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Effects of Nebivolol on Skin Flap Survival: A Randomized Experimental Study in Rats

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ABSTRACT

BACKGROUND: Skin flaps are among the basic treatment options in the reconstruction of soft tissue defects. To improve skin flap survival, a variety of methods, including pharmacologic agents, have been investigated. The effectiveness of anticoagulants, antioxidants, anti-inflammatory drugs, and vasodilatory drugs in improving flap survival has been studied. Nebivolol is a new-generation selective β_1-adrenoreceptor blocking agent that has vasodilatory, antithrombotic, antioxidative, and anti-inflammatory effects.

OBJECTIVE: The aim of this experimental study was to investigate the effects of nebivolol (50 mg/kg/d) on random pattern skin flap survival in rats.

METHODS: Male Wistar rats weighing 290 to 310 g were randomly divided into 2 groups—the nebivolol group and the control group. Random patterned, caudally-based, ~3 × 10-cm skin flaps were elevated on the back of each rat. In the nebivolol group, nebivolol 50 mg/kg/d (1 mL of a racemic solution of nebivolol) was administered orally 2 days before surgery to reach steady-state drug blood concentrations and was continued for 6 days. In the control group, 1 mL/d of sterile saline solution was orally administered 2 days before surgery and was continued for 6 days. To observe the effects of nebivolol, cutaneous blood flow was examined using a laser Doppler flowmeter before and after surgery on days 1, 3, 5, and 7, and flap tissue, malondialdehyde (MDA) and glutathione (GSH) concentrations, and superoxide dismutase (SOD) activity were measured 7 days postsurgery. Flap viability was evaluated 7 days after surgery by measuring necrotic flap area and total flap area.

RESULTS: All 20 rats (nebivolol group, n = 10; control group, n = 10) survived throughout the study period. Mean (SD) MDA concentration was significantly lower in the nebivolol group than in the control group (69.25 [5.82] vs 77.67 [6.87] nmol/g tissue; $P = 0.009$). GSH concentration was significantly higher in the nebivolol group than in the control group (2.14 [0.15] vs 1.88 [0.22] nmol/mg tissue; $P = 0.004$). SOD activity was significantly greater in the nebivolol group than in the control

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group (49.28 [5.49] vs 42.09 [4.95] U/g tissue; $P = 0.007$). The percentage of the flap that was necrotic was significantly lower in the nebivolol group than in the control group (40.27 [4.08] vs 48.87 [6.35]; $P = 0.007$).

CONCLUSIONS: This small, experimental, in vivo animal study found that nebivolol was associated with reduced necrotic random pattern skin flap area. Further studies are needed to clarify these findings. (*Curr Ther Res Clin Exp.* 2008;69:449–458) © 2008 Excerpta Medica Inc.

KEY WORDS: nebivolol, skin flap survival, cutaneous blood flow.

INTRODUCTION

Skin flaps are commonly used to treat soft tissue defects in reconstructive surgery, but necrosis remains a serious complication.[1,2] While the pathophysiology of skin necrosis is complex, 2 mechanisms have been implicated in promoting postsurgical flap damage: (1) neutrophil infiltration caused by ischemia-reperfusion (I/R) injury; and (2) vascular thrombosis caused by surgical trauma.[1–5] Many factors may be involved in I/R injury, including neutrophils and superoxide radicals.[1–8] An incomplete ischemic state accompanied by tissue damage is seen in the distal region of random pattern skin flaps.[9] Moreover, cutaneous blood flow initially decreases immediately after skin flap surgery in the distal region of random pattern skin flaps but increases significantly within 24 hours after flap elevation, as seen in I/R injury.[10,11] Sufficient blood flow is essential for survival of these flaps, and poor flap design, injury to the flap vascular system, or I/R injury may cause partial or complete flap loss.[10]

Reperfusion injury refers to the cellular damage that occurs when an insufficient amount of blood is supplied to a region of the body, followed by the resumption of sufficient blood flow.[6] During this process, a large amount of molecular oxygen is supplied to the tissues, and an abundant amount of reactive oxygen species (ROSs) is produced.[6,7] In biological systems, during multiple physiologic and pathophysiologic processes, ROS molecules that are generated are thought to be involved in I/R injury.[6,10,12] Reperfusion of ischemic flaps increases the effect of early ischemic injury by releasing ROSs and accumulating activated neutrophils.[3,6,9,10] Proteins, carbohydrates, lipids, and nucleic acids can be damaged by these species.[6,13] Organisms have enzymatic (superoxide dismutase [SOD], glutathione [GSH] peroxidase, and catalase) and nonenzymatic (GSH, carotenes, ascorbic acid, and ubiquinol) antioxidative defense mechanisms.[6,7] When the equilibrium between the production and the destruction of ROSs is upset, excess ROSs are produced and tissues are exposed to oxidative injury.[6,7] Numerous studies have investigated methods for improving survival, many of which have focused on improving viability through pharmacologic manipulation.[8–20]

Nebivolol is a selective β_1-adrenoreceptor blocking agent that possesses a pharmacodynamic profile and a chemical structure that are different from traditional β-blockers.[21,22] Nebivolol has peripheral vasodilating properties that are mediated by the modulation of the endogenous production of nitric oxide (NO), as well as other antioxidative effects.[21–25] The drug has also been reported to have antiaggregatory and

anti-inflammatory properties.[24,25] Although nebivolol has been reported to have antioxidative, anti-inflammatory, antiaggregatory, and vasodilatory effects, its effect on ischemic flap survival has not, to our knowledge, been investigated. A search of the literature using the PubMed database did not find any articles on the effects of nebivolol on ischemic flap survival.

This experimental study was designed to investigate the effects of nebivolol on random pattern skin flap survival in rats.

MATERIAL AND METHODS

ANIMALS AND SURGICAL TECHNIQUE

Male Wistar rats weighing 290 to 310 g were supplied by Haydarpasa Numune Education and Research Hospital's Animal Laboratory, Istanbul, Turkey. This study was approved by The Animal Research Committee of the Haydarpasa Numune Education and Research Hospital. The protocols and laboratory standards were in accordance with the local Animal Care Committee.

The rats were randomly divided into 2 groups—the nebivolol group and the control group—using a computer-based number-generation technique. In the nebivolol group, oral nebivolol 50 mg/kg/d (1 mL of a racemic solution of nebivolol) was started 2 days before surgery to reach steady-state drug blood concentrations and was continued for 6 days. In the control group, oral saline 1 mL/d was started 2 days before surgery and was continued for 6 days. The surgeon was blinded to the treatment group. The rats were anesthetized with ketamine (50 mg/kg intraperitoneally [IP]) and xylazine (5 mg/kg IP). The dorsal skin of each rat was prepared by clipping the hair and applying hair remover. The caudally-based dorsal flap was located in reference to anatomic landmarks and extended from the tip of the scapula to the hip joints. The lateral margins were 1.5 cm to either side of the midline, making the flap ~3 × 10 cm. An additional triangular 3-cm segment of skin was removed from the apex of the wound. This segment was positioned in the wound near the base of the flap after removing the panniculus carnosus from its underside. The wound was then closed, with this skin incorporated in the closure as a graft. In this manner, complete isolation of the base of the flap was obtained without kinking the flap pedicle, while allowing a contoured closure of the cephalic portion of the wound. The flap, now completely isolated from the underlying bed, was positioned on top of the closed wound.[26]

LASER DOPPLER FLOWMETRY

Laser Doppler flowmeter (Laserflo BPM2, Vasamedics LLC, St. Paul, Minnesota) measurements were obtained in all rats by a second investigator (F.U.) who was blinded to treatment group. The measurements were taken at a standardized flap location (3 cm distal from the base of the flap at the midline). After each animal was anesthetized with pentobarbital, the output was recorded in milliliters per minute per 100 g of tissue and was a relative measurement of microvascular nutrient perfusion. The laser Doppler flow was zeroed on the neutral-colored faceplate. All measurements were done when the probe, which was heated to 40°C, had been placed flat on the skin

for 60 seconds. Standard probe adhesive strips were used to keep the probe on skin. The laser Doppler flow recordings were performed immediately before and after the flap surgery and 1, 3, 5, and 7 days after surgery. The mean of these 6 measurements was used for each rat.

BIOCHEMICAL ANALYSES

A full-thickness, 3-mm punch biopsy of the skin flap was taken from the center of each flap 12 hours after the onset of reperfusion while the rats were lightly anesthetized.[3,10] Tissue samples were stored at –80°C. A third investigator (G.B.) who was blinded to the study groups performed the biochemical analyses. Fifty milligrams of frozen flap tissue specimen cut into pieces using blades on dry ice were homogenized in 1.15% potassium chloride buffer (1:9 w/v) using a manual glass homogenizer for ~5 minutes and flushed by centrifugation for ~10 seconds to remove large pieces of debris. The supernatant was used for analysis.[10]

Malondialdehyde (MDA) concentration of the homogenates was determined spectrophotometrically by measuring the presence of thiobituric acid-reactive substances.[27] Results were expressed as nanomoles per gram of tissue.

GSH concentration was measured spectrophotometrically using Elman's reaction.[28] Results were expressed as nanomoles per milligram of tissue.

SOD enzyme activity was measured based on the production of hydrogen peroxide from xanthine by xanthine oxidase and the reduction of nitroblue tetrazolium, as previously described.[29] Results were expressed as units per gram of tissue.

SKIN FLAP SURVIVAL

Skin flap survival was the primary end point in this study. A fourth investigator (R.E.) who was blinded to the study groups assessed skin flap survival on postoperative day 7. Gross observation was used to identify the demarcation between viable tissue (characterized by soft, rosy, warm skin with hair) and necrotic tissue (characterized by stiff, dark, cool skin without hair).[30] The entire flap and the necrotic and viable portions were traced onto a transparent cellophane sheet and transferred onto graph paper with a 1-mm^2 grid.[2] The area of necrosis was expressed as a percentage of the entire flap area (necrotic flap percentage = necrotic flap area/total area × 100). After skin flap survival was assessed, the rats were euthanized on postoperative day 7 using an overdose of sodium pentothal injected IP.

STATISTICAL ANALYSIS

Data were reported as mean (SD). SPSS for Windows, version 10.0 (SPSS, Inc., Chicago, Illinois) was used for statistical analysis. Because the results of laser Doppler flow were distributed normally according to the Kolmogorov-Smirnov test, a *t* test was used to compare the laser Doppler flows between the nebivolol and the control groups. However, because the results of the survival proportions and biochemical analyses were not distributed normally according to the Kolmogorov-Smirnov test, they were compared using the nonparametric Mann-Whitney *U* test. $P < 0.05$ was considered significant.

RESULTS

Twenty rats (mean [SD] weight, 200 [7.1] g) were randomly divided into 2 groups—the nebivolol group (n = 10) and the control group (n = 10). All rats survived throughout the study period.

LASER DOPPLER FLOWMETRY

The results of the laser Doppler flowmeter measurements are summarized in Table I. Blood flow was significantly greater in the nebivolol group than in the control group at all 6 measurements (all, $P = 0.001$). Nebivolol was associated with increased skin blood flow and improved blood circulation in the skin flaps.

BIOCHEMICAL ASSAYS

MDA concentration was significantly lower in the nebivolol group than in the control group (69.25 [5.82] vs 77.67 [6.87] nmol/g tissue; $P = 0.009$). GSH concentration (2.14 [0.15] vs 1.88 [0.22] nmol/mg tissue; $P = 0.004$) and SOD enzyme activity (49.28 [5.49] vs 42.09 [4.95] U/g tissue; $P = 0.007$) were significantly higher in the nebivolol group than in the control group (Table II).

Table I. Mean (SD) laser Doppler flowmeter findings in the nebivolol and the control groups (N = 20). All measurements were mL/min per 100 g tissue.

Time Point	Nebivolol (n = 10)	Control (n = 10)
Immediately before surgery	97.13 (2.04)*	90.64 (2.83)
Immediately after surgery	37.99 (2.23)*	29.60 (1.45)
Day 1	45.67 (2.04)*	35.20 (2.39)
Day 3	46.69 (1.94)*	36.86 (2.50)
Day 5	54.70 (1.41)*	42.00 (1.62)
Day 7	56.93 (1.56)*	43.97 (1.44)

*$P = 0.001$ versus control group.

Table II. Mean (SD) tissue malondialdehyde (MDA) and glutathione (GSH) concentrations and superoxide dismutase (SOD) activity in the nebivolol and the control groups 12 hours after reperfusion (N = 20).

Concentration	Nebivolol (n = 10)	Control (n = 10)
MDA, nmol/g tissue	69.25 (5.82)*	77.67 (6.87)
GSH, nmol/mg tissue	2.14 (0.15)†	1.88 (0.22)
SOD, U/g tissue	49.28 (5.49)‡	42.09 (4.95)

*$P = 0.009$ versus control group.
†$P = 0.004$ versus control group.
‡$P = 0.007$ versus control group.

FLAP SURVIVAL

A significantly smaller percentage of the skin flap was necrotic in the nebivolol group than in the control group (40.27 [4.08] vs 48.87 [6.35]; $P = 0.007$) (**Table III**).

DISCUSSION

Cutaneous blood flow initially decreases immediately after skin flap surgery in the distal region of random pattern skin flaps but increases significantly within 24 hours after flap elevation.[10,11] An incomplete ischemic state with tissue damage occurs in the distal region of a random pattern skin flap.[9] An ischemic zone that may undergo necrosis is also likely in the distal portion.[2,10] Tissue ischemia induced by leukocyte inflammation and inadequate blood flow was believed to be the principal factor predisposing a patient to flap tissue necrosis.[1–5] Since an ischemic flap is a major surgical problem that may cause prolonged hospitalization, pharmacologic agents that decrease the detrimental tissue and vascular changes may provide great benefits.

Several treatments may improve skin flap survival, including anti-inflammatory drugs such as prednisolone[31]; vasodilatory agents such as prostacyclin, nifedipine, nitroglycerin, and NO[15,18,32]; and the fibrinolysis of clotted blood in flap vessels with streptokinase.[33] Other approaches include restoration of high levels of energy-rich phosphate compounds such as fructose-1,6-diphosphate[20]; removal of damaging free radicals by scavengers such as SOD, allopurinol, L-arginine, melatonin, and vitamins A and E[11,13,34,35]; immunosuppression with cyclosporin A and azathioprine[31]; inhibition of coagulation with anticoagulants such as aspirin, clopidogrel, and heparin[1,2]; and modulation of neutrophil activity with agents such as fucoidin.[3] In the present study, nebivolol was associated with a significant increase in the survival of skin flaps. We think that this improvement may be a result of its antioxidative, anti-inflammatory, anticoagulant, and vasodilating properties and its protective effects against I/R injury. Improving flap survival using anticoagulants and vasodilatory drugs has been widely studied.[15,18,32] Nebivolol has been reported to decrease platelet activation and increase cutaneous blood flow.[23,24] However, the anticoagulant properties of nebivolol were not examined in this study; instead, its effects on cutaneous blood flow were assessed using a laser Doppler flowmeter. Throughout the study, the nebivolol-treated group was found to have significantly higher cutaneous blood flow in flaps than the control group. In our opinion, higher blood flow with nebivolol treatment immediately after surgery may reduce ischemic injury in the early period after flap placement and may be one of the major mechanisms by which nebivolol improved skin flap survival in this model.

Table III. Mean (SD) percentage of the necrotic flap tissue in the nebivolol and the control groups (N = 20).

	Nebivolol (n = 10)	Control (n = 10)
Necrotic flap tissue, %	40.27 (4.08)*	48.87 (6.35)

*$P = 0.007$ versus control group.

It has been well established that ROSs and inflammatory cells are major mediators of I/R injury in experimental random pattern flaps and free flaps.[1,3,5–11,31–38] Therefore, the current study focused on investigating the protective effects of nebivolol on survival of random pattern skin flaps by measuring MDA and GSH concentrations and SOD activity. Additionally, cutaneous blood flow was measured using a laser Doppler flowmeter to explore the flap circulation.

The MDA concentration was significantly higher in the control group than in the nebivolol-treated group. As an end product of lipid peroxidation, MDA is an indicator of the damage caused by free radicals to components of the cell membrane.[21] This may be due to 2 mechanisms: (1) blood flow increased by nebivolol, probably resulting in a decrease in the size of the ischemic zone in the distal flap region and decreased ischemic injury; and (2) nebivolol probably attenuated the increase of MDA concentration in the flap tissue by directly eliminating ROSs and increasing antioxidant enzyme activity. In our opinion, the combination of these 2 mechanisms decreased the MDA concentration because nebivolol therapy was previously found to suppress lipid peroxidation, activate antioxidant enzymes, and increase cutaneous blood flow.[21,23] As nebivolol increased blood flow and reduced ischemic and oxidative injury to cellular structures, the concentration of the intracellular antioxidant GSH, which is otherwise oxidized when inactivating free radicals, was significantly higher in the nebivolol group than the control group. Therefore, it appears that the antioxidant effects of nebivolol on lipid peroxidation do not involve the expenditure of tissue GSH stores, but that the antioxidant pool is further sustained by the action of nebivolol. Moreover, the reduction in antioxidant capacity caused by I/R injury may have been reversed by nebivolol treatment.

SOD is one of the most important endogenous free radical scavengers, and exogenous SOD is one of the treatments that has been found to improve skin flap survival.[34] In this study, SOD enzyme activity in the nebivolol group was found to be significantly higher than in the control group, supporting the protective effect of nebivolol on skin flap survival in tissue injury. We did not investigate the anti-inflammatory effects of nebivolol in skin flap survival, but nebivolol was found to reduce the expression of proinflammatory genes in endothelial cells and vascular smooth muscle cells in vitro, whereas metoprolol did not.[25] Nebivolol has also been found to inhibit neointima formation by reducing smooth muscle cell proliferation and macrophage accumulation.[25]

Considering the increased blood flow and reduced ischemic and oxidative damage associated with nebivolol treatment, nebivolol's protective actions in the current study are believed to be a result of its antioxidative and vasodilating activities. Our results are consistent with studies that found that nebivolol administration reduced MDA concentration and increased GSH concentration, SOD activity, and cutaneous blood flow.[21–25] In this study, nebivolol was associated with a significantly reduced proportion of skin flap necrosis. The antiaggregatory and anti-inflammatory effects of nebivolol may play a synergistic role with its antioxidant and vasodilating effects in improving random pattern skin flap survival.

STUDY LIMITATIONS

Assessing the percentage of skin flap necrosis by tracing on a transparent sheet and transferring onto graph paper was a limitation of this study because the method is less precise than using a computer program to analyze a digital picture. However, the method we used is among the methods commonly used in making this type of assessment.[2] Another limitation of this study was that the antiaggregatory and anti-inflammatory effects of nebivolol were not investigated. We found that the initial blood flows were higher in the nebivolol-treated group immediately before the surgery. It is well known that nebivolol is associated with cutaneous vasodilatation.[23] Accordingly, in our opinion, the higher blood flows were due to nebivolol being administered 2 days prior to surgery.

CONCLUSIONS

The present study found that nebivolol, a new generation selective β_1-adrenoreceptor blocker, was associated with significantly reduced random pattern skin flap necrosis in this animal model. Further investigations with larger animal models are needed, especially studies of the mechanisms of the antiaggregatory and anti-inflammatory effects of nebivolol in an I/R injury model.

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