ORIGINAL ARTICLE

# Inhibitors of Nitric Oxide Promote Microvascular Thrombosis

*Babak Azizzadeh, MD; Georgette M. Buga, PhD; Gerald S. Berke, MD; Babak Larian, MD; Louis J. Ignarro, PhD; Keith E. Blackwell, MD*

**Background:** Microvascular free tissue transfer is a widely utilized method of head and neck reconstruction. Despite advances in the field, reports of experienced microvascular surgeons on large series of free flap procedures reveal that the incidence of free flap failure varies between 5% and 9%. Most cases of free flap failure are initiated by platelet-mediated events that result in thrombosis at the microvascular anastomoses. Recent evidence indicates that nitric oxide (NO) plays a critical role in preventing thrombosis by inhibiting platelet adhesion and aggregation. The role of NO in microvascular anastomotic thrombosis has not been studied.

**Objective:** To determine the role of NO in microvascular thrombosis using an in vivo rabbit model.

**Methods:** An arterial inversion graft (AIG)–induced microvascular thrombosis model was utilized in New Zealand white rabbits. The femoral arteries were used bilaterally to create 3-mm AIGs. Intravenous NO donor, NO inhibitor, or isotonic sodium chloride solution (control) was administered for 1 hour following the completion of the AIG, and vessel patency was then checked using a direct "milking test." Sixteen rabbits (32 AIGs) were used as controls. A potent NO inhibitor, N(w)-nitro-L-arginine methylester (L-NAME), was administered to 13 rabbits (26 AIGs) and L-arginine, a NO precursor/donor, was given to 10 rabbits (20 AIGs).

**Results:** The control animals had a thrombosis rate of 46.9%. The rate of thrombosis in animals exposed to an NO inhibitor (L-NAME) was significantly higher, at 76.9% ($P<.05$, $\chi^2=4.23$). The L-arginine group did not show a statistical difference with the control in the rate of thrombosis (50.0%).

**Conclusions:** Nitric oxide plays a role in microvascular anastamotic thrombosis. Intravenous NO inhibitors appear to increase the short-term rate of microvascular thrombosis. L-arginine, an NO precursor, does not appear to produce the opposite effect. Further studies using local NO donors and antagonists as well as more potent NO precursors are needed to further evaluate NO's role in microvascular thrombosis. The results of this study may have applications to human microvascular surgery.

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*From the Division of Head and Neck Surgery (Drs Azizzadeh, Berke, Larian, and Blackwell) and the Department of Molecular and Medical Pharmacology (Drs Buga and Ignarro), UCLA School of Medicine, Los Angeles, Calif.*

MICROVASCULAR free tissue transfer has become a popular method of head and neck reconstruction. Despite advances in free flap surgery, reports of experienced microvascular surgeons on large series of free flap procedures reveal that the incidence of free flap failure ranges between 5% and 9%, and that thrombosis at the microvascular anastomoses plays a key role in most cases of failure.[1-5] Thrombosis can be initiated by various events, including technical errors resulting from an imperfect microvascular anastomosis; extrinsic compression; infection; recipient and donor vessel size mismatch; and changes in systemic hemodynamics.[6-8] Regardless of the triggering cause, platelet-mediated events are thought to be critical to the initiation of thrombus formation.

Mounting evidence indicates that nitric oxide (NO) plays a crucial role in platelet-mediated thrombosis. Nitric oxide is synthesized from the amino acid L-arginine (**Figure 1**), in a reaction mediated by one of several isoforms of the enzyme nitric oxide synthase (NOS). The constitutive isoform (cNOS) is expressed in endothelium cells, neurons, platelets, and megakaryoblastic cells, whereas the inducible isoform (iNOS) is expressed in macrophages and vascular smooth muscle cells.[9-12] Although many authors have studied the role of NO in microvascular ischemia-reperfusion injury, to our knowledge, none have looked specifically at its role in microvascular thrombosis.[13-17]

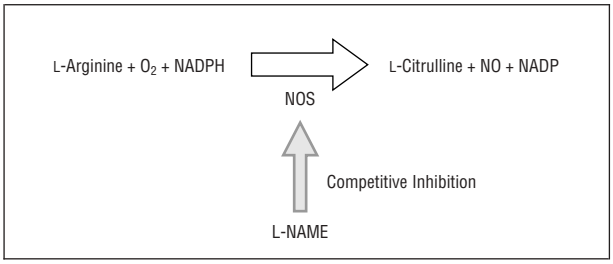


**Figure 1.** Nitric oxide is synthesized from the amino acid L-arginine in a reaction mediated by one of several isoforms of the enzyme nitric oxide synthase (NOS). The constitutive isoform is expressed by endothelium, neurons, platelets, and megakaryoblastic cells. The inducible isoform is expressed in macrophages and vascular smooth muscle cells. L-NAME is a commonly used competitive inhibitor of NOS. NADPH indicates the reduced state of nicotinamide adenine dinucleotide phosphate and NADP its oxidized form; NO, nitric oxide; and L-NAME, N(w)-nitro-L-arginine methylester.

## METHODS

### SURGICAL TECHNIQUE: ARTERIAL INVERSION GRAFT

Institutional guidelines regarding animal experimentation were followed according to the UCLA Chancellor's Animal Research Committee. New Zealand white rabbits weighing between 2 and 3 kg were anesthetized with pentobarbital (30 mg/kg) via the lateral ear vein. The arterial inversion graft (AIG)–induced microvascular thrombosis model described by Kersh et al[18] was utilized. The inguinal area was prepared and draped in standard aseptic surgical technique. The femoral artery was exposed in the right inguinal area between the epigastric branch and the great saphenous artery. Using a surgical microscope, the side branches were cauterized by bipolar cauterization and the adventitia was gently and meticulously cleaned. A microvascular approximating clamp was applied and a 3-mm segment of artery was excised in an area free of side branches, yielding a free segmental graft. This free segment was carefully turned inside out with minimal manipulation and sewn back into its native position using 10 to 12 interrupted 10-0 nylon sutures (**Figure 2**). The adventitia was therefore placed within the lumen of the interposed segment. Heparinized isotonic sodium chloride solution was not introduced into the field. The approximating clamp was removed and blood flow restored through the AIG. L-arginine (Alexis Biochemical, Lausanne, Switzerland), N(w)-nitro-L-arginine methylester (L-NAME; Alexis Biochemical), or a 0.90% sodium chloride solution (Baxter International Inc, Deerfield, Ill) was infused via an intravenous catheter for 1 hour following the completion of the anastomoses (**Table**).

In previous experimental models, L-arginine has been used as a donor or agonist of NO, whereas L-NAME is a commonly used inhibitor of NOS.[19-20] The concentration and infusion rate of L-arginine and L-NAME were determined according to previous experimental models. Vessel patency was checked 1 hour after clamp removal using the standard direct "milking test." The results were recorded in a computer database. Animals were killed with anesthetic overdose.

### EXPERIMENTAL GROUPS AND STATISTICAL ANALYSIS

Thirty-nine rabbits were randomly assigned to 3 experimental groups in a blinded fashion (Table). Bilateral femoral arteries were used to limit the number of test animals required to complete the experiment. The right and left femoral arteries were randomly assigned as the first vessel to undergo AIG in each animal. Both femoral arteries were dissected at the beginning of the experiment, and the second vessel was clamped proximally and distally to the site of AIG to avoid exposure to the drug treatment of the first vessel. Both vessels were exposed to the same treatment to avoid additional variability. Standard $\chi^2$ tests were used to analyze the difference between the control group and NO donor/inhibitor groups. The right and left vessels were also analyzed for any discrepancies between the 2 sides.

## RESULTS

There were no perioperative complications. The control animals had a thrombosis rate of 46.9% (**Figure 3**). The rate of thrombosis in animals exposed to an NO inhibitor (L-NAME) was significantly higher, at 76.9% ($P<.05$; $\chi^2=4.23$). The L-arginine group did not show a statistical difference with the control in the rate of thrombosis (50.0%). The rate of microvascular right and left vessel thrombosis in each experimental group was not significantly different.

## COMMENT

Microvascular free tissue transfer has become the standard of care for extensive head and neck reconstruction. Despite advances in free flap surgery, reports of experienced microvascular surgeons on large series of free flap procedures reveal that the incidence of free flap failure varies between 5% and 9%. Recently, Finical et al[21] examined the records of 121 patients who had undergone free flap reconstruction for recurrent head and neck squamous cell carcinoma and found a 14% rate of flap loss.

Most cases of free flap failure are initiated by platelet-mediated events that result in thrombosis at the microvascular anastomoses. Disruption of the vascular endothelium results in the exposure in the lumen of subendothelial collagen and fibronectin that act as strong ligands for platelet adhesion. The attachment of platelets to this matrix results in its activation, which leads to contractile and secretory processes that attract more platelets and promote the formation of a clot. Activated platelets metabolize arachidonic acid into thromboxane $A_2$, a potent vasoconstrictor and platelet aggregator. Activated platelets also express prothrombinase, which converts prothrombin to thrombin. Thrombin then mediates the conversion of fibrinogen into fibrin, which stabilizes the latticework of the growing platelet thrombus.[22]

Recent evidence indicates that NO plays a 2-fold role in thrombosis by inhibiting platelet adhesion and aggregation. Intact endothelium releases NO and prostaglandin $I_2$, which act synergistically to inhibit platelet aggregation and adhesion.[23] Endothelium NO activates guanylate cyclase, an enzyme that inhibits platelet expression of glycoprotein IIb/IIIa by increasing levels of cyclic guanosine monophosphate, a process required for platelet adhesion and aggregation.[24,25] Once the vascular endothelium is disrupted and platelets are activated, platelet-derived NO down-regulates thrombus formation.[26,27]

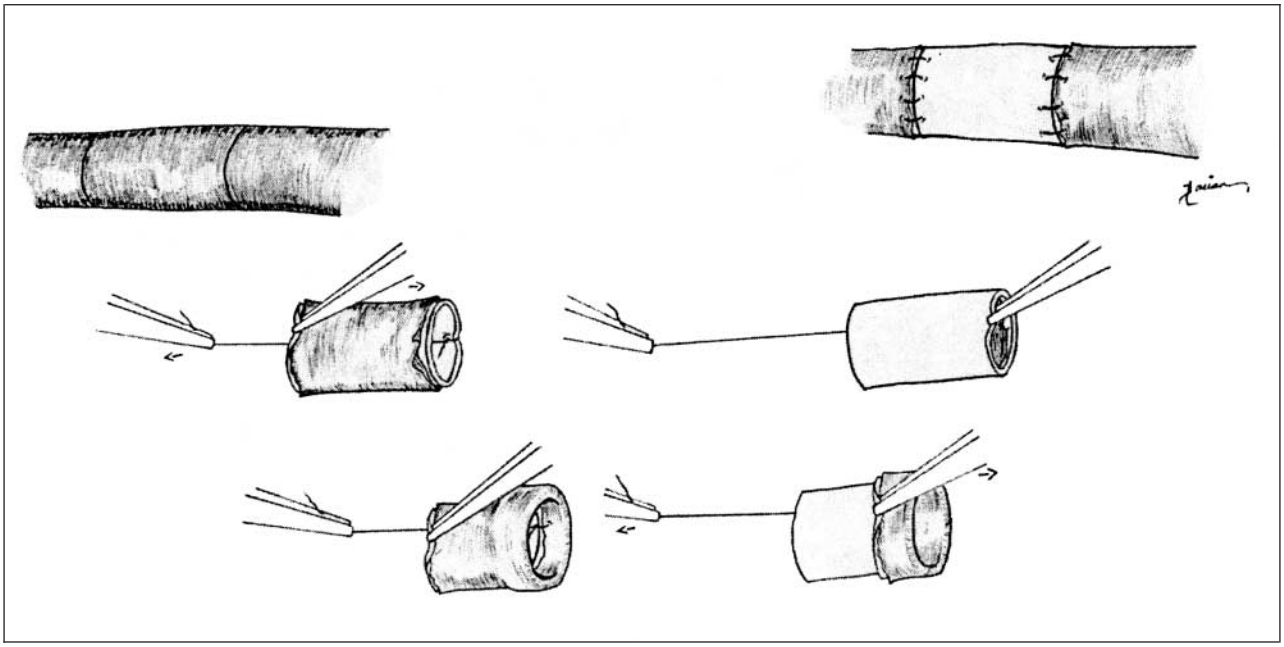

**Figure 2.** In the arterial inversion graft thrombosis model, a 3-mm segment of artery excised in an area free of side branches yields a free segmental graft. This free segment is carefully turned inside out with minimal manipulation and sewn back into its native position using 10 to 12 interrupted 10-0 nylon sutures. The vessel adventitia is therefore placed within the lumen of the interposed segment.

**Experimental Groups**

| Groups | Compound | Dose/Rate | No. of Rabbits | No. of AIGs |
|---|---|---|---|---|
| Control | Saline | 10 mL/h | 16 | 32 |
| NO donor | L-arginine | 10 mg · $kg^{-1}$ · $min^{-1}$ | 10 | 20 |
| NO inhibitor | L-NAME | 10 mg · $kg^{-1}$ · $min^{-1}$ | 13 | 26 |

Abbreviations: AIG, arterial inversion graft; NO, nitric oxide; L-NAME, N(w)-nitro-L-arginine methylester; saline, 0.9% isotonic sodium chloride solution.

In this study, we show that L-NAME, a potent NO inhibitor, results in a significant increase in microvascular thrombosis ($P<.05$). These findings are consistent with previous experiments by Provost et al[28] who showed that under physiologic conditions, L-NAME increases mural platelet deposition. Others have demonstrated that systemic L-NAME induces platelet adhesion and aggregation in the subendothelium of the injured carotid artery of rabbits.[26]

L-arginine did not decrease the rate of microvascular thrombosis in this experiment. There may be several explanations for this phenomenon. In our model, the AIGs' endothelia are not in the lumen, and therefore do not release in the lumen the endothelial cNOS necessary to convert L-arginine to NO in the microcirculation. Furthermore, there may be sufficient preexisting L-arginine in the plasma to ensure the endothelial cNOS operation. The $K_m$ of L-arginine (5-10 µmol/L) for the endothelial cNOS is below the plasma levels of L-arginine (0.1-0.3 mmol/L); and unless the animal has arginine deficiency, sepsis, atherosclerosis, or other chronic vascular disorders, exogenous L-arginine will not have an impact. The effect of L-arginine on iNOS is necessarily limited in this study because it takes about 4 hours for iNOS messenger RNA to be expressed, and 12 to 15 hours for detection of increased systemic NO to be possible. Nitric oxide was not used as the primary pharmacological agent in this study because of its short half-life, potential toxicity, and volatility. L-arginine and L-NAME were used because of their stability, safety, and documented utility in previous animal experiments.[19,20] Experiments with more active NO donors are necessary to study this matter further.

The AIG procedure that we used is the well-established model for provoking microvascular thrombosis described by Kersh et al.[18] The arterial graft is turned inside out to project adventitia intraluminally, and hence initiate and promote platelet-mediated thrombosis. This model has been reproduced and studied in several experiments involving rats and rabbits.[29-32] Basile et al[29] used the AIG model to demonstrate that ticlopidine as a single agent or with aspirin significantly improves the 1-hour patency rate. Greenberg et al[30] used this model to determine the anastomotic patency rates between intra-arterial and intravenous heparin infusion. Others have also used AIGs to elucidate the role of human tissue plasminogen activator in preventing astomotic microvascular thrombosis.[32]

Clinical pharmacotherapy plays a role in preventing microvascular thrombosis in both experimental and clinical models. Many pharmacological agents have been shown to reduce the incidence of microvascular throm-

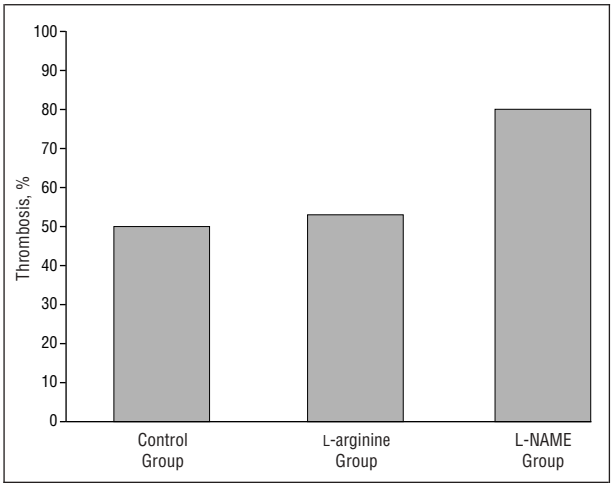


**Figure 3.** Rate of microvascular thrombosis in various nitric oxide experimental groups. L-NAME indicates N(w)-nitro-L-arginine methylester.

bosis in animal models.[25,33-36] These drugs are thromboxane $A_2$ inhibitors (including aspirin, ibuprofen, and indomethacin); prostacyclin and its analogue iloprost; anticoagulants (including dextran, heparin, and hirudin); and thrombolytic agents (streptokinase and urokinase). Several clinical studies have shown improved microvascular anastomotic rates with prophylactic aspirin.[37-39] A 1991 survey of 83 centers performing free flap surgery indicated that aspirin, heparin, and dextran are the most commonly used prophylactic antithrombotic agents.[40] Most recently, a phase 2 trial of intraluminal irrigation with recombinant human tissue factor pathway inhibitor showed promising results in reducing free flap thrombosis and postoperative hematoma.[41] Overall, the incidence of microvascular thrombosis is reduced in centers that use prophylactic pharmacotherapy compared with those that do not use it.

## CONCLUSIONS

Most cases of free flap failure are initiated by platelet-mediated events that result in thrombosis at the microvascular anastomoses. Nitric oxide plays a critical role in thrombosis by inhibiting platelet adhesion and aggregation. In this experiment, we have shown that systemic NO inhibitors increase the short-term rate of microvascular thrombosis. L-arginine, an NO precursor, does not appear to exert the opposite effect. Further studies using local NO donors and antagonists as well as more potent NO precursors are needed to further evaluate the role of NO in microvascular thrombosis. The results of this study may have applications to human microvascular surgery.

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*Corresponding author: Keith E. Blackwell, MD, UCLA Division of Head and Neck Surgery, 10833 Le Conte Ave, CHS 62-132, Los Angeles, CA 90095 (e-mail: kblackwe@ucla.edu).*

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***Quotable***

In higher education today corporations not only sponsor a growing amount of research—they frequently dictate the terms under which it is conducted. Professors, their image as unbiased truth-seekers notwithstanding, often own stock in the companies that fund their work. And universities themselves are exhibiting a markedly more commercial bent. Most now operate technology-licensing offices to manage their patent portfolios. . . . Schools with limited budgets are pouring money into commercially oriented fields of research, while downsizing humanities departments and curbing expenditures on teaching.

*Eyal Press and Jennifer Washburn*
*"The Kept University"*
The Atlantic Online, *March 2000*
*Available at http://www.theatlantic.com /issues/2000/03/press.htm*