Far Red/Near Infrared Light Treatment Promotes Femoral Artery Collateralization in the Ischemic Hindlimb

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Abstract

Nitric oxide (NO) is a crucial mediator of hindlimb collateralization and angiogenesis. Within tissues there are nitrosyl-heme proteins which have the potential to generate NO under conditions of hypoxia or low pH. Low level irradiation of blood and muscle with light in the far red/near infrared spectrum (670 nm, R/NIR) facilitates NO release. Therefore, we assessed the impact of red light exposure on the stimulation of femoral artery collateralization. Rabbits and mice underwent unilateral resection of the femoral artery and chronic R/NIR treatment. The direct NO scavenger carboxy-PTIO and the nitric oxide synthase (NOS) inhibitor L-NAME were also administered in the presence of R/NIR. DAF fluorescence assessed R/NIR changes in NO levels within endothelial cells. In vitro measures of R/NIR induced angiogenesis were assessed by endothelial cell proliferation and migration. R/NIR significantly increased collateral vessel number which could not be attenuated with L-NAME. R/NIR induced collateralization was abolished with c-PTIO. In vitro, NO production increased in endothelial cells with R/NIR exposure, and this finding was independent of NOS inhibition. Similarly R/NIR induced proliferation and tube formation in a NO dependent manner. Finally, nitrite supplementation accelerated R/NIR collateralization in wild type c57Bl/6 mice. In an eNOS deficient transgenic mouse model, R/NIR restores collateral development. In conclusion, R/NIR increases NO levels independent of NOS activity, and leads to the observed enhancement of hindlimb collateralization.

Keywords

Hindlimb Collateralization; Nitric Oxide; Angiogenesis; Low level light therapy; Nitrosyl-hemoglobin

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1.0. Introduction

Peripheral arterial disease (PAD) is an enormous clinical problem with 8 million Americans being treated for symptoms such as intermittent claudication, and critical limb ischemia [1]. Therapies have focused on risk reduction, anti-platelet medications, and vasodilators such as cilostazol [2–4]. Surgical intervention with stent placement or bypasses of affected blood vessels is reserved for patients with severe critical limb ischemia and is often ineffective, therefore the risk for limb amputation remains high.

One treatment would be to stimulate collateral blood vessels within the ischemic limb and restore blood flow to the distal extremity. NO (nitric oxide) mediates collateralization through the production of growth factors e.g. vascular endothelial growth factor (VEGF), angiopoietin, and basic fibroblast growth factor (bFGF) [5–7]. NO is predominantly produced by enzymatic reduction of L-arginine to L-citrulline via nitric oxide synthases (NOS). However there is increasing interest in NOS- independent NO generation, particularly during hypoxia or anoxia, when NOS is inactive [8,9]. Nitrite can be enzymatically reduced to NO by molecules such as deoxyhemoglobin [10–13], deoxymyoglobin [14], xanthine oxidoreductase [9,15], cytochrome c oxidase [16] and NOS itself [17]. The heme moiety in these proteins binds the newly formed NO, thus generating nitrosyl storage pools of potentially bio-available NO.

Previously, we identified that light in the far red/near infrared spectrum (670 nm, R/NIR) releases NO from nitrosyl hemoglobin and nitrosyl myoglobin. NO generated in this fashion was responsible for R/NIR induced cardioprotection, thereby supporting the concept these storage pools of NO can be manipulated for therapeutic purposes [13]. Light exposure between 630nm and 830nm has been extensively studied *in vivo* and *in vitro* [18–21]. In this range there is improved tissue penetration due to limited melanin absorption [22]. Radiant heat production in R/NIR exposed tissue is below 0.5°C, because of limited water absorption by light [23,24]. Based on this evidence, we propose repetitive application of R/NIR (670 nm) will stimulate collateral blood vessel development independent of NOS. We further propose this increase in NO is physiologically significant so as to allow peripheral vascular collateralization in a NO deficient model.

2.0. Materials and Methods

All experimental procedures and protocols used in this investigation were reviewed and approved by the Animal Care and Use Committee of the Medical College of Wisconsin. Furthermore, all conformed to the *Guiding Principles in the Care and Use of Animals* of the American Physiologic Society and were in accordance with the *Guide for the Care and Use of Laboratory Animals*.

2.1. R/NIR Sources

The 670nm LED light source with power output up to $100mW/cm^2$ was obtained from Quantum Devices and $60mW/cm^2$ light source from NIR Technologies. Power output was measured with a light meter (X97, GigaHertz-Optik) at the surface to report actual irradiance intensity. The light sources were placed 2.5 cm from their target in all animal experiments. In vitro, culture dishes and slides were placed directly on the light source. The irradiance intensities listed below reflect the values obtained at the skin surface for in vivo studies, or the bottom of the culture dish for in vitro studies.

2.2. Rabbit Model of Hind-limb Ischemia

New Zealand White rabbits underwent unilateral femoral artery excision according to previously published protocols [25]. After a 5-day recovery, rabbits were randomized into 6 groups. Group 1 (n=6) underwent ischemia alone and group 2 (n=6) underwent ischemia with a daily R/NIR exposure (60mW/cm^2 × 10 min = 36 J/day) for 14 days. Group 3 (n=6) received 0.5g/L of L-NAME (Sigma) in their drinking water for 14 days and Group 4 (n=6) received L-NAME with R/NIR exposure. Group 5 (n=6) received infusions of 0.17mg/kg/min carboxy-PTIO (c-PTIO, Alexa chemical), a NO scavenger 1 min prior to R/NIR through 2 min post R/NIR. Group 6 (n=6) received daily infusion of c-PTIO without R/NIR. After 14 days, rabbits were anesthetized, the left carotid was cannulated, and both femoral arteries were injected with 10cc Omnipaque contrast media, and imaged with radiographic fluorography. Collateral blood vessel number was directly counted by 2 independent and blinded reviewers.

2.3. Endothelial Cell Proliferation Assay

Human umbilical vein endothelial cells (HUVEC) were grown to passage 4–6 in EGM media (Lonza), and then seeded (10,000 cells/well) on a 24 well culture plate (Costar). Cells were exposed to R/NIR (100mW/cm^2/day) for 4 days. The exposure duration varied to generate an energy dose response curve for cell proliferation. After the last exposure, cells were washed with PBS, trypsinized and counted using a hemocytometer. Experiments were performed in quadruplicate

2.4. Western Analysis

Vessels from the ischemic and control limbs were excised, snap frozen in liquid nitrogen, and isolated using protease inhibitor supplemented NP40 lysis buffer supplemented with protease inhibitors. Protein extracts were measured by the BCA method (Pierce), resolved using standard SDS-PAGE, and transferred to nitrocellulose. Blots were probed with mouse anti-VEGF antibody (1:200, Santa Cruz). Membranes were stripped and re-probed with mouse alpha- actin (1:200, Santa Cruz). Densitometry was performed using Image J software. Four samples in each group were analyzed. Data was normalized to the non-ischemic limb.

2.5. Endothelial Cell Tube Formation

Human coronary endothelial cells (HUVECs) were grown to passage 4–6 in HUVEC media (Cell Applications). Fibrin gels were prepared according to previously published protocols [26,27]. HUVECs (40,000 cells/well), were seeded on the surface of the three-dimensional matrix and incubated 1 hr prior to exposure with HUVEC media alone, Group 2 with 50 ng purified VEGF/well, Group 3 with L-NAME (1 mM), and Group 4 with c-PTIO (100μM). One plate was exposed to R/NIR (50mW/cm^2 × 150s =7.5J), and a control plate was not exposed. Thirty minutes post-exposure, the media was exchanged for standard media in order to avoid cell death secondary to extreme chronic NO depletion. This process was repeated daily for a total of 4 exposures. Experiments were performed in quadruplicate.

2.6. NO Detection by DAF-2 (diaminofluorescein) Fluorescence

HUVECs (passage 4–6) were seeded (25,000 cells/chamber) on chamber slides (Nunc) and grown to 90% confluence. Individual cell chambers incubated for 30 min in DMEM, 1mM L-NAME, or 100 μM carboxy-PTIO, before 4-amino-5methylamino-2′7′-diamiofluorescein diacetate (2 μM DAF-2, Invitrogen) was added prior to exposure. Cells were then irradiated for 7.5J (150 s at 50mW/cm^2), incubated for 30 min at 37°C, and then

mounted for confocal microscopy. Images were acquired using a Nikon Eclipse TE2000-U microscope equipped with EZC12 software. Fluorescence was monitored with excitation at 488 nm and emission at 510 nm. Images were analyzed using Nikon Element software to measure mean fluorescence intensity. Five images from each group were measured.

2.7. Mouse Hind-limb Collateralization Model

C57Bl/6 mice (n=6) or endothelial NOS knock-out mice (eNOS$^{-/-}$, n=6) underwent femoral artery ligation and excision distal to the femoral profundus and proximal to the saphenous and popliteal arteries [28,29]. The mice were placed within a box containing a transparent floor which rested directly on the light source. Beginning immediately after surgery, C57 Bl/ 6 or eNOS$^{-/-}$ mice were exposed to R/NIR light (60mW/cm^2 × 10 min = 36J/day) for 14 days. Additional groups of C57Bl6 and eNOS −/− mice underwent femoral artery ligation and excision with sodium nitrite supplementation (8.2 µg/kg/day, for 14 days, i.p.), and then further divided into receiving daily R/NIR or not receiving R/NIR [30].

2.8. Collateral Blood Flow

Laser doppler flow imaging was performed on all mice for noninvasive measurement of blood flow (Moor Instruments, UK). Mice were anesthetized with 1.25% isoflurane, allowed to acclimate on a heating pad to maintain body temperature, and then scanned [28]. Flow intensities in both limbs were measured on days 0, 3, 7, and 14. Data was expressed as the ratio of mean intensity between the ischemic limb and the contra-lateral control.

2.9. Statistical Analysis

All values are expressed as mean ± SEM. Comparisons were made using a one way ANOVA with post-hoc Bonferroni t-test, or a Student's t-test. Values for $p < 0.05$ were considered significant.

3.0. Results

3.1. Application of R/NIR stimulates femoral artery collateralization

To assess the effect of R/NIR on femoral artery collateralization, a rabbit hind limb ischemia model was employed. Figure 1 summarizes angiographic data collected at day 19 (5 day recovery plus 14 day protocol). Ischemia stimulated collateral vessels (11.7 ± 0.47; mean ± SEM) vs. control, (5.2 ± 0.23; p<.001), but R/NIR further increased blood vessel growth as compared to ischemia alone (15.9 ± 0.67 vs. 11.7 ± 0.47, p<0.001). Inhibition of NOS with L-NAME abolished collateral development stimulated by ischemia alone (5.6 ± 0.37 vs. 11.7 ± 0.47, p<0.001), however, R/NIR induced collateralization was unaffected (14.9 ± 0.81 vs. 15.9 ± 0.67). When c-PTIO was administered, ischemia (6.7 ± 0.42 vs. 11.7 ± 0.47, p<0.001) and R/NIR collateralization (8.9 ± 0.72 vs. 15.9 ± 0.67, p<0.001) was abolished. These studies suggest daily R/NIR enhances collateral development through an NO mediated mechanism, but does not require NOS as a source of the NO.

3.2. HUVECs produce NO in response to R/NIR

Cultured HUVECs were exposed to R/NIR in the presence of DAF-2 to quantify relative changes in NO. One exposure of R/NIR to HUVECs in Figure 2 (60 mW/cm^2 × 150 s = 7.5J/cm^2) increased fluorescence intensity (181 a.u. vs. 95 a.u., p<0.001). Pre-incubation with L-NAME, lead to a significant decrease in fluorescence when compared to control (39 a.u. vs. 95 a.u., p<0.05), but after R/NIR exposure the fluorescence intensity significantly increased (105 a.u. vs. 39 a.u., p <0.01). NO baseline levels were not affected by c-PTIO, but c-PTIO attenuated R/NIR stimulated increases in NO (121 a.u. vs 181 a.u., p<0.001)

confirming NO increases by R/NIR are independent of functional NOS. The finding that c-PTIO did not affect baseline fluorescence reflects the oxidation of NO to nitrite by c-PTIO. In the presence of NO abundance and nitrite, N_2O_3 is produced, which reacts with DAF-2 to generate fluorescence [31,32].

3.3. R/NIR increases Vascular Endothelial Growth Factor (VEGF)

One mechanism by which NO mediates collateralization is through stimulation of growth factors [27,33]. VEGF expression increased 1.2 fold in the collateral limb over the non-ischemic control limb (Figure 3). With R/NIR collateralization, VEGF expression increased 2.5 fold ($p < 0.01$). Although L-NAME abolished ischemia induced VEGF expression it did not attenuate R/NIR increases in VEGF. VEGF expression was completely abolished with c-PTIO.

3.4. R/NIR stimulates cellular proliferation

Endothelial cell proliferation is a hallmark of angiogenesis. HUVECs were repetitively exposed to variable doses of R/NIR (Figure 4). Doses of R/NIR below 6 J could not stimulate proliferation, however at 6 J, cell number increased almost 60% ($p < 0.05$). The proliferative effect of light plateaued at 30 J, indicating the optimal response to light is between 6J and 30J.

3.5. R/NIR stimulates cellular tube formation

Cell migration is an additional measure of angiogenesis therefore HUVECs were seeded on a fibrin matrix gel and repetitively exposed to R/NIR. HUVECs formed significant tubes (28 intersecting squares vs. 0; $p < 0.05$; Figure 5) when stimulated with R/NIR. L-NAME did not significantly impair endothelial cell migration (23 vs. 28 intersecting squares), but c-PTIO (100 µM) abolished cell migration in response to R/NIR stimulation, again confirming a key role for NO in light induced collateralization (12 vs. 28 intersecting squares, $p < 0.05$). Purified VEGF stimulated HUVECs to form tubes, thus verifying the integrity of the cells in culture.

3.6. R/NIR restores collateralization in eNOS deficient mice

Hind limb collaterals were stimulated in C57 Bl/6 mice after 14 days of ischemia. Blood flow in the C57Bl/6 group was significantly elevated at Day 7 (0.48 (±0.1) vs. 0.17 (±0.02, $p < 0.001$ vs. day 0), n=7) and increased to 0.71 (±0.08 $p < 0.001$ vs day 0, n=7) by day 14 (Figure 6A). Daily R/NIR treatment could not significantly increase collateral flow over ischemia alone. Since R/NIR treatment is presumed to work through release of NO from nitrosyl-hemeproteins, sub-stimulatory doses of sodium nitrite (8 µg/kg/day) were combined with daily R/NIR irradiation were performed. As expected collateral blood flow did not significantly increase with nitrite alone when compared to the C57Bl/6 control (Figure 6A). When nitrite was administered with daily R/NIR, an increase in collateral blood flow was observed at day 7 (0.76±0.09, n=7) and was significantly increased when compared to ischemia (0.48±0.1, $p < 0.01$), or R/NIR (0.46±0.04, $p < 0.01$, n=7) (Figure 6A). This observed increased in collateral blood flow in the combined nitrite and R/NIR groups was no longer present at Day 14 when compared to ischemia, nitrite or R/NIR alone.

If R/NIR exposure stimulates NO production independent of NOS, then it would be expected to restore collateralization in NOS deficient states. Mice which contain a transgenic deletion of eNOS underwent femoral artery ligation. Blood flow in the ischemic limb was significantly decreased at all time points, with a peak flow ratio of 0.45 (n=9; $p < 0.05$), consistent with impaired collateralization (Figure 6B) [6]. Daily R/NIR exposure significantly increased blood flow in eNOS knockout mice (0.70, n=10; $p < 0.05$ vs eNOS

control) at Day 14. Chronic administration of sodium nitrite did not stimulate increases in collateral blood flow. When sodium nitrite was combined with R/NIR treatment, collateral flow could not be significantly increased over R/NIR treatment alone. Thus, R/NIR can effectively stimulate collateral blood flow in the absence of eNOS.

4.0. Discussion

The findings of this investigation indicate that repeated R/NIR exposure stimulates angiogenesis *in vivo* and *in vitro*. In a hind limb model, R/NIR significantly augmented collateralization in a NO dependent manner, since NO scavenging can abolish R/NIR collateralization. These results also indicate the NO source was independent of NOS, since chronic NOS inhibition was unable to inhibit R/NIR collateralization. In the presence of chronic nitrite treatment and R/NIR, ischemia induced angiogenesis was accelerated in wild type c57Bl/6 by day 7. Although R/NIR exposure could restore collateral blood flow in a model of transgenic deletion of eNOS, sodium nitrite treatment could not further enhance collateralization. It may be that eNOS$^{-/-}$ mice require higher doses of nitrite to overcome their chronic NO deficiency.

In vitro, R/NIR increased intracellular NO in HUVECs that was independent of NOS. While a specific intracellular source of NO was not identified in this study, the argument for many potential NO donors can be proposed. Cytochrome c oxidase reversibly binds NO as a nitrosyl- adduct or as nitrite, depending on the concentration of reduced cytochrome c and O_2 [16]. It absorbs light in the far red/near infrared spectrum, and is the likely photoacceptor modulating neuroprotection from methanol toxicity [34]. Cytochrome c complexed with mitochondrial cardiolipin binds NO, which is also photosensitive [35]. Guanylate cyclase, the chemoreceptor for NO mediated vascular relaxation, is activated when NO binds its heme site and is photosensitive [36]. Cytoglobin, the newest member of the globin family, is a hexacoordinate heme protein with nitrite reductase capable of reducing nitrite to NO in the presence of hypoxia [37]. It's abundantly expressed in vascular smooth muscle and endothelial cells [37,38]. Nitrite reduction and NO binding to the heme iron by cytoglobin is possible when the heme-histidine bond is in a five-coordinate state, which results in a free binding site for nitrite or NO respectively. Approximately 0.6% of cytoglobin exhibits this binding affinity when the pentacoordinate state is at equilibrium with the hexacoordinate state [37]. Since cytoglobin is present in micromolar amounts in cells, there is still potential for it to be a source of NO generated by R/NIR. S-nitrosothiols are an additional source to be studied because they have photosensitivity at wavelengths between 550- 600nm [39,40]. In the presence of hematoporphyrins, there is increased photosensitivity of some nitrosothiol compounds at 650 nm [40]. It is critical to note that photolysis of sodium nitrite to yield NO does not occur at 670 nm, but rather in the ultraviolet range of wavelengths [41]

An effective collateral circulation relies on vascular remodeling, for which endothelial cell proliferation and migration is critical. Growth factor expression is necessary for this process, and this study focused on VEGF specifically, as it is well characterized in collateral development and can be stimulated by NO [27]. Recently, cytochrome c oxidase derived NO contributes to HIF-1–α, thus leading to accumulation of this transcription factor, and ultimately an increase in VEGF expression. [42]. The modest expression of VEGF in the ischemic group reflects the decreased hypoxic stimulus at day 14 when collateral development, and presumably perfusion had increased. R/NIR stimulated increases in VEGF suggest the continued generation of NO provides a chronic stimulus for VEGF expression.

There are several limitations to the current study. Although the action of R/NIR light appears independent of NOS derived NO, we did not directly identify a sole source of NO which is essential for angiogenesis. As evidenced from the discussion above, there are

numerous and abundant intracellular sources of nitrosyl-heme proteins which could generate free NO upon light stimulation. This will require further analysis using genetically modified protein expression. Additionally, the current conditions for R/NIR remain only broadly defined. Within the literature R/NIR has been used to reduce infarct size, protect neurons from methanol toxicity, heal chemotherapy induced mucositis, and stimulate angiogenesis [18,21,34,43–47]. However the irradiance and dosing parameters, i.e. wavelength, power output, distance, density, and time vary in each study. Another factor is tissue penetration, which reflects the lower doses of energy required for single cell monolayers in culture, when compared to multiple layers of tissue in vivo. There is increasing interest to "tune" these parameters to specific endpoints, e.g. measured reactive oxygen species, NF-κB, and caspase activity [48,49].

4.1.Conclusion

R/NIR light in low doses can stimulate peripheral collateralization via increases in NO. This investigation provides further evidence that biologically significant amounts of NO do not require functional NOS. In fact, the novelty of R/NIR treatment is that NO generation occurs despite conditions, like hypoxia and acidosis, which inactivate NOS. Thus, an alternative and redundant system to NOS exists to generate NO. Ischemic tissues may be able to increase nitrosyl storage pools by the addition of nitrite, and R/NIR can subsequently be used to stimulate NO release in a site specific manner. Safe NO delivery by R/NIR offers considerable therapeutic advantages.

Acknowledgments

Abbreviations and Definitions

R/NIR	Red/Near Infrared Light wavelength peak at 670 nm
NO	Nitric oxide
NOS	Nitric oxide synthase
HUVEC	Human umbilical vein endothelial cell
VEGF	Vascular endothelial growth factor

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Highlights

Low levels of red/near infrared light stimulate collateral formation in the hindlimb.

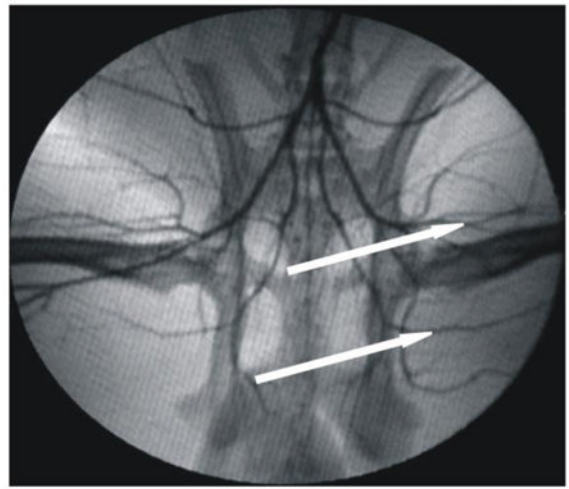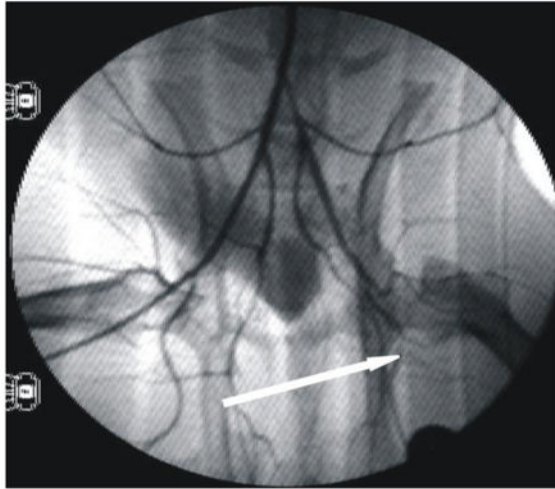Nitric oxide levels increase in response to light.

Nitric oxide increases are independent of functional nitric oxide synthases.

Endothelial cells proliferate and migrate in response to light.

Repetitive doses of red light stimulate collateralization in an impaired model of angiogenesis where eNOS is deficient.
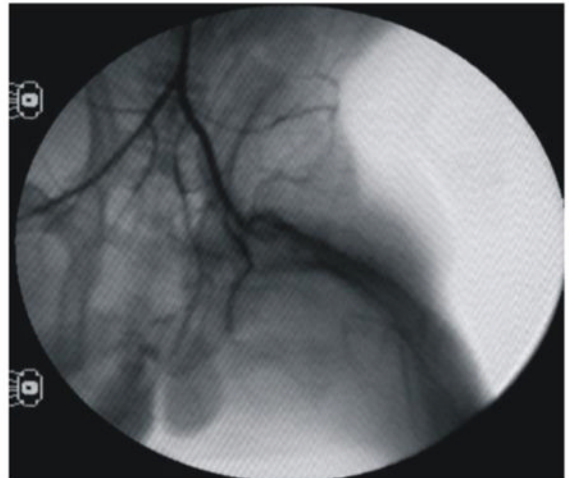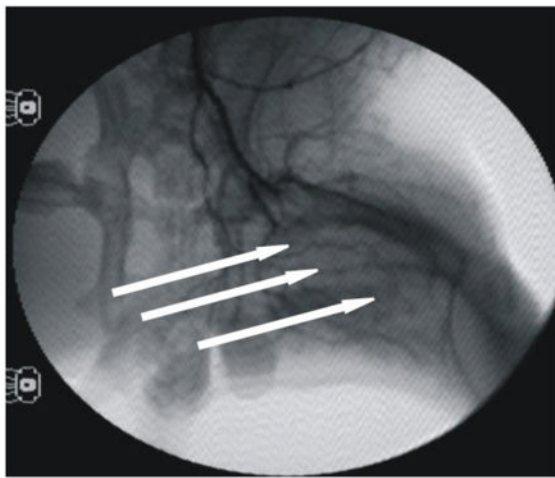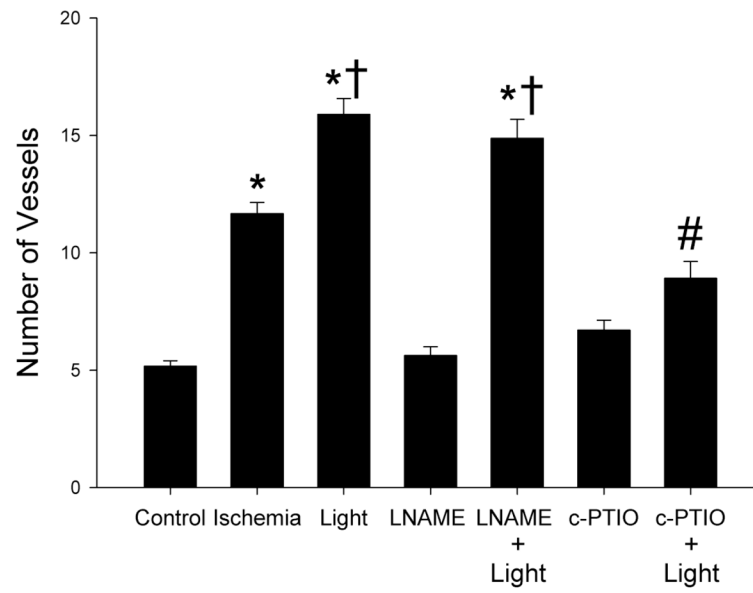
Figure 1A

Figure 1B

Figure 1. R/NIR increases collateral vessels in rabbits
A) Representative images from each group (n=6). B) R/NIR increases collateral vessel
number over ischemia. *p<0.001 vs. control, †p< 0.001 vs. ischemia, #p<0.001 vs. R/NIR.
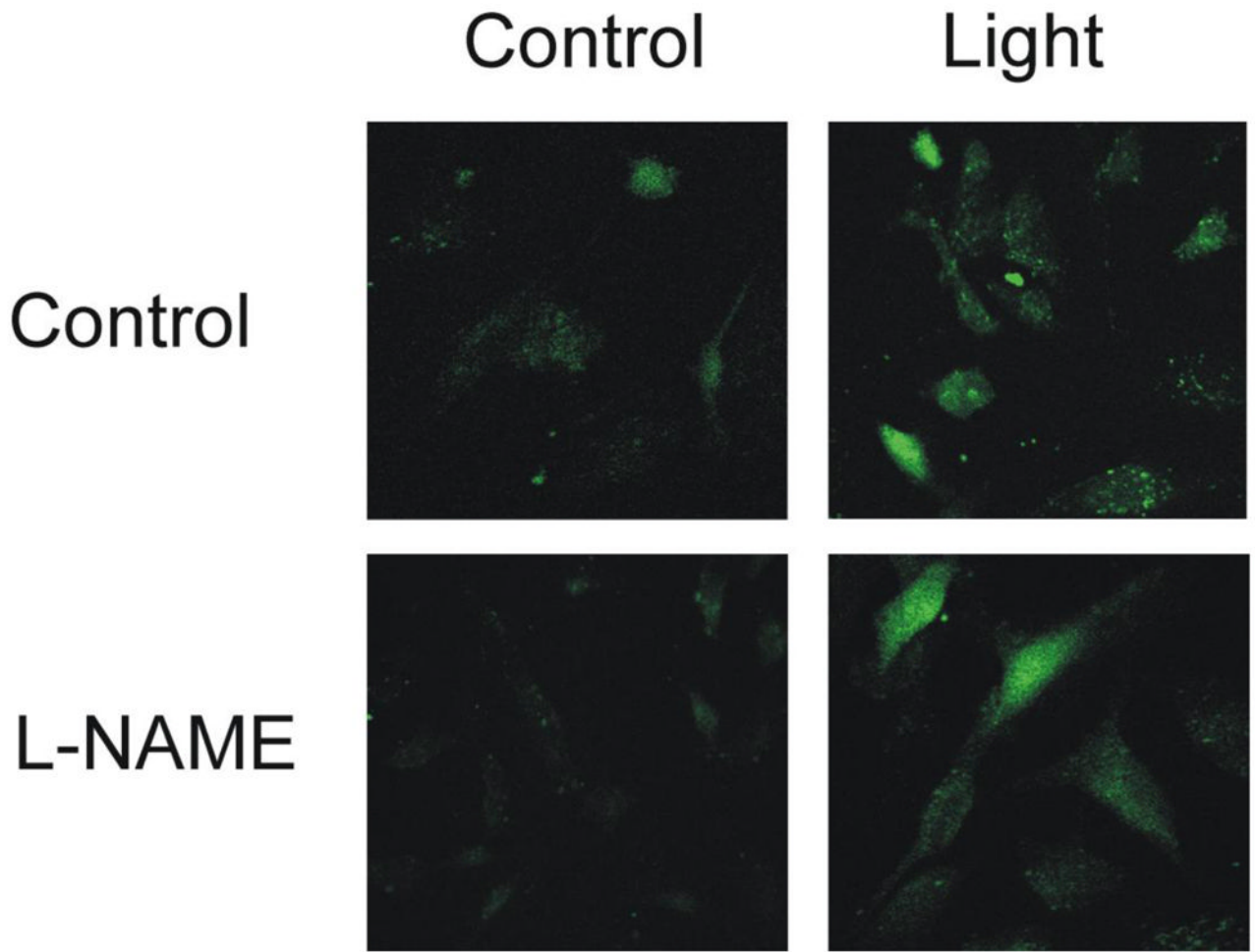
Figure 2A
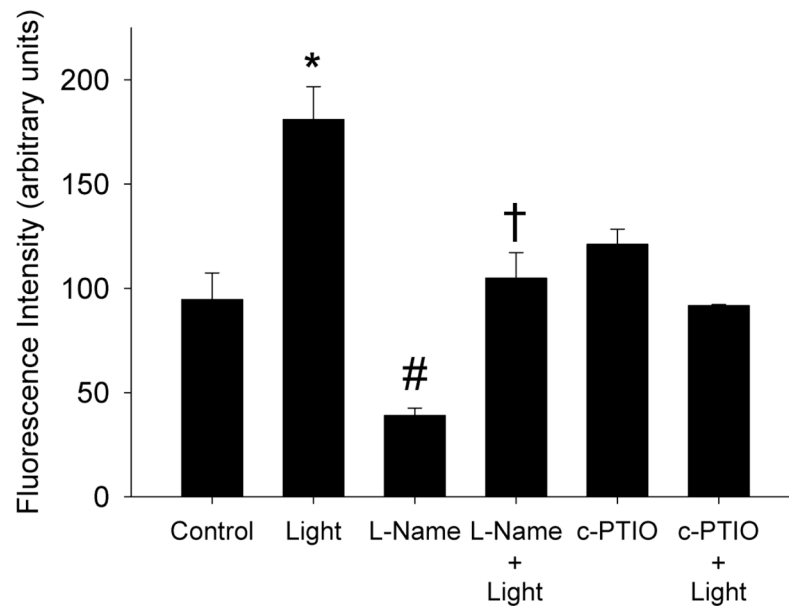
Figure 2B

Figure 2. R/NIR increases DAF-2 fluorescence
A) Representative confocal images of HUVECs pretreated with and without L-NAME and
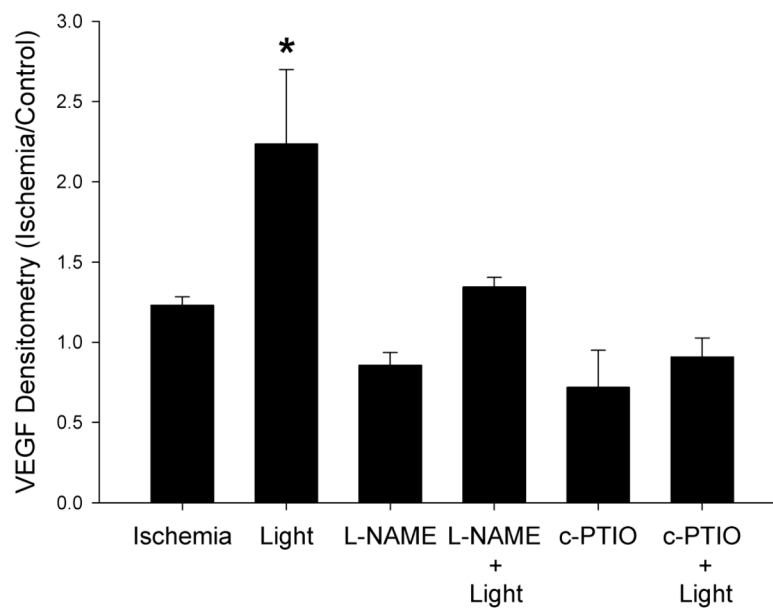R/NIR B). Mean fluorescence intensity quantification. *p<0.001 vs control, #p<0.05 vs.
control, †p< 0.01 vs. L-NAME.
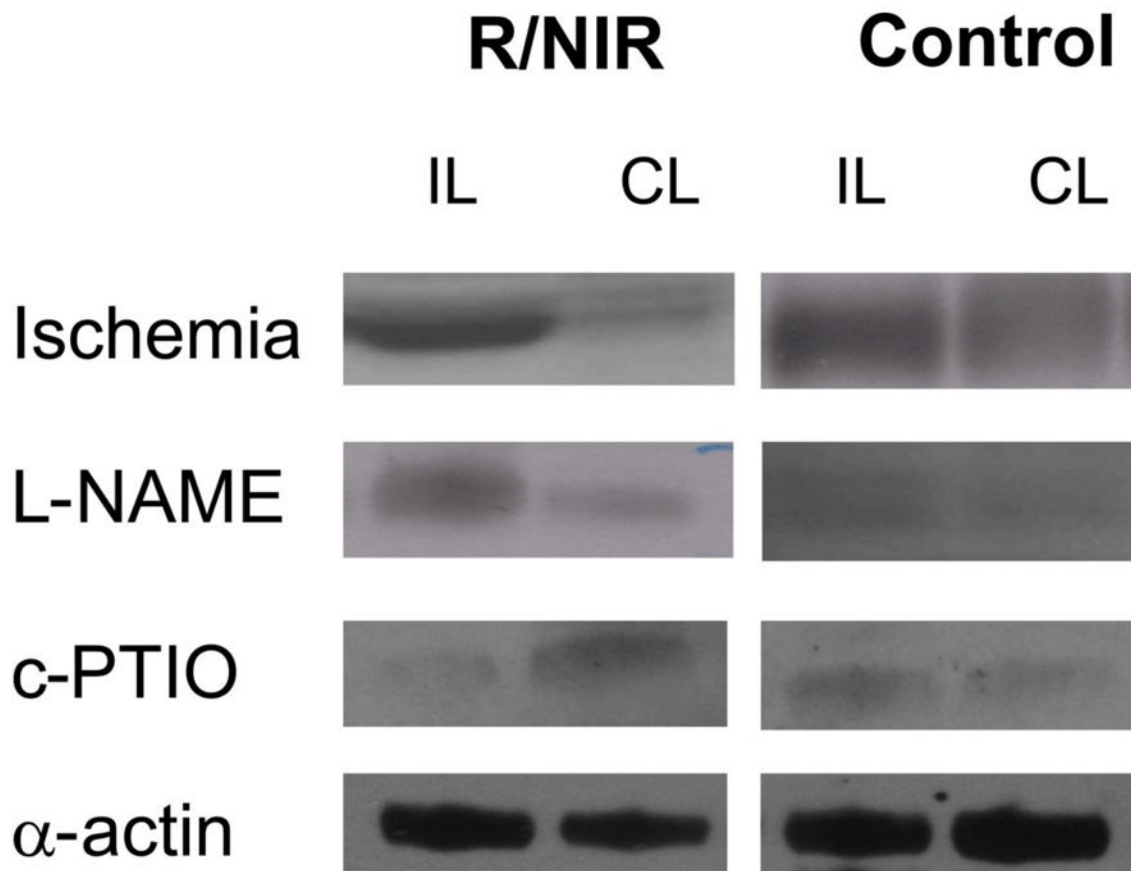
Figure 3A

Figure 3B

Figure 3. VEGF expression in collateral dependent hind limb (n=4)

A) Representative western blots. B) R/NIR increased VEGF expression in the ischemic limb (IL) vs. non ischemic control (CL). *p<0.01 vs. ischemia.
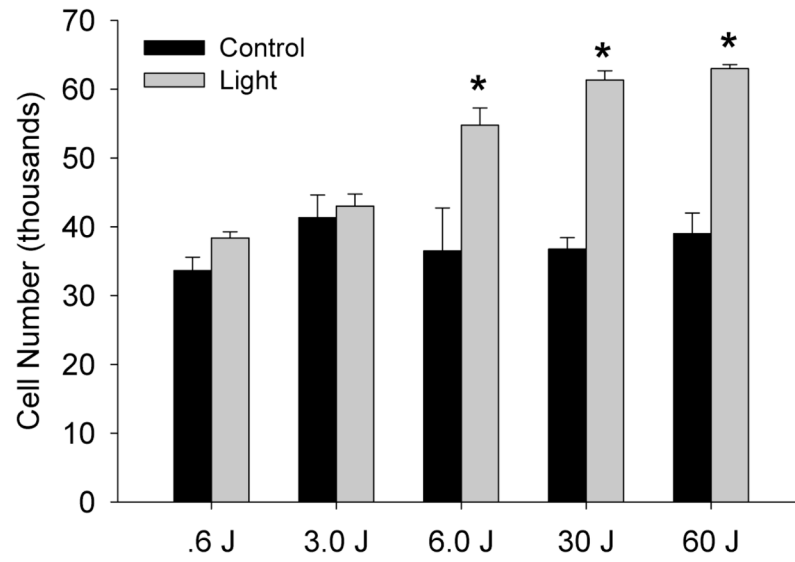
Figure 4. R/NIR stimulates endothelial proliferation
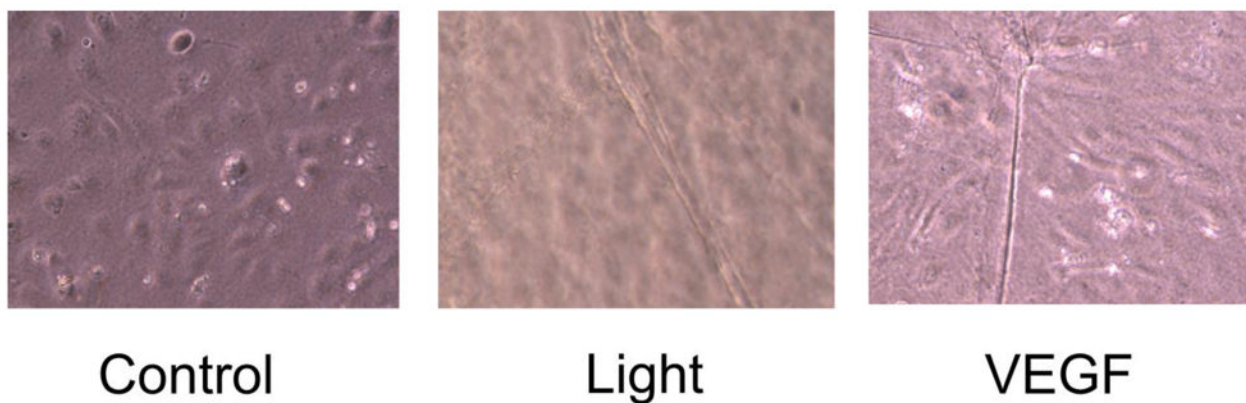Increasing energy dose of R/NIR stimulates HUVEC proliferation. n=4 *p<0.05 vs. control.

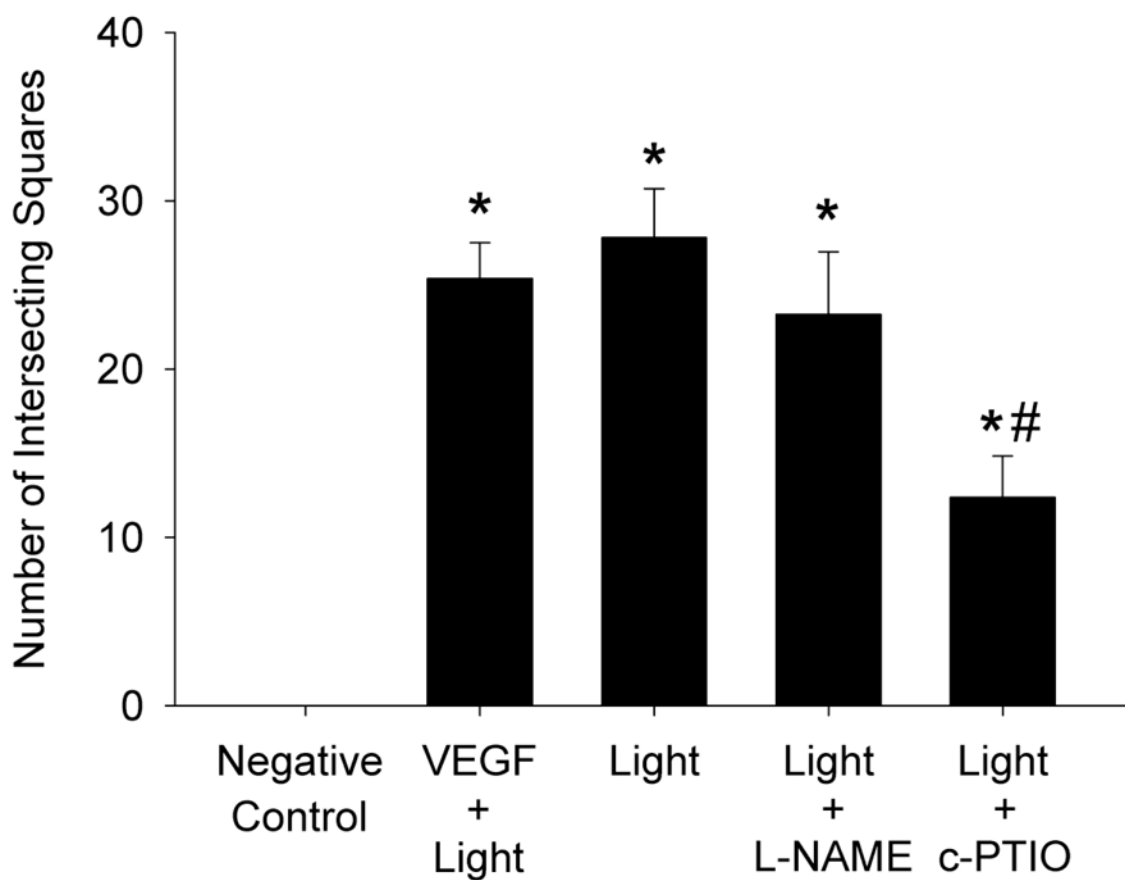Figure 5A

Figure 5B

Figure 5. R/NIR stimulates endothelial migration
A) Representative images HUVECs ± R/NIR treatment and VEGF positive control. B)
Quantified intersecting squares reflect R/NIR tube formation in the presence of NOS

inhibitor (L-NAME) and the NO scavenger (c-PTIO). $*p<0.05$ vs. control; $\#p<0.05$ vs. R/ NIR.
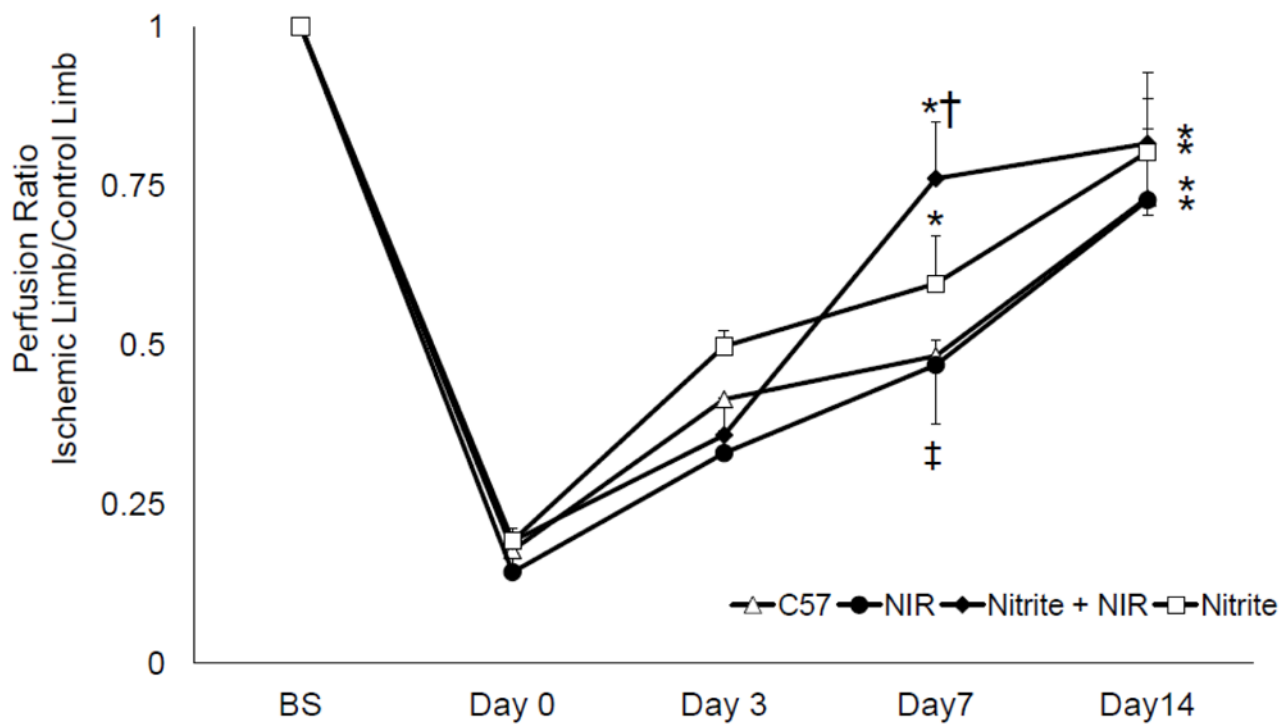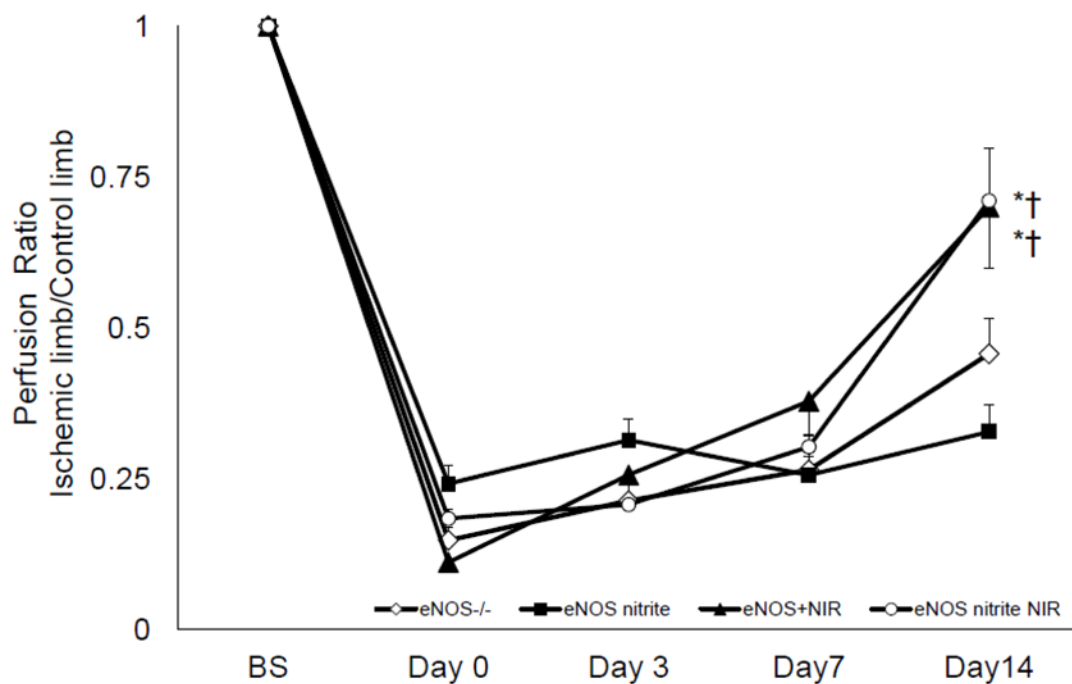
Figure 6A

Figure 6B

Figure 6. R/NIR increases collateral flow in eNOS deficient mice

A) Blood flow intensity ratio ischemic/control for c57Bl/6 with nitrite and R/NIR. *p<0.001 vs. Day 0, ‡p<0.05 vs Day O †p<0.01 vs. R/NIR B). Intensity ratio ischemic/control for eNOS$^{-/-}$ with nitrite and R/NIR. *p<0.001 vs. Day 0.