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*Vasc Endovascular Surg* 2002; 36; 357
DOI: 10.1177/153857440203600505

The online version of this article can be found at:
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Electrical Stimulation Promotes Angiogenesis in a Rabbit Hind-Limb Ischemia Model

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In previous investigations, it was shown that applying a modest regimen of electrical stimulation (ES), even in severely ischemic tissue, improves the healing process, accelerates neovascularization, and enhances angiogenesis in muscle tissue. Our objective in this current report was to further understand ES as a potential alternative treatment for severe muscle ischemia. Immediately after the left distal external iliac artery and the femoral artery were excised, ES (30 contractions per minute [cpm], 2 V, single impulses per burst) was applied to rabbit adductor muscle near the site of the excised femoralis artery for 24 hours daily over 1 month. Three other series served as controls: ES without arterial excision; arterial excision without ES or lead implantation; and arterial excision with lead implantation but no ES. Histologic study of capillary density was performed by angiography (employing a grid template) and by measuring the lower limb-calf blood pressure ratio. At the end of 30 days in the ES series, 10.5 ±1.2 contrast-medium opacified arteries (COAs) crossed a specific grid section segment compared with 7.2 ±1.5 in the control series without ES (p<0.05); 68.2 ±9.3 COAs crossed a grid section compared with 43.2 ±6.4 in controls (p<0.05); 27.3 ±1.2 grids contained COAs compared with 29.3 ±3.5 in controls (p<0.05); lower limb-calf blood pressure ratio was 0.81 ±0.06 compared with 0.31 ±0.07 in controls (p<0.05); and capillary density was 283.7 ±24.5 mm² compared with 91.4 ±20.9 mm² in controls (p<0.001). These preliminary results show that cautious ES enhances and accelerates muscle revascularization in severely ischemic tissue.

Introduction

Critical limb-threatening ischemia develops in approximately 150,000 patients per year in the United States or 500 to 1,000 individuals per million each year worldwide, particularly in patients who have atherosclerosis related to diabetes mellitus.1 Non-diabetic patients who have critical limb ischemia undergo only 200 lower limb amputations per million annually, but 3,900 diabetic patients undergo such amputations per million.2

In many cases, the extent and distribution of advanced peripheral arterial disease precludes
any operative or percutaneous revascularization, and an inexorable downhill course follows. Amputation—despite its associated morbidity, mortality, and functional disabilities—is often recommended at this end stage of limb ischemia.3,4

When surgical management is not possible, the future development of the disease depends on the balance between further progression of peripheral artery disease and the natural growth of compensative collateral vessels. Accordingly, disease management attempts to change this balance in favor of collateral vessel growth.

One of the newest pathways in this direction is augmentation of the patient's native collateral vessels and subsequent restoration of perfusion to the affected limb. This approach combines therapeutic angiogenesis, defined as an extension of primitive vasculature through sprouting of new capillaries from an existing network, and arteriogenesis, defined as development of a preexisting arterial connection into true vessels.5-7

Several studies using experimental models of hind-limb ischemia have shown that specific angiogenic factors (vascular endothelial growth factors, fibroblast growth factors) are effective.8-13 Moreover, growth factors have been used clinically to treat critical limb ischemia with reports of improved clinical status.14-18 A drawback is the requirement to deliver the angiogenic growth factors through either multiple injections or an invasive procedure.13

Toward the end of finding a simpler, more practical approach to therapeutic angiogenesis, in 1999, Kanno and associates19 reported that low voltage electrical stimulation (ES) of skeletal muscle induced de novo synthesis of vascular endothelial growth factor (VEGF) protein, promoted local angiogenesis, and restored blood flow in ischemic tissue. Their investigations were based on several previous studies of ES-induced angiogenesis in skeletal muscle.20-22 Those studies, however, were aimed at producing maximal muscle contraction; the finding of increased capillary density only confirmed improvement in muscle resistance to fatigue but was not in itself a study goal.

During the same period (1997–1998), we investigated low-frequency ES and showed that even in severely ischemic tissues, the healing process can be improved, neovascularization accelerated, and angiogenesis in muscles enhanced.23,24 The objective of this current study was to further our understanding of ES as a potential alternative treatment for severe hind-limb muscle ischemia using a rabbit model.

Materials and Methods

Animal studies reported here conform with the “Guiding Principles Regarding the Care and Use of Animals” of the American Physiological Society and with all federal laws and regulations regarding animal use and were approved by our institution’s Animal Care Committee.

General Anesthesia and Antibiotic Therapy

Twenty-four (24) adult New Zealand White rabbits (males, mean weight 4 kg) were used in this study and were randomly assigned to one of four series: one experimental group (series 2) and 3 control groups (series 1, 3, and 4) (Table I).

<table>
<thead>
<tr>
<th>Series</th>
<th>Femoral Artery Excision</th>
<th>Lead Implantation</th>
<th>Stimulator Implantation</th>
<th>Electrical Stimulation</th>
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Table I. Experimental and control series.
Before surgery, catheterization, or lower limb blood pressure measurements, rabbits were anesthetized with a cocktail of ketamine (25 mg/kg IM), acepromazine (1 mg/kg IM), and glycopyrrolate (0.22 mg/kg IM). Once sedated, rabbits were placed on a semi-open, non-re-breathing ventilation system with halothane gas (0.75%–2%) mixed with 2 to 3 L/min O₂ via mask, then given buprenorphine (0.05 mg/kg IM) for pain control (two thirds of the dose after induction of anesthesia, one third after recovery from surgery).

Additional pain medication was given as needed (buprenorphine, 0.02–0.05 mg/kg twice daily). Antibiotic therapy included use of enrofloxacin (Baytril, 5–10 mg/kg IM twice daily for 14 days) to treat Pasteurella pneumonia if necessary, and chloramphenicol succinate (30 mg/kg IM once daily for 5–7 days) for prophylaxis against infection. Incision sites were checked at least once daily for any abscess formation.

Surgical Procedures

In series 1 (control), a small incision was made on the left thigh solely for electrode implantation. In series 2 to 4, a longitudinal incision was made from the left inguinal ligament to a point just proximal to the patella. The left distal external iliac artery and femoral artery were dissected free (series 2–4). All major branches of the femoral artery (deep femoral, lateral circumflex, inferior epigastric, and superficial epigastric) were ligated, as well as the distal external iliac artery. The femoral artery was excised from the point of the external iliac artery to the bifurcation into the saphenous and popliteal arteries, making blood flow to the hind-limb dependent on flow through the internal iliac artery. One electrode was implanted into the adductor muscle near the site of the excised femoral arteries (series 2, 3), and a stimulator (Thera, Medtronic) was implanted in a separate pocket (series 1 and 2). The incision was closed in three layers using 3.0 silk.

Electrical Stimulation Protocol

In our preliminary study, we investigated the influence on skeletal muscle of different regimens of ES in the onset of acute ischemia, varying contractions per minute (30, 60, or 120), voltages (2, 3, or 4 V), and number of impulses per burst (1, 3, or 6).23–25 Our results showed that ES could be applied to skeletal muscle safely using a regimen of 30 cpm, 2 V amplitude, and single impulses. In series 1 and 2 reported here, we started ES immediately postoperatively at 30 cpm, 2.0 V, and single impulses 24 hours daily and continued this regimen for 1 month.

Angiography

To establish the anatomy of the collateral vessels and to demonstrate that the femoral artery stump continued to be an end artery, angiography was performed in all 18 rabbits (series 2–4) before and immediately after ligation and excision of the femoral artery, and 1 month after surgery. In the six rabbits in series 1 (no artery excision), angiography was performed before ES and 30 days later. A 22-gauge infusion catheter was introduced into the right femoral artery and advanced to the lower abdominal aorta under fluoroscopic guidance. After intraarterial injection of nitroglycerin (0.25 mg), 5 mL of contrast media (Optiray 350, Mallinckrodt Inc, St. Louis, MO) was injected at the rate of 1 mL/sec. Serial images of left hind-limb were recorded for 10 seconds at one image per second.

Collateral development was analyzed according to Tufts University School of Medicine recommendations9 using a template of 30 1-cm² grids placed on the film taken 4 seconds after injection of the contrast media in each series of angiograms (Figure 1). This template was placed over the area of interest with the center dot over the middle part of the femur and the thick centerline parallel to the central line of the body. Three separate observers blinded to the treatment regimen counted the following: number of contrast-opacified arteries (COAs) crossing a specified segment of the grid (third parallel line); total number of grid intersections (from 71 segments total) crossed by COAs; and total number of grids with COAs (from 30 total). An angiographic score for each film was calculated as the ratio of grids with COAs divided by the total number of grid intersections in the investigated area. Another score was calculated for the ratio of grids with COAs divided by the total number of grids (30) in the investigated area. This angiographic analysis reflected vascular density in the medial thigh.

Lower Limb–Calf Blood Pressure Ratio

Calf blood pressure was measured using a Model 1050-C Doppler Flowmeter (Parks Medical Electronics, Inc, Aloha, OR) immediately after surgery (series 2–4), and on days 10, 20, and 30 (all series). A Doppler probe was placed over the
posterior tibial artery and a special 2.5-cm-wide cuff was placed over the upper calf. The cuff was inflated to 100 mm Hg, then the first appearance of the Doppler signal during cuff deflation was recorded as the systolic pressure. Calf blood pressure was measured on both nonischemic limbs in series 1, on ischemic and nonischemic limbs in series 2 to 4, then the lower limb–calf blood pressure ratio was calculated, that is, the ratio of systolic pressure of the left (ischemic) limb to systolic pressure of a right (normal) limb. A single observer who was blinded to the particulars and aims of the experiment performed all procedures and calculations.

Histologic Determination of Capillary Density

Biopsy specimens for histologic determination of capillary density were taken from the adductor and semimembranous muscles immediately after the animal was killed, and these samples were frozen for 30 seconds in a bath of liquid nitrogen. Multiple frozen sections were cut (10 μm thickness) on a cryostat and placed on glass slides. Next, the sample was stained for alkaline phosphatase using the indoxyl-terazolin method. A pathologist who was blinded to the series of experiment counted the number of capillaries under 20× objective in a chosen field (a total of 10 fields from two different sections of the muscle tissue per sample). Capillary density was calculated as the number of capillaries per square millimeter of muscle.

Statistical Analysis

All data are presented as mean ± SEM. Comparisons within the same animal were performed by a paired Student t test. For comparison between groups, a one-way ANOVA was used followed by an unpaired Student t test. A p value of less than 0.05 was considered statistically significant.

Results

All rabbits tolerated both the limb ischemia and effects of ES well. No animal lost weight nor did we find signs of muscle atrophy or necrosis in the nails or the muscle, perhaps because the external iliac artery was not excised. In two related investigations (not discussed here), excising the external iliac artery (above the peritoneum) was not tolerated by the rabbits.

Angiographic Assessment

Baseline angiography before surgery and ES showed 5.3 ±1.3 COAs crossing a specified segment of the grid, which decreased significantly after surgery to 3.2 ±10 (p < 0.05). A month

Figure 1. Photograph shows 30-grid template (each grid 1 cm²) placed on film taken 4 seconds after injection of contrast medium.
later, without treatment or intervention (ES), this increased to 7.2 ±1.5 (p < 0.05 vs after surgery but p > 0.05 vs baseline; series 4). Electrode implantation alone (without ES) had no effect on this data (6.9 ±1.1; p > 0.05 vs baseline and vs series 4). When ES was applied (series 2), the number of COAs crossing a specified segment increased to 10.5 ±1.2 (p < 0.001 vs after surgery; p < 0.05 vs baseline and series 1, 3, and 4). When ES was applied to normal nonischemic tissue, the number of COAs increased to 6.1 ±0.8 but this was not statistically significant compared with baseline data (p > 0.05; Figure 2).

The same change was seen in grid intersections crossed by COAs: 30.2 ±6.5 at baseline vs 19.3 ±4.8 after surgery (p < 0.05). This increased significantly compared with postsurgery data (p < 0.05) in both control series: no ES with electrode implantation (41.8 ±4.3) and no ES without electrode implantation (43.2 ±6.4); but these changes were not significant compared with baseline (p > 0.05). In the ES series, grid intersections crossed by COAs were 68.2 ±9.3 (p < 0.001 vs after surgery; p < 0.05 vs baseline, series 1, 3, and 4). When ES was applied to the normal nonischemic limb this increased to 38.1 ±5.2 (p < 0.05 compared with baseline; Figure 3).

The angiographic score for grid intersections crossed by COAs was 42.5 at baseline, 27.1 after surgery, 53.6 in series 1, 96.0 in series 2 (p < 0.05 compared with all series), 58.8 in series 3, and 60.8 in series 4.

In the normal nonischemic muscle a decrease was seen from 18.3 ±3.8 COAs in 30 occupied grids before surgery to 12.2 ±2.5 (p < 0.05) after surgery. In both control series (no ES), the number of grids occupied increased after surgery to 21.4 ±3.75 in series 3 and to 22.3 ±3.5 in series 4 (p > 0.05 compared with each other; p > 0.05 vs baseline; p < 0.05 vs after surgery). In ES-treated series 2, we found 27.3 ±1.2 grids occupied by COAs (p < 0.05 vs series 1, 3, and 4). In nonischemic ES-treated series 1, this increased to 20.1 ±4.3 but was statistically insignificant compared with baseline (p > 0.05; Figure 4).

In nonischemic muscle, the angiographic score for grids occupied by COAs was 61 at baseline, 40.6 after surgery, 67 in series 1, 91 in series 2, 71.3 in series 3, and 74.3 in series 4 (p < 0.05 in series 2 vs series 1, 3, and 4).

In all series (even in the control group undergoing femoral excision without ES), angiography revealed progressive linear extension of collateral arteries from the origin stem artery to the distal point of the reconstituted parent ves-

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**Figure 2.** Contrast opacified arteries crossing a specific segment of the grid.

**Figure 3.** Contrast opacified arteries crossing grid intersection.

**Figure 4.** Contrast opacified arteries in grid.
Electrical stimulation

Figure 5. Angiography before surgery (top), immediately after surgery (center), and 1 month after surgery (bottom) in control series (femoralis artery excision but no ES).

Figure 6. Angiography before surgery (top), immediately after surgery (center), and 1 month after surgery (bottom) in experimental series (femoralis artery excision plus ES).

sels (Figure 5). However, in series 2 (the experimental group) a great many vessels were seen emerging from the pelvis and running through the thigh (Figure 6). One or two of these anastomoses obliquely connected to the distal part of the excised femoral artery to supply the hind-limb with blood.

Lower Limb Calf-Blood Pressure Ratio

A Doppler flow signal could not be detected immediately after surgery and on day 10 in series 2 to 4. In series 1 (no femoralis artery excision), on day 10 the blood pressure ratio was 0.95 ± 0.06 (practically the same as the other limb). On day 20, a flow signal was visible in all four series. However, in both series without ES, the blood pressure ratio was only 0.25 ± 0.04 (series 3) and 0.24 ± 0.03 (series 4, p > 0.05). In the ES-treated limbs with an excised femoral artery, blood pressure ratio increased to 0.63 ± 0.08 (p < 0.05 vs series 2 and 3). In the limb with ES but no artery excision, the blood pressure ratio increased to 1.09 ± 0.08 (however, this was statistically non-significant compared with the normal limb).

At day 30, blood pressure ratio was further improved in all series, but this was statistically significant only in series 2 (excised artery and ES) (0.81 ± 0.06 vs 0.63 ± 0.08 on day 20). In the series with ES but no excised artery, blood pressure ratio increased to 1.16 ± 0.07 (p < 0.05 vs normal limb; Figure 7).

Capillary Density

To further evaluate the effect of ES on revascularization of an ischemic hind-limb, the thigh muscles were histologically examined at day 30 as described previously. Light microscopy revealed no frank necrosis in series 4. Capillary density for ES-treated ischemic muscle (series 2) was 283.7 ± 24.5/mm² vs 91.4 ± 20.9/mm² in control series 4 without electrode implantation and ES (p < 0.001) and vs 87.4 ± 31.4/mm² in
control series 3 with electrode implantation but no ES. In series I (no femoral artery excision but with ES) capillary density was greater than that in normal muscle (201.0 ± 29.3/mm² vs 183.5 ± 32.2/mm²), but this was statistically non-significant (p > 0.05) (Figure 8).

Discussion

The treatment of peripheral artery disease, although greatly improved in recent decades by surgical and interventional techniques, remains limited by the proliferation of vascular lesions and our inability to slow progression of native disease. Therapeutic options are limited for patients who have lower extremity vascular obstructive disease. 3,26

Recently, several approaches have been investigated in an attempt to improve limb perfusion. Conservative approaches include vacuum compression therapy and heated mattresses to increase skin microcirculation 27; surgical approaches include reperfusion therapy with hypertonic or hyperonetic perfusates containing antioxidants.28 However, these approaches only slightly improve total perfusion and oxygenation but do not improve nutritive microcirculation.

During the past 5 years, growth factors have been used clinically to treat critical limb ischemia by accelerating angiogenesis and arteriogenesis.14-18 Benefits from this approach may be of major importance for patients; however, the need for multiple injections with perhaps an invasive procedure to deliver the growth factors, and the resultant side effects may limit use of this promising approach. Electrical stimulation, a simple and safe procedure, could revolutionize treatment for patients with severe peripheral arterial disease if it could restore both macrocirculation and microcirculation.

Chronic low-frequency ES was first used to decrease fatigue in fast-twitch muscles.29,30 Along with increased fatigue resistance based on increased activity of oxidative enzymes, this approach was shown to increase capillary supply.20,31 Capillary density in muscle was found to increase after only 2 to 4 days of ES.32 Continuing ES increased the total capillary surface area by 30% 33 and the numerical density of arterioles by 100% 34 whereas, after 7 days, both arteriole diameter and responsiveness to vasodilation returned to baseline.35

Following these investigations, Hudlicka and Price 36 hypothesized that the increase in capillary density in the skeletal muscle was related to hypoxia that resulted from muscle contraction. Kanno and associates19 discovered the mechanism for this response in 1994, reporting that low voltage ES induced de novo synthesis of VEGF protein, which, in turn, promoted local angiogenesis and restored blood flow to ischemic tissue. Patterson and Runge 37 summarized the benefits of this approach as follows:

“It can be applied transcutaneously and is thus remarkably safe, relatively inexpensive, and easy to administer. For contrast with intravascular injection of VEGF, induction of endogenous VEGF by electrical stimulation could be performed noninvasively and repeatedly. Unlike gene therapy, electrical stimulation has been used successfully for
decades and untoward effects are unlikely if this technique is used to stimulate VEGF expression for the treatment of ischemic vascular disease."

In our preliminary experiments with ES,23-25 applying it to the latissimus dorsi muscle following subtotal mobilization and heart wrap for cardiac bioassist, we found that the healing process can be improved, neovascularization accelerated, and angiogenesis in muscle tissue is enhanced, even in severely ischemic tissue. We also found that tissue could be damaged or healed depending on the ES regimen applied: ES at a rate of 60 to 120 contractions per minute was injurious (i.e., seriously damaging endothelial cells by degenerating intracellular components) whereas a cautious rate of 15 to 30 cpm safely increased the number of capillaries in ischemic tissue (i.e., the endothelial cells appeared normal). Interestingly, we found no evidence of new vessel formation in nonischemic tissue subjected to ES.24,25

On the basis of these results, we hypothesized that a modest regimen of ES would improve muscle revascularization in limb-threatening ischemia. The control series, in which the femoral artery was excised and angiography was performed 1 month later, showed interesting, if not intriguing, results. Although limb ischemia was immediately evident after the artery was excised and there were considerably decreased COAs in the area of investigation, in a period of just 1 month, blood flow in the limb was restored without any intervention to either accelerate angiogenesis or to vascularize the limb. The number of vessels crossing a specified grid segment more than doubled (from 3.2 ±1.0 to 7.2 ±2.5) and even exceeded the number of vessels before excision (5.3 ±1.3). Vessel density in the investigated area also doubled, with grid intersections increasing from 19.3 ±4.8 to 43.2 ±6.4, and the number of grids with COAs increasing from 12.2 ±2.5 to 22.3 ±3.5. The increase in vessel density was above baseline before ligation (30.2 ±6.5 vs 43.2 ±10.7 grids crossed by COAs and 18.3 ±3.8 vs 22.3 ±3.5 grids with COAs).

However, angiography showed that the process of arteriogenesis had reopened collaterals that had existed without function in the normal limb before the femoralis artery was excised. The actual extent of angiogenesis can be determined by calculating capillary density. Less than 1 month after surgery, the number of capillaries on angiography was three times that of baseline (91.4 ±20.9/mm² vs 183.5 ±32.2/mm²), showing that productive angiogenesis cannot be expected to restore compromised blood supply.

Hershey and associates12 showed that the improvement in angiogenic response did not translate to improved collateral blood flow. In our investigation, 1 month postoperatively, the blood pressure ratio was 0.31 ±0.07; Bauters and associates13 reported a ratio of 0.49 ±0.05 at day 30 whereas Pu and associates18 reported 0.55 ±0.02 on day 20. Takeshita and associates17 reported an even lower ratio (0.25 ±0.25) and capillary density (83.1 ±31.4/mm²). These data support the views of Ito and associates5 who concurred with the statement of Hershey and associates12 that "although capillary sprouting may deliver some relief to the underperfused territory, only true collateral arteries are principally capable of providing a large enough amount of blood flow to the ischemic area at risk for necrosis of lack of function."

Our results with ES were impressive. The number of COAs crossing a specific grid segment more than doubled over baseline to 10.5 ±1.2 (p <0.001); there were 68.2 ±9.3 grid intersections crossed by COAs vs 30.2 ±6.5 at baseline (p <0.01) and with 43.2 ±6.5 in control (p <0.05); there were 27.3 ±1.2 of 30 grids occupied by COAs vs 18.3 ±3.8 at baseline (p <0.05) and with 22.3 ±3.5 in control (p <0.05). Capillary density was 283.73 ±24.5/mm².

ES had a greater effect on the process of angiogenesis than on the process of arteriogenesis, i.e., the number of COAs in the ES series was only 1.5 times that of the control series, but the number of capillaries was three times more than that in controls (91.4 ±20.9/mm²; p <0.001). In the control series, ES resulted in both of these processes returning to near-normal levels (blood pressure ratio 0.81 ±0.06 vs the ratio in control 0.31 ±0.07; p <0.001). Two additional control series were in our investigation. In series 3, we investigated the effect of electrode implantation without ES on ischemic tissue and found no histologic evidence of an inflammation reaction. All angiographic parameters were practically the same as those in series 4 (control without electrode implantation). Also practically the same were capillary density and lower limb calf-blood pressure ratio.

Another control series (series 1) was used to study the effect of ES on normal nonischemic tissue. In this case, ES acted like intensive exercise in causing the muscle to repeatedly contract. One
month after ES, all parameters increased over baseline but these increases were not significant \(p < 0.05\): COAs crossing a specified segment (6.1 ± 0.8 vs 5.3 ± 1.3); grid intersections crossed by COAs (38.1 ± 5.2 vs 30.2 ± 6.5); grids occupied by COAs (20.1 ± 4.3 vs 18.3 ± 3.8); and capillary density (20.01 ± 29.3/mm² vs 183.5 ± 32.2/mm²). The only statistically significant change \(p < 0.05\) was an increase in blood pressure ratio (1.15 ± 0.07 vs 0.95 ± 0.06).

**Conclusion**

Currently, ES therapies are used primarily to treat chronic pain syndromes and soft tissue wounds. ES may be used clinically to augment angiogenesis for patients who have ischemic vascular disease. Our data showed that when appropriately applied to ischemic tissues, ES improves the status of ischemic tissues by enhancing and accelerating muscle revascularization. In all cases of lower limb ischemia, angiography revealed new vessels arising from the caudal artery in the direction of the distal femoral artery, connecting with it to restore distal blood flow. These preliminary results may be interpreted cautiously to suggest use of ES as a new approach to treat subacute limb ischemia, perhaps widening the horizon for patients who have severe localized peripheral artery disease.

**Acknowledgments**

The authors would like to acknowledge Brian Miller and Brian Schurrer for their technical assistance in preparing figures for this manuscript, Michelle Maternowski for statistical analysis, and Rob Henderson for his editorial assistance.

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