of the wounds were taken on days 1, 2, 3, 5, and 7 to measure the wound size after crust removal. Wound sizes were measured using the ImageJ program (National Institutes of Health, Bethesda, MD, USA) from the digital photographs. The wound tissues were excised after measurement on day 7 to identify the migration of

ADSCs to the wound site. The wounded tissue and the surrounding skin were carefully excised and fixed in 10% formalin overnight at 4°C. All experiments of this study was approved by the Institutional Animal Care and Use Committee of our institution [18-0037-S1A0 (4)].

Fluorescence microscopy

Homing of ADSCs was detected by observing the DAPI-stained tissues using fluorescence microscopy [19]. The number of recruited PKH-26-stained ADSCs wascounted from three randomly selected areas on each slide.

In vivo Imaging System (IVIS)

ADSCs were labeled with PKH26 (Mini26-1Kt, Sigma-Aldrich) prior to injection, which results in a yellow-orange fluorescence identified at 551 nm excitation and 567 nm emission [21]. The Lumina system (Perkin Elmer, USA) was used to detect the PKH26 signal after ADSC injection for confirmation.

Statistical analysis

All statistical analyses were performed using the IBM SPSS Statistics 23 package (Chicago, IL, USA). Student's t-test was used to compare data between two groups in each case.

RESULTS

Histologic analysis

Fluorescence microscopy showedthe red-colored PKH-26-stained ADSCs scattered throughout the DAPI-stained background tissues (Figure 2).

The average number of recruited ADSCs at the NTGO-applied wounds and conventional ointment-applied wounds was 11.7 ± 6.5 and 6.2 ± 4.4, respectively, representing a statistically significant difference ($p < 0.004$).

In vivo imaging

Migration of ADSCs to the wound site was tracked using IVIS at days 1, 3, 5, and 7. Increased signals from PKH-26-labeled ADSCs were observed at the wound site from day 3 and were maintained until day 7 (Figure 3.)

Wound size

The size of NTGO-applied wounds was not significantly different

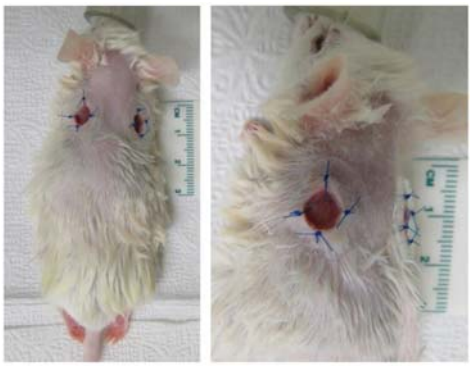

Figure 1: Wound model in 5-week-old male ICR mice. After epilation, two identical wounds were created on the back of each mouse using a 6-mm punch biopsy device. Silicone splints with a 6-mm-diameter hole were fixed on each wound.

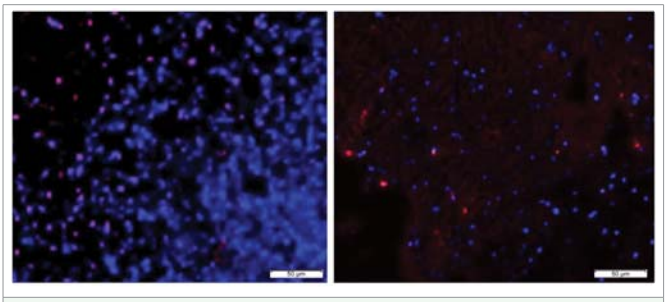

Figure 2: Fluorescence microscopy of wound tissues at 7 days after systemic injection of PKH-26-labeled ADSCs. PKH-26-labeled ADSCs can be seen (red) among the cells stained with DAPI identified in the wound tissue (blue).

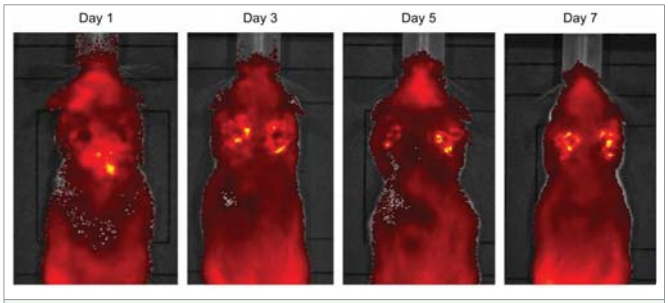

Figure 3: Tracking the migration of ADSCs to the wound site with an *in vivo* imaging system. The signal from PKH-26-labeled ADSCs was tracked at days 1, 3, 5, and 7.

compared with that of the control from day 1 to day 7 in the groups with saline injection (Table 1). However, the size of NTGO-applied wounds was significantly narrower than that of the control at day 4 and day 7 in the groups that received ADSC injection (Table 2, Figure 4).

DISCUSSION

ADSCs have been widely used in the field of plastic and reconstructive surgery, which overcome some of the limitations of bone marrow-derived mesenchymal stem cells, such as morbidity of the donor site and a low cell number upon processing [22,23]. ADSCs have been used in several trials to reconstruct soft tissue defects [24,25] and enhance the wound-healing process [26,27].

ADSCs can be injected directly as local implants, although this may disrupt the highly complex microenvironment of the targeted sites [28].The therapeutic effect of ADSCs is achieved by their differentiation ability, as well as by the paracrine effect of their released cytokines [3,29]. Therefore, some researchers suggested systemic administration as the preferred method of ADSC delivery. Although the passive mechanism of ADSC homing is unclear, Walczak, et al. [10] explored this mechanism during the intra-arterial delivery of ADSCs, and found that engraftment was higher when the microvascular circulation was compromised. In the group with preserved microvasculature, the engraftment rate was lower and distribution to non-desirable sites was increased.

NTGO was first described for the management of angina pectoris [30] and is now widely used for treating anal fissures, pressure sores, and peripheral tissue ischemia [31-34]. In the context of wound healing, NTGO has been reported as effective in preventing skin flap necrosis [35-38]. NTGO relaxes the smooth muscles in the vessel wall and dilates both the veins and arteries. NTGO also stimulates the synthesis of prostacyclin, a potent vasodilator and inhibitor of

Table 1: Measurement of the wound area in mice without ADSC injection.

		Day 1	Day 2	Day 3	Day 5	Day 7
Wound area (%)	**N**	100	84.1 ± 16.6	53.0 ± 9.8	43.1 ± 15.8	24.3 ± 12.9
	C	100	78.1 ± 21.6	52.4 ± 25.4	26.0 ± 11.3	15.8 ± 6.5
***P*-value**			0.409	0.967	0.182	0.274

ADSC: Adipose-Derived Stem Cell; N: Nitroglycerin-applied wound; C: Control
Values are presented as the mean ± SD.

Table 2: Measurement of the wound area in the groups with ADSC injection.

		Day 1	Day 2	Day 3	Day 5	Day 7
Wound area (%)	**N**	100	81.4 ± 11.7	65.7 ± 14.8	56.5 ± 18.1	45.2 ± 19.4
	C	100	86.4 ± 10.1	74.4 ± 13.0	65.6 ± 14.8	56.9 ± 12.3
***P*-value**			0.189	0.036	0.050	0.002

ADSC: Adipose-Derived Stem Cell; N: Nitroglycerin-applied wound; C: Control
Values are presented as the mean ± SD.

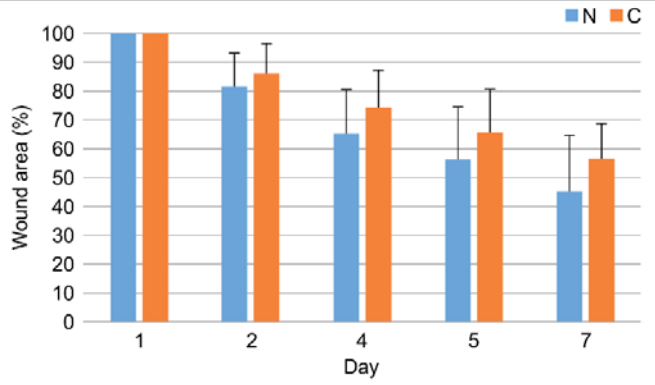

Figure 4: Accelerated wound healing of NTGO-applied wounds after ADSC injection. N, NTGO-applied wounds; C, conventional ointment-applied wounds.

platelet aggregation [39]. We have focused on the mechanical aspect of ADSC entrapment into end-vessels, where the fate of entrapment is determined by the relative size of ADSCs to the vessels. Moreover, we considered that the diameter of the end-vessels near the wound site is relatively smaller than the mean diameter of stem cells [40,41]. Accordingly, we assumed that vasodilator ointments such as NTGO would enlarge the vessel size near the wound bed, and may enable the recruitment of ADSCs by preventing proximal entrapment before reaching the wound sites. We designed an animal study using mice to assess our hypothesis. The healing process of skin in mice involves contraction and epithelialization. We applied a silicone splint in our wound model to prevent contraction, as suggested by Galiano, et al. [22] to mimic the nature of human skin.

The results of our study support this hypothesis. The density of ADSCs at the wound bed was found to be higher in NTGO-applied wounds compared with that of the controls, and the healing rates were also higher in NTGO-applied wounds. More specifically, the group without ADSC injection showed no significant differences in the wound-healing rate between the NTG-applied wound and control. However, significant acceleration in the wound-healing rate was observed in the NTG-applied wound compared with that of the control in the group that received ADSC injection. These results suggest that a combination of systemic ADSC injection and NTGO application is a superior option compared with injecting ADSCs alone.

The main limitation of this study is that NTGO might affect other nearby control wounds as a paracrine effect, which should be evaluated. Further, for clinical translation, the study needs to be repeated in other animal models such as pigs, because the skin properties of mice differ from those of humans.

CONCLUSION

Homing of ADSCs was increased to the NTGO-applied wound compared with the control. Further, the wound-healing rate was accelerated in the NTGO-applied wound compared with that in the control.

Data Availability

All the data generated in this study can be made available upon request to the corresponding author.

Conflicts of Interest

The authors declare that there is no conflict of interest regarding the publication of this paper.

Funding Statement

There are no funding or grant received for this study.

REFERENCES

1. AM Hocking, NS Gibran. Mesenchymal stem cells: paracrine signaling and differentiation during cutaneous wound repair. Experimental Cell Research. 2010; 316: 2213-2219. https://doi.org/10.1016/j.yexcr.2010.05.009
2. PA Zuk, M Zhu, P Ashjian. Human adipose tissue is a source of multipotent stem cells. Molecular Biology of the Cell. 13: 2002; 42794295. DOI: https://doi.org/10.1091/mbc.E02-02-0105
3. H Zhang, X. Qiu, AW Shindel. Adipose tissue-derived stem cells ameliorate diabetic bladder dysfunction in a type II diabetic rat model. Stem Cells and Development. 2011; 21: 1391-1400. DOI: https://doi.org/10.1089/scd.2011.0244
4. X Qiu, J Villalta, L Ferretti. Effects of intravenous injection of adipose-derived stem cells in a rat model of radiation therapy-induced erectile dysfunction. The Journal of Sexual Medicine. 2012; 9: 1834-1841. DOI: https://doi.org/10.1111/j.1743-6109.2012.02753.x
5. JM Quimby, TL Webb, E Randall, A Marolf, A Valdes-Martinez, et al. Assessment of intravenous adipose-derived allogeneic mesenchymal stem cells for the treatment of feline chronic kidney disease: a randomized, placebo-controlled clinical trial in eight cats. Journal of Feline Medicine and Surgery. 2016; 18: 165-171. DOI: https://doi.org/10.1177/1098612X15576980.
6. G Du, Y Liu, M Dang. Comparison of administration routes for adipose-derived stem cells in the treatment of middle cerebral artery occlusion in rats. ActaHistochemica. 2014; 116: 1075-1084. DOI: https://doi.org/10.1016/j.acthis.2014.05.002
7. T Kato, M Okumi, M Tanemura. Adipose tissue-derived stem cells suppress acute cellular rejection by TSG-6 and CD44 interaction in rat kidney transplantation. Transplantation. 2014; 98: 277-284. DOI: https://doi.org/10.1097/tp.0000000000000230
8. JM Karp, GSL Teo. Mesenchymal stem cell homing: The devil is in the details. Cell Stem Cell. 2009; 4: 206-216. DOI: https://doi.org/10.1016/j.stem.2009.02.001
9. K Ley, C Laudanna, MI Cybulsky, S Nourshargh. Getting to the site of inflammation: The leukocyte adhesion cascade updated. Nature Reviews Immunology. 2007; 7: 678-689. DOI: https://doi.org/10.1038/nri2156
10. P Walczak, J Zhang, AA Gilad. Dual-modality monitoring of targeted intraarterial delivery of mesenchymal stem cells after transient ischemia. Stroke. 2008; 39: 1569-1574. DOI: https://doi.org/10.1161/STROKEAHA.107.502047

11. AM Bailey, MB Lawrence, H Shang, AJ Katz, SM Peirce. Agent-based model of therapeutic adipose-derived stromal cell trafficking during ischemia predicts ability to roll on P-selectin. PLoSComputational Biology. 2009; 5: e1000294. https://doi.org/10.1371/journal.pcbi.1000294

12. S Brenner, N Whiting-Theobald, T Kawai. CXCR4-transgene expression significantly improves marrow engraftment of cultured hematopoietic stem cells. Stem Cells. 2004; 22: 1128-1133. DOI: https://doi.org/10.1634/stemcells.2003-0196

13. YCLin, S Leu, CK Sun. Early combined treatment with sildenafil and adipose-derived mesenchymal stem cells preserves heart function in rat dilated cardiomyopathy. "Journal of Translational Medicine. 2010; 88. DOI: https://doi.org/10.1186/1479-5876-8-88

14. E Chavakis, C Urbich, S Dimmeler. Homing and engraftment of progenitor cells: a prerequisite for cell therapy. Journal of Molecular and Cellular Cardiology. 2008; 45: 514-522. DOI: https://doi.org/10.1016/j.yjmcc.2008.01.004

15. BM. Bennett GS. Marks. How does nitroglycerin induce vascular smooth muscle relaxation? Trends in Pharmacological Sciences. 1984; 5: 329-332. DOI: https://doi.org/10.1016/0165-6147(84)90461-9

16. AT Blei, S Friedman, J Gottstein, G Robertson, HL Fung, et al. Pharmacokinetic-hemodynamic interactions between vasopressin and nitroglycerin: Comparison between intravenous and cutaneous routes of nitrate delivery. Hepatology. 5: 1985; 264-270. DOI: https://doi.org/10.1002/hep.1840050219

17. E Blomain, E Manders, G Saggers, T Davis, W Graham. Topical nitroglycerin ointment enhances the survival of skin flaps. Surgical Forum. 1982; 33: 594-595.

18. H Karanlik, R Akturk, H Camlica, O Asoglu. The effect of glyceryl trinitrate ointment on posthemorrhoidectomy pain and wound healing: results of a randomized, double-blind, placebo-controlled study. Diseases of the Colon & Rectum. 2009; 52: 280-285. DOI: https://doi.org/10.1007/dcr.0b013e31819c98a7

19. A Mildmay-White, W Khan. Cell surface markers on adipose-derived stem cells: A systematic review,"Current Stem Cell Research &Therapy. 2017; 12: 484-492. DOI: https://doi.org/10.2174/1574888x11666160429122133

20. H Kim, MR Hyun, SW Kim. The effect of adipose-derived stem cells on wound healing: Comparison of methods of application. Stem cells international. 2019. DOI: https://doi.org/10.1155/2019/2745640

21. RY Poon, BM Ohlsson-Wilhelm, CB Bagwell, KA Muirhead. Use of PKH membrane intercalating dyes to monitor cell trafficking and function. In: Living Color, Springer. 2000; 302-352.

22. RD Galiano, J Michaels 5, M Dobryansky, JP Levine, QC Gurtner. Quantitative and reproducible murine model of excisional wound healing. Wound Repair and Regeneration. 2004; 12: 485-492. DOI: https://doi.org/10.1111/j.1067-1927.2004.12404.x

23. PA. Zuk, M Zhu, H Mizuno. Multilineage cells from human adipose tissue: implications for cell-based therapies. Tissue Engineering. 2001; 7: 211-228. DOI: https://doi.org/10.1089/107632701300062859

24. N Seyhan, D Alhan, AU Ural, A Gunal, MC Avunduk, N Savaci. "The effect of combined use of platelet-rich plasma and adipose-derived stem cells on fat graft survival,"Annals of Plastic Surgery. 2015; 74: 615-620. DOI: https://doi.org/10.1097/sap.0000000000000480

25. YC Bae, JS. Song, SH Bae, JH Kim. Effects of human adipose-derived stem cells and stromal vascular fraction on cryopreserved fat transfer. Dermatologic Surgery. 2015; 41: 605-614. DOI: https://doi.org/10.1097/dss.0000000000000342

26. G Rigotti, A Marchi, M Galie. Clinical treatment of radiotherapy tissue damage by lipoaspirate transplant: a healing process mediated by adipose-derived adult stem cells. Plastic and Reconstructive Surgery. 2007; 119: 1409-1422. DOI: https://doi.org/10.1097/01.prs.0000256047.47909.71

27. WS Kim, BS Park, JH Sung. Wound healing effect of adipose-derived stem cells: a critical role of secretory factors on human dermal fibroblasts. Journal of Dermatological Science. 2007; 48: 1524. DOI: https://doi.org/10.1016/j.jdermsci.2007.05.018

28. S Khaldoyanidi. Directing stem cell homing. Cell Stem Cell. 2008; 2: 198200. DOI: https://doi.org/10.1016/j.stem.2008.02.012

29. H Zhang, H Ning, L Banie. Adipose tissue-derived stem cells secrete CXCL5 cytokine with chemoattractant and angiogenic properties. Biochemical and Biophysical Research Communications. 2010; 402: 560-564. DOI: https://doi.org/10.1016/j.bbrc.2010.10.090

30. P Needleman. Drugs used for the treatment of angina. The Pharmacological Basis of Therapeutics. 1985; 806-816.

31. C Fenton, K Wellington, SE Easthope. 0.4% nitroglycerin ointment. Drugsvol. 2006; 66: 343-349.

32. RL Nelson, K Thomas, J Morgan, A Jones. Non surgical therapy for anal fissure. Cochrane Database of Systematic Reviews. 2012. DOI: https://doi.org/10.1002/14651858.CD003431.pub3

33. JT Jones, MA Stenson, BD Spannagel, DD Robinson. Treatment of pressure ulcers with nitropaste. Journal of the American Geriatrics Society. 1997; 45: 895.

34. AF Wong, LM McCulloch, A Sola. Treatment of peripheral tissue ischemia with topical nitroglycerin ointment in neonates. The Journal of Pediatrics. 1992; 121: 980-983. DOI: https://doi.org/10.1016/s0022-3476(05)80356-7

35. Z Fan, J He. Preventing necrosis of the skin flaps with nitroglycerin after radical resection for breast cancer. Journal of Surgical Oncology. 1993; 53: 210. DOI: https://doi.org/10.1002/jso.2930530319

36. S Kutun, AA Ay, H Ulucanlar. Is transdermal nitroglycerin application effective in preventing and healing flap ischaemia after modified radical mastectomy? South African Journal of Surgery. 2010; 48: 119-121. PubMed: https://pubmed.ncbi.nlm.nih.gov/22353276/

37. LS Nichter, MW Sobieski, MT Edgerton. Efficacy of topical nitroglycerin for random-pattern skin-flap salvage. Plastic and Reconstructive Surgery. 1985; 75: 847-852. DOI: https://doi.org/10.1097/00006534-198506000-00016

38. S. Scheuer, MK Hanna. Effect of nitroglycerin ointment on penile skin flap survival in hypospadias repair. Experimental and Clinical Studies Urology. 1986; 27: 438-440. DOI: https://doi.org/10.1016/0090-4295(86)90410-3

39. RI Levin, EA Jaffe, BB Weksler, K Tack-Goldman. Nitroglycerin stimulates synthesis of prostacyclin by cultured human endothelial cells. The Journal of Clinical Investigation. 1981; 67: 762-769. DOI: https://doi.org/10.1172/JCI110093

40. D Furlani, M Ugurlucan, L Ong. Is the intravascular administration of mesenchymal stem cells safe?: Mesenchymal stem cells and intravital microscopy. Microvascular Research. 2009; 77: 370-376. DOI: https://doi.org/10.1016/j.mvr.2009.02.001

41. HN Mayrovitz. Skin capillary metrics and hemodynamics in the hairless mouse. Microvascular Research. 1992; 43: 46-59. DOI: https://doi.org/10.1016/0026-2862(92)90005-a