

BASIC RESEARCH STUDIES

Polydeoxyribonucleotide (PDRN) restores blood flow in an experimental model of peripheral artery occlusive disease

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Objective: This study investigated whether polydeoxyribonucleotide (PDRN) may be efficacious in the treatment of peripheral artery occlusive diseases, which are a major cause of morbidity in Western countries and still lack standardized treatment.

Methods: We investigated the effects of PDRN, a mixture of deoxyribonucleotides, in an experimental model of hind limb ischemia (HLI) in rats to stimulate vascular endothelial growth factor (VEGF)-A production and to avoid critical ischemia. The femoral artery was excised to induce HLI. Sham-operated on rats (sham HLI) were used as controls. Animals were treated daily with intraperitoneal PDRN (8 mg/kg) or its vehicle. Animals were euthanized at day 7, 14, and 21 after the evaluation of blood flow by laser Doppler. Dissected muscles were used to measure VEGF-A messenger RNA (mRNA) and protein expression, to evaluate edema, and to assess histologic damage.

Results: Administration of PDRN dramatically increased VEGF mRNA throughout the study (day 14: HLI, 7 ± 2.2 n-fold/ β -actin; HLI + PDRN, 13.3 ± 3.8 n-fold/ β -actin; $P < .0001$) and protein expression (HLI, 11 ± 3.4 integrated intensity; HLI + PDRN, 16 ± 3.8 integrated intensity; $P < .0001$). The compound stimulated revascularization, as confirmed by blood flow restoration ($P < .005$ vs HLI + vehicle), and blunted the histologic damage and the degree of edema. PDRN did not modify VEGF-A expression and blood flow in sham HLI animals. Furthermore, the concomitant administration of 3,7-dimethyl-1-propargilxanthine (DMPX), a selective adenosine A_{2A} receptor antagonist, abolished the positive effects of PDRN, confirming that PDRN acts through this receptor.

Conclusion: These results led us to hypothesize a role for PDRN in treating peripheral artery occlusive diseases. (*J Vasc Surg* 2008;48:1292-1300.)

Clinical Relevance: The compound polydeoxyribonucleotide (PDRN), acting through the adenosine receptors, which are expressed during ischemia, can stimulate therapeutic angiogenesis during ischemic conditions without any adverse effect. Owing to this peculiar frame of activity, its use is also safe in diabetic patients where an augmented vascular endothelial growth factor production can be disadvantageous, as we have already demonstrated.

Peripheral arterial occlusive disease (PAOD) of the lower extremities is becoming more prevalent worldwide. The general prognosis for patients with peripheral arterial disease is particularly negative; in fact, such patients have a high prevalence of coronary heart disease and cerebrovascular disease.¹ Nonsurgical treatment options provide the foundation for management; in this effort, several therapeutic agents have been used with success and others are

still undergoing testing. Definitive recommendations cannot be made on the use of specific drugs until further evaluation is completed.^{2,3}

Ongoing research with new strategies for angiogenesis and the use of progenitor cells has yielded encouraging results, particularly for patients with critical limb ischemia and limited options.^{4,5} The most effective angiogenic molecule is vascular endothelial growth factor (VEGF), and especially the isoform 165 of VEGF-A (hereinafter VEGF), which is able to stimulate new vessels formation also during adult life. Different cell types release VEGF in response to an ischemic injury, and it is of crucial importance to direct angiogenesis towards nonperfused tissue.

To study the possible beneficial effects of therapies, several animal models have been described in recent years; however, ligation and excision of the animal's left femoral artery, which results in a significant decrease of blood flow in the ischemic limb, has been recognized one of the most

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Competition of interest: none.

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effective to reproduce PAOD.⁶ Furthermore, these experimental conditions make it possible to assess the hypoxia-driven angiogenic response and to determine the residual perfusion by laser Doppler analysis.⁷

An increase in VEGF has been shown as a result of adenosine A_{2A} receptor stimulation. This seems to be mediated directly by increasing endothelial cell migration and microvascular endothelial cell VEGF production^{8,9} and also indirectly through promotion of VEGF production by macrophages.¹⁰

Polydeoxyribonucleotide (PDRN) is a compound that, acting through adenosine receptors, is able to stimulate VEGF production during pathologic conditions of low tissue perfusion such as diabetes mellitus and thermal injury.^{11,12} PDRN is the active fraction of a preparation used in therapy as an agent to stimulate tissue repair and is extracted from the sperm of trout bred for human consumption. The drug is obtained by an extraction process with purifying and high temperature sterilizing procedures to obtain an >95% pure active principle without pharmacologically active proteins and peptides (Registration Dossier, Italian Ministry of Health).¹³ This compound holds a mixture of deoxyribonucleotides polymers with chain lengths ranging between 50 and 2000 bp and may also represent the source of purine and pyrimidine deoxynucleosides/deoxyribonucleotides and bases.

The aim of our work was to study the possible beneficial role of PDRN in a rat model of hind limb ischemia (HLI). More in detail, we investigated the angiogenic process through VEGF expression and laser Doppler evaluation.

MATERIALS AND METHODS

This investigation conforms with the *Guide for the Care and Use of Laboratory Animals* (National Institutes of Health Publication No. 85-23, revised 1996).

Animals. The study used male Sprague-Dawley rats (250 to 300 g) fed on a standard diet and with tap water ad libitum and kept in a 12-hour light-dark cycle. The temperature of the animals was maintained at approximately 37°C using an overhead lamp during the experiment. Environmental conditions (light, temperature and humidity) have been constantly maintained in the vivarium for all the time of the experiment and especially during laser Doppler evaluations.

Experimental procedures. The surgical procedures have been performed as previously described.¹⁴ Briefly, rats were anesthetized with an intraperitoneal (IP) injection of sodium pentobarbital (50 mg/kg). Hair was removed from the hindquarters with a depilating cream, with care taken to avoid erythema. The left femoral artery was exposed aseptically through a 2-mm incision and isolated from the femoral vein and nerve, with care taken to avoid damage to vessels or nerve, then was ligated with 7-0 suture just distal to the bifurcation of the anterior epigastric and lateral caudal femoral arteries. Finally, the femoral artery was excised from the ligation to the point distally where it bifurcates into the saphenous and popliteal arteries. As a consequence, blood flow to the ischemic limb becomes

completely dependent on the collateral vessels (HLI animals). Control animals underwent sham operations (sham HLI) that consisted of all surgical procedures except for artery ligation and consequent excision.

Animals were randomized to receive daily IP PDRN or its vehicle (100 µL 0.9% saline). To establish the most effective dose of PDRN under these experimental conditions, we performed a dose-response experiment using 42 rats that were randomized as follows: sham HLI + vehicle (n = 7), HLI + vehicle (n = 7), and HLI treated with PDRN at 2, 4, 8 and 16 mg/kg (n = 7 in each experimental group). Rats underwent laser Doppler evaluation at day 0, 7, 14, and 21 after the surgical procedure to estimate changes in perfusion.

A total of 82 animals were then used in the experiment done with the most effective dose. After surgery, rats were randomly assigned to four different groups: sham HLI + vehicle, sham HLI + PDRN, HLI + vehicle, and HLI + PDRN. Immediately after ischemia, animals were treated thereafter daily with the most effective dose of PDRN (8 mg/kg IP) or with its vehicle. Groups of 28 animals (7 for each treatment) were used to evaluate laser Doppler perfusion imaging, VEGF messenger RNA (mRNA), and protein at 7, 14, and 21 days.

To clarify whether PDRN activity was mediated by the A_{2A} receptor, three additional groups of 28 animals were treated daily either with 8-mg/kg PDRN IP or 0.1-mg/kg 3,7-dimethyl-1-propargilxanthine (DMPX) IP (sham HLI + DMPX, HLI + DMPX, and HLI + PDRN + DMPX). In these last groups, we analyzed the changes in perfusion, the time course of VEGF mRNA and protein expression, and tissue edema. Tissue edema was evaluated only at day 7 in the adductor longus and histologic damage only at day 21. Evaluations of VEGF mRNA and protein expression and the histologic analysis were performed in the adductor magnus muscle, sectioned in two parts, one for histology and one for molecular analysis.

Laser Doppler perfusion imaging. After anesthesia, hair was removed from both legs using a depilatory cream. Hind limb perfusion was measured using a PIM 1.0 laser Doppler perfusion imager (Perimed/Periscan, Stockholm, Sweden). Measurements were obtained over the same regions of interest (leg and foot) on the day of surgery (baseline), 10 minutes after arterial excision, and at 7, 14 and 21 days after surgery. The laser source was mounted onto a movable rack exactly 20 cm above the rat limbs, the laser beam (780 nm) reflecting from moving red blood cells in vessels was detected and processed to provide a computerized color-coded photographs. The recorded images were analyzed by the instrument's software, and the average perfusion of the ischemic and nonischemic limb was calculated. Low or no perfusion is displayed as dark blue, whereas the highest perfusion interval is displayed as red. To account for variables such as ambient light and temperature, the results were expressed as the ratio of perfusion in the left (ischemic) vs right (nonischemic) limb.

Isolation of cytoplasmic proteins and determination of VEGF. Muscle samples were homogenized in lysis buffer (1% Triton, 20 mM Tris/HCl [pH 8.0], 137 mM NaCl, 10% glycerol, 5 mM ethylenediaminetetraacetic acid, 1 mM phenylmethylsulfonyl fluoride, 1% aprotinin, and 15- μ g/mL leupeptin). Protein samples (30 μ g) were denatured in reducing buffer (62-mmol/L Tris [pH 6.8], 10% glycerol, 2% sodium dodecyl sulfate [SDS], 5% β -mercaptoethanol, and 0.003% bromophenol blue) and separated by electrophoresis on a SDS (12%) polyacrylamide gel. The separated proteins were transferred on to a nitrocellulose membrane using the transfer buffer (39-mmol/L glycine, 48 mmol/L-Tris [pH 8.3], and 20% methanol) at 200 mA for 1 hour. The membranes were stained with Ponceau (0.005% in 1% acetic acid) to confirm equal amounts of protein and blocked with 5% nonfat dry milk in Tris-buffered saline (TBS)-0.1% Tween for 1 hour at room temperature, washed three times for 10 minutes each in TBS-0.1% Tween, and incubated with a primary phosphorylated antibody for VEGF (Chemicon, Temecula, Calif) in TBS-0.1% Tween (1:1000) overnight at 4°C. After being washed three times for 10 minutes each in TBS-0.1% Tween, the membranes were incubated with a specific peroxidase-conjugated secondary antibody (1:20,000; Pierce, Chester, UK) for 1 hour at room temperature.

The membranes were washed and analyzed by the enhanced chemiluminescence system according to the manufacturer's protocol (Amersham Bioscience, Amersham, UK). The VEGF protein signal was quantified by scanning densitometry using a bioimage analysis system (Bio-Profil, Celbio, Milan, Italy). The results from each experimental group were expressed as relative integrated intensity compared with control measured with the same batch. Equal loading of protein was assessed on stripped blots by immunodetection of β -actin with a rabbit monoclonal antibody (diluted 1:500; Cell Signaling, Danvers, Mass) and peroxidase-conjugated goat antirabbit immunoglobulin G (diluted 1:15000; Pierce). All antibodies were purified by protein A and peptide-affinity chromatography.

RNA extraction and real-time polymerase chain reaction. Adductor muscles were processed for isolation of total RNA using Trizol Reagent (Invitrogen, Milan, Italy), and the procedure was performed according to the protocol provided by the manufacturer. The RNA from each sample was dissolved in 50 μ L of sterile RNase-free water, quantified spectrophotometrically, and either stored at -80°C or used immediately for real-time polymerase chain reaction (PCR). The concentration and purity of RNA were estimated by calculating the ratio of absorbance at 260 nm to absorbance at 280 nm using an ultraviolet spectrophotometer (BioPhotometer, Eppendorf, Hamburg, Germany). The total RNA isolated with this protocol had an A260/A280 ratio of 1.8:2.2.

Total RNA (5 μ g) from each sample was reverse transcribed using the High Capacity complementary DNA (cDNA) Archive Kit according to the manufacturer's manual (Applied Biosystem). The cDNA from each sample

(5 ng) was amplified by real-time PCR with 2X TaqMan universal PCR Mastermix (Applied Biosystem), 20X target primer, and probe. β -Actin was used as the housekeeping gene. Each sample was analyzed in duplicates using SDS 7300 (Applied Biosystem). The results were expressed as the n-fold difference relative to normal controls (relative expression levels).

Measurement of malondialdehyde content. Determination of malondialdehyde (MDA) was done to estimate the extent of lipid peroxidation in adductor muscle. The assay was done by using a colorimetric commercial kit (lipid peroxidation assay kit, Calbiochem-Novabiochem Corp, San Diego, Calif). We used for our experiments the MDA-586 method, which is specific to assay free MDA, or after a hydrolysis step, total MDA. The assay serves to minimize interference from other lipid peroxidation products, such as 4-hydroxyalkenals.

Determination of tissue edema. Skeletal muscle edema was quantified in adductor longus muscles by measuring tissue wet weight-dry weight ratio at day 7. Immediately after harvest, tissue samples were blotted, weighed, and placed in a drying oven at 55°C until a constant weight was obtained. Muscle edema was quantified by measuring the wet weight-dry weight ratio.

Histologic evaluation. The adductor magnus muscles were obtained from both ischemic and nonischemic leg, fixed in 4% buffered formaldehyde, and then embedded in paraffin. The paraffin-embedded samples were sectioned and stained with hematoxylin and eosin for routine histologic studies. Slides were evaluated by three different pathologists blinded to the treatment. The variables examined and scored¹⁵ were presence of necrosis, tissue edema, presence of hemorrhagia, inflammatory cell infiltration, and tissue architecture. A score of 0 represents the intact muscle and a score of 5 a necrotic muscle, as specified in the Table.

Immunohistochemistry. Paraffin-embedded tissues from animals sacrificed at day 14 were sectioned, and antigen retrieval was performed using 0.05M sodium citrate buffer. Tissues were treated with primary antibody against platelet-endothelial cell adhesion molecule 1 (PECAM-1; Santa Cruz Biotechnology, Santa Cruz, Calif) or VEGF (Upstate Biotech, Lake Placid, NY). Secondary antibody was provided by Innovex (Richmond, Calif), and the location of the reaction was visualized with 3,3'-diaminobenzidine tetrahydrochloride (Sigma, St Louis, Mo). Slides were then mounted with coverslips. Histologic assays were all batch processed and used quality controls. A negative control (nonimmune serum plus detection reagent) and positive control (ileum for CD31, breast angiosarcoma for VEGF) was performed on each run.

The angiogenic response was determined by counting the number of PECAM-1-positive capillaries/mm² in at least 10 fields per specimen (original magnification $\times 40$). The assessor was blinded to the specimen identity.

Drugs. PDRN was a kind gift of Mastelli Srl, Sanremo, Italy. It was freshly prepared and dissolved in 0.9% saline solution.

Table. Criteria for histologic score

Score	Presence of necrosis	Tissue edema	Presence of hemorrhagia	Inflammatory cell infiltration	Tissue architecture
0	No fiber damage	Not present	Not present	Not present	Highly conserved
1	Localized and/or sparsely scattered fiber destruction	Slightly present	Not present	Present	Mostly conserved
2	More extensive necrosis after major connective tissue planes and involving few muscle fascicles	Present	Slightly present	Sparsely present	Partially conserved
3	More extensive necrosis after major connective tissue planes and involving numerous muscle fascicles	Present	Present between fibers	Present	Sparsely conserved
4	Destruction of almost all the entire muscle mass in the eye-field	Present	Present in most of the eye-field	Present	Not conserved
5	Destruction of essentially the entire muscle mass in the eye-field.	Present	Present in most of the eye-field	Present	Not conserved

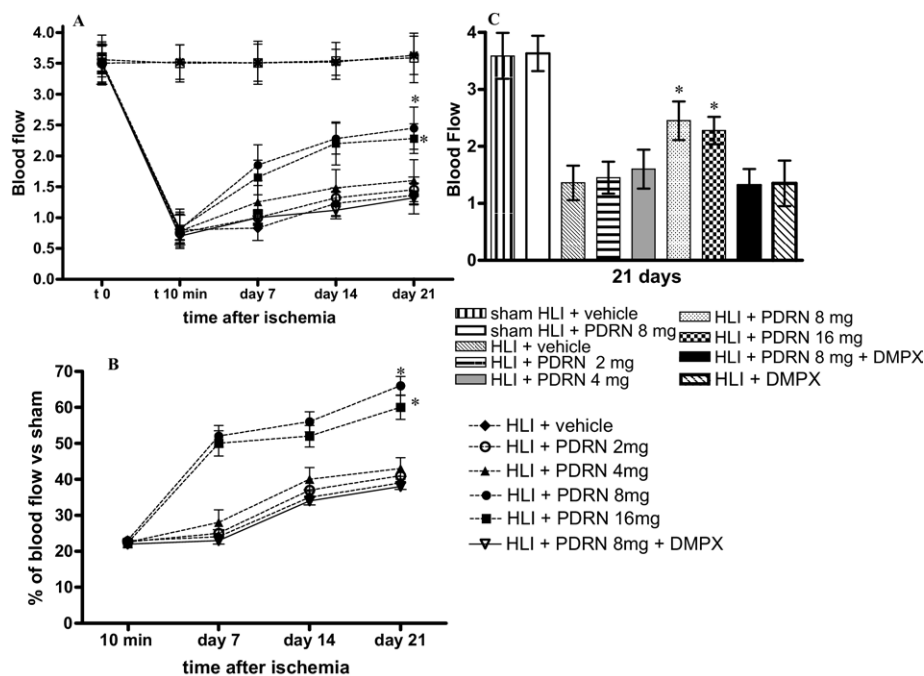


Fig 1. A, Blood flow evaluated by laser Doppler at 10 minutes, and at 7, 14, and 21 days after hind limb ischemia (HLI) in the various treatment groups. Rats were administered the vehicle or intraperitoneal polydeoxyribonucleotide (PDRN), at 2, 4, 8, or 16 mg/kg daily, starting immediately after surgery. PDRN (8 and 16 mg/kg) restored blood flow in treated rats. The blood flow of ischemic hind limb is expressed as a relative amount compared with the uninjured limb. * $P < .005$ vs HLI + vehicle. B, Percentage of blood flow vs animals undergoing sham operations (sham HLI) evaluated by laser Doppler at 7, 14, and 21 days after hind limb ischemia. Rats were administered the vehicle or intraperitoneal PDRN (2, 4, 8, and 16 mg/kg daily) starting immediately after surgery. PDRN (8 and 16 mg/kg) restored blood flow in treated animals. * $P < .005$ vs HLI + vehicle. C, Blood flow evaluated by laser Doppler at day 21 after HLI in the experimental groups. The blood flow of ischemic hind limb was expressed as a relative amount compared with the uninjured limb. * $P < .005$ vs HLI + vehicle. Error bars in the panels represent the standard deviation. DMPX, 3,7-dimethyl-1-propargilxanthine.

Statistical analysis. The results were expressed as means \pm standard deviation. Differences in skeletal muscle damage between treatment groups were examined for significance by using the χ^2 test, and values of $P < .05$ were considered

significant. Agreement between examiners' scorings yielded a rank correlation coefficient of 0.93 for the entire study. For all other data, one-way or two-way analysis of variance (when a time \times treatment interaction occurs) was used.

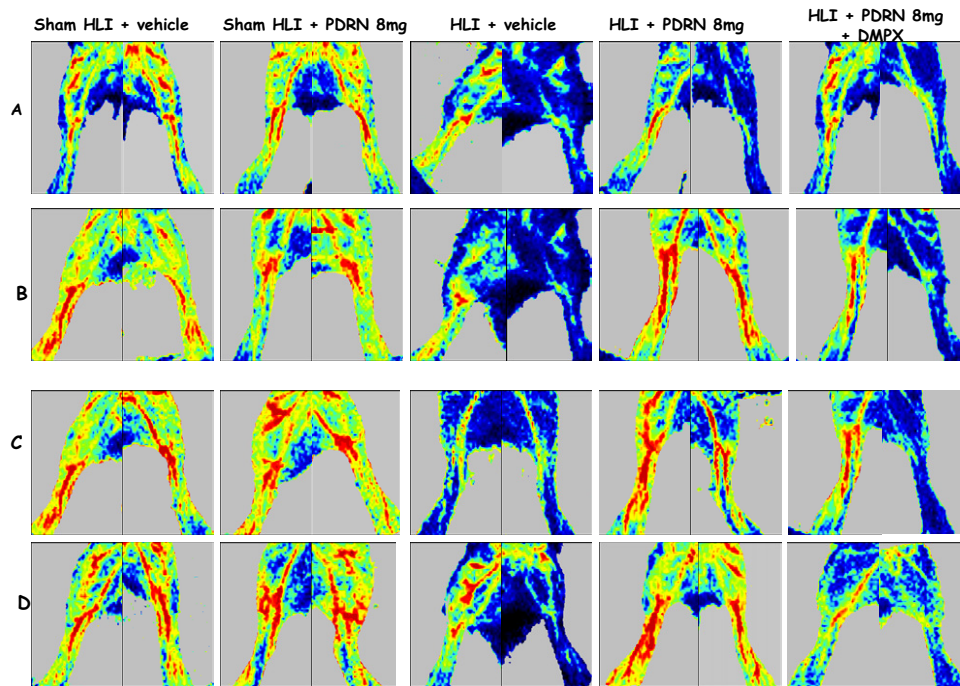


Fig 2. Laser Doppler images at (Row A) 10 minutes, and at (Row B) 7, (Row C) 14, and (Row D) 21 days after hind limb ischemia (HLI). Polydeoxyribonucleotide (PDRN), at 8 mg/kg, restored blood flow in treated animals. DMPX, 3,7-dimethyl-1-propargilxanthine.

RESULTS

PDRN improves tissue perfusion of ischemic hind limb. All animals recovered well from anesthesia and were normally moving in the cage the day after surgery. As a consequence of femoral artery excision, blood flow significantly diminished in all the ischemic groups 10 minutes after surgery (Fig 1, A and B). The untreated ischemic animals showed an almost not significant increase in blood flow evaluated 7, 14 and 21 days after surgery (Fig 1, C), whereas a slight increase in blood flow over time was observed in the ischemic hind limbs of animals treated with PDRN at 2 and 4 mg/kg (Fig 1; $P = .4$ vs ischemic at all time points). In contrast, a marked increase in blood flow was noticed in ischemic limbs treated with PDRN at 8 mg/kg after 7 days and gradually over time (Fig 1; $P < .0001$ at 7, 14, and 21 days). No further increase in blood flow was observed with the 16-mg/kg dose of PDRN. Thus for the subsequent assessment, we used the 8-mg/kg dose. The concomitant administration of DMPX abolished the positive effects of PDRN. DMPX alone did not modify blood flow in sham HLI or HLI animals (Fig 1, C).

Fig 2 shows laser Doppler perfusion images of tissue perfusion of the several study groups at 10 minutes, and 7, 14 and 21 days after surgical procedures. The fall in perfusion, shown by the blue color, was apparent 10 minutes after artery excision, with no differences between the ischemic groups. In the ischemic group, the laser Doppler perfusion images show a partial recovery after 21 days,

whereas the ischemic animals treated with PDRN had a complete recovery starting from day 7, as highlighted by the red color (Fig 2). A moderate ischemia occurred also in the contralateral hind limb of untreated ischemic animals, as observed in laser Doppler perfusion images shown in Fig 3. PDRN treatment was also able to abate the ischemic damage in the contralateral limb. PDRN did not affect blood flow in sham HLI animals (Fig 2). The concomitant administration of DMPX abolished the positive effects of PDRN in HLI rats, but DMPX alone did not affect blood flow in sham HLI and HLI animals (Fig 2). Moreover, blood flow was still increased in PDRN-treated HLI animals 1 month after surgical procedures (data not shown).

PDRN stimulates VEGF expression in ischemic hind limb. We examined the expression of VEGF mRNA and protein in adductor muscles by real-time PCR and Western blotting (Fig 3). In the sham HLI rats, VEGF was not detectable throughout the experiment. In HLI animals treated with vehicle, VEGF mRNA and protein slightly increased. In contrast, treatment with PDRN significantly enhanced the amount of VEGF mRNA and protein in HLI rats at day 7, with a further increase at day 14. PDRN administration did not modify VEGF expression in sham HLI rats (Fig 3). The concomitant administration of DMPX abolished the positive effects of PDRN on VEGF mRNA and protein synthesis. DMPX alone did not modify VEGF expression in sham HLI and HLI animals (Fig 3).

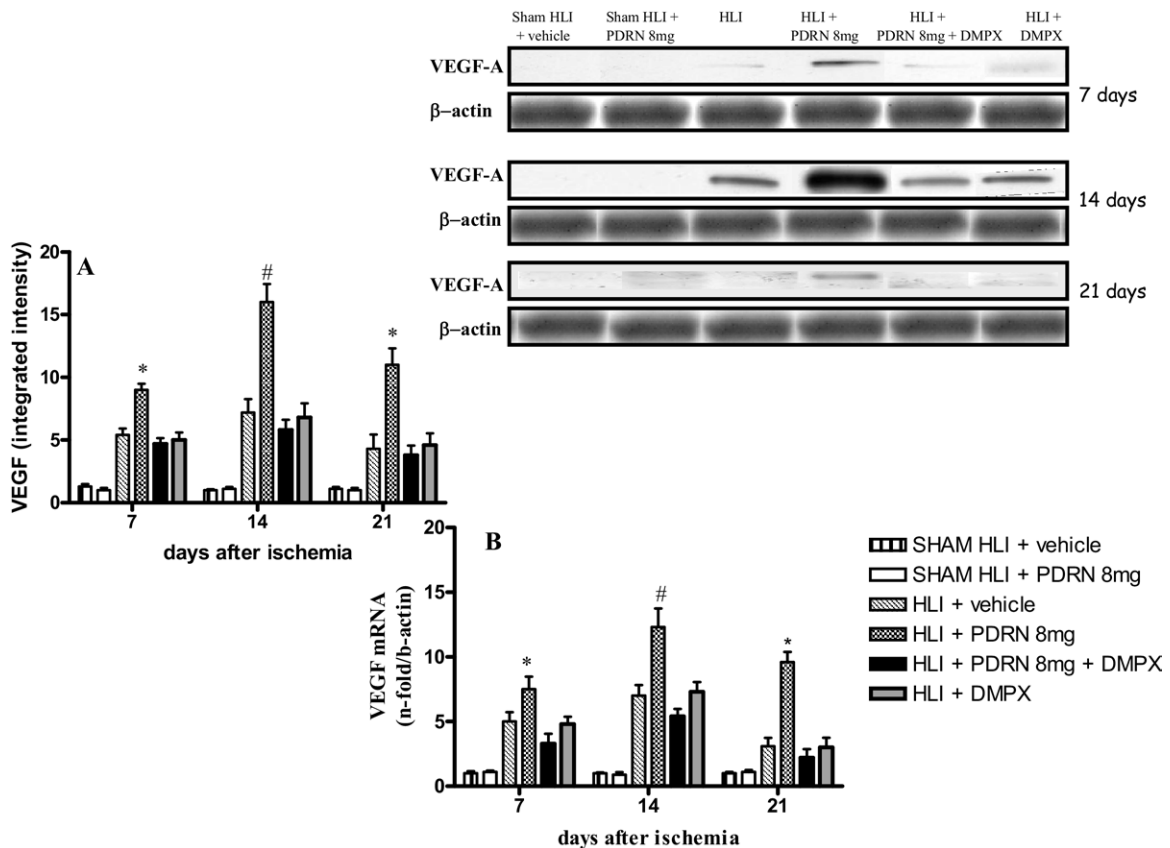


Fig 3. **A**, Western blots for vascular endothelial growth factor (*VEGF*) at 7, 14, and 21 days after hind limb ischemia (*HLI*). **Upper panel**, Representative autoradiography highlighting *VEGF* expression. **Lower panel**, quantitative data representing the mean \pm SD of seven experiments. * $P < .005$ and # $P < .0001$ vs *HLI* + vehicle. **B**, Expression of messenger RNA for *VEGF* at 7, 14, and 21 days after *HLI*. **Bars** represent the mean \pm SD of seven experiments. * $P < .005$ and # $P < .0001$ vs *HLI* + vehicle. *DMPX*, 3,7-dimethyl-1-propargilxanthine; *PDRN* polydeoxyribonucleotide.

PDRN decreases tissue edema and oxidative stress in ischemic hind limb. As a consequence of critical limb ischemia, untreated rats developed a marked edema (5.8 ± 0.6 ; $P < .0001$ vs sham, Fig 4, E) 7 days after surgery. *PDRN* treatment significantly reduced the degree of tissue edema detected in adductor muscles 7 days after surgical procedures (3.5 ± 0.8 ; $P > .0001$ vs *HLI* + vehicle; Fig 4, E). The concomitant administration of *DMPX* reversed the effects of *PDRN* in *HLI* animals. *DMPX* alone did not modify tissue edema in sham *HLI* and *HLI* animals (results not shown). *PDRN* did not modify the wet weight-dry weight ratio in sham *HLI* rats (Fig 4, E).

Oxidative stress was measured by the means of *MDA* levels in both the ischemic (left) and contralateral (right) adductor muscles as a consequence of ischemia: After 7 days, *MDA* levels increased dramatically in *HLI* animals (left limb, 7.1 ± 2 nmol/mg of tissue; right limb, 3.5 ± 0.8 nmol/mg of tissue; $P < .01$ vs sham *HLI*). *PDRN* daily administration blunted this increase in both limbs (left, 2.5 ± 0.7 nmol/mg of tissue; right, 1.8 ± 0.5 nmol/mg of tissue; $P < .05$ vs *HLI*). The concomitant administration of *DMPX* reversed the beneficial effect of *PDRN*.

Histologic evaluation. A complete histologic examination was performed after 21 days. The histologic pictures and the histologic score shown in Fig 4 (A-D) clearly demonstrate a marked edema with cellular infiltration, primarily macrophages and necrotic degeneration of muscle fibers of the adductor in *HLI* animals (Fig 4, B), when compared with the sham *HLI* animals (Fig 4, A). Animals treated with *PDRN* showed a marked decrease in edema, with no sign of cellular infiltration or muscular necrosis (Fig 4, C). The concomitant administration of *DMPX* reversed the effects of *PDRN* in *HLI* animals (results not shown). *DMPX* alone did not modify the histologic pattern in sham *HLI* and *HLI* animals (results not shown). *PDRN* did not modify histology in sham *HLI* rats (results not shown).

Moreover, the immunostaining for *PECAM-1* (Fig 5, A and B), performed to identify new capillaries between fibers, revealed a sustained angiogenesis in *PDRN*-treated *HLI* animals. When compared with *HLI* + vehicle animals, *DMPX* administration reversed the angiogenic effect of *PDRN* (Fig 5, C). In fact, angiogenesis in *PDRN*-treated animals was sustained by an increased *VEGF* production by the macrophages present in the site of ischemia, as demon-

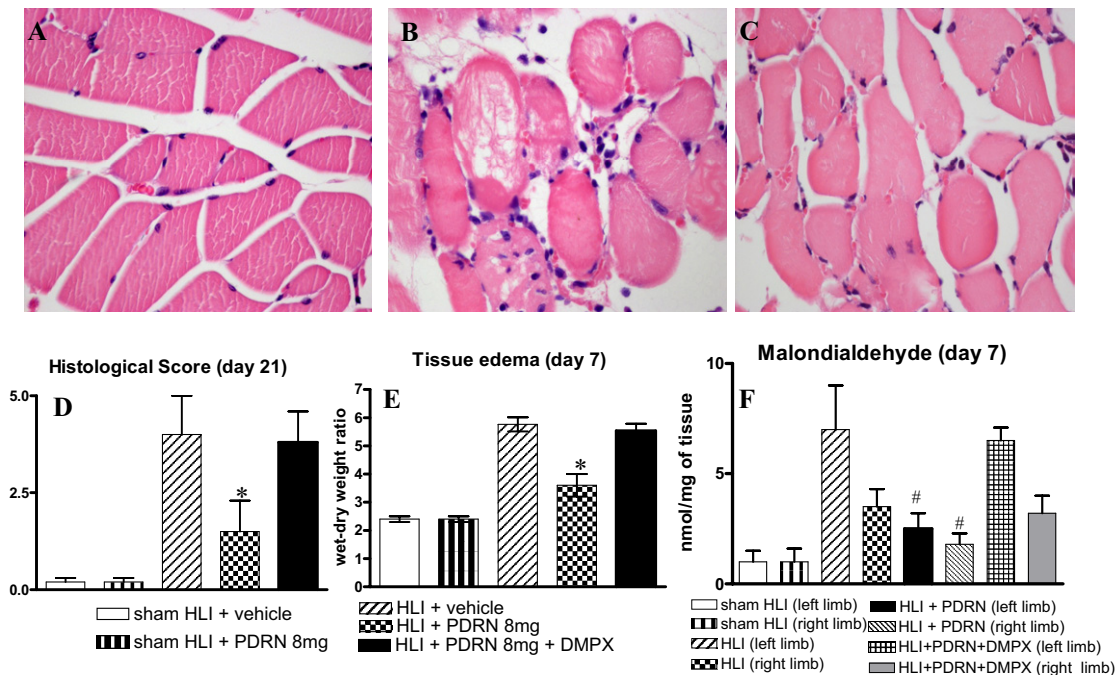


Fig 4. Histologic evaluation of adductor muscles after hind limb ischemia (HLI) at day 21 (original magnification $\times 20$). **A**, Sham HLI animal adductor muscle showing normal muscular architecture. **B**, HLI animal adductor muscle showing edema with marked cellular infiltration and necrosis. **C**, Adductor muscle from HLI rats treated with intraperitoneal polydeoxyribonucleotide (PDRN), at 8-mg/kg daily, showing reduced edema and cellular infiltration with no sign of necrosis. **D**, The means \pm SD of the histologic score obtained from seven animals. $*P < .005$ vs HLI + vehicle. **E**, Tissue edema evaluated in adductor muscles at day 7 after ischemia. PDRN (8 mg/kg) diminished the degree of edema in treated animals. $*P < .005$ vs HLI + vehicle. **F**, Malondialdehyde levels evaluated in adductor muscles of both legs at day 7 after ischemia. PDRN diminished the degree of edema in treated animals. $\#P < .05$ vs HLI. DMPX, 3,7-dimethyl-1-propargilxanthine.

strated by Western blotting and real-time PCR evaluation and confirmed by immunostaining (Fig 5, D and E).

DISCUSSION

The clinical consequences of PAOD include pain on walking, pain at rest, and loss of tissue integrity in the distal ischemic limbs. Although development of beneficial drugs and intervention devices have contributed to the treatment of this disease, critical limb ischemia is estimated to develop in 500 to 1,000 individuals per million each year.¹⁶ Because angiogenic growth factors can stimulate the development of collateral arteries, a concept called "therapeutic angiogenesis" is now being evaluated in various clinical fields.

Recent progress in molecular biology has led to the development of gene therapy as a new strategy to treat a variety of cardiovascular diseases using angiogenic growth factors such as VEGF.¹⁷ Therapeutic angiogenesis using angiogenic growth factors, particularly VEGF, is expected to be a new treatment for patients with severe PAOD. The most used approaches have been based on VEGF local delivery but failed to meet the primary objective of significant amputation reduction.¹⁸ As summarized in a recent review,¹⁷ other treatments have been studied in patients with critical limb ischemia, such as the delivery of cytokine

growth factor, but larger clinical studies have failed to achieve concrete results.

An increased understanding of the complex mechanisms involved in angiogenesis has resulted in gene therapy¹⁹ and cell therapy²⁰ being moved to the forefront of therapeutic angiogenesis, but we are still far from the identification of a definitive treatment. To date, no other drug treatment has been demonstrated efficacious in stimulating angiogenesis in the ischemic limb; furthermore, it has been shown that adenosine receptor stimulation can induce the expression of VEGF in many types of cells,²¹ and this may be achieved by stimulating the A_{2A} or A_{2B} receptor, or both, following the signaling pathways activated by hypoxia.^{21,22} In addition, A_{2A} receptors are expressed on different cell types, especially under ischemic conditions.²³⁻²⁷

These observations, together with our previous findings on the ability of PDRN to induce VEGF expression during altered healing conditions such as diabetes¹¹ and burn injury,¹² acting through the A_{2A} receptor, led us to hypothesize that PDRN could be also efficacious in stimulating VEGF and revascularization in the ischemic limb. Indeed, in this study we demonstrated the beneficial role of

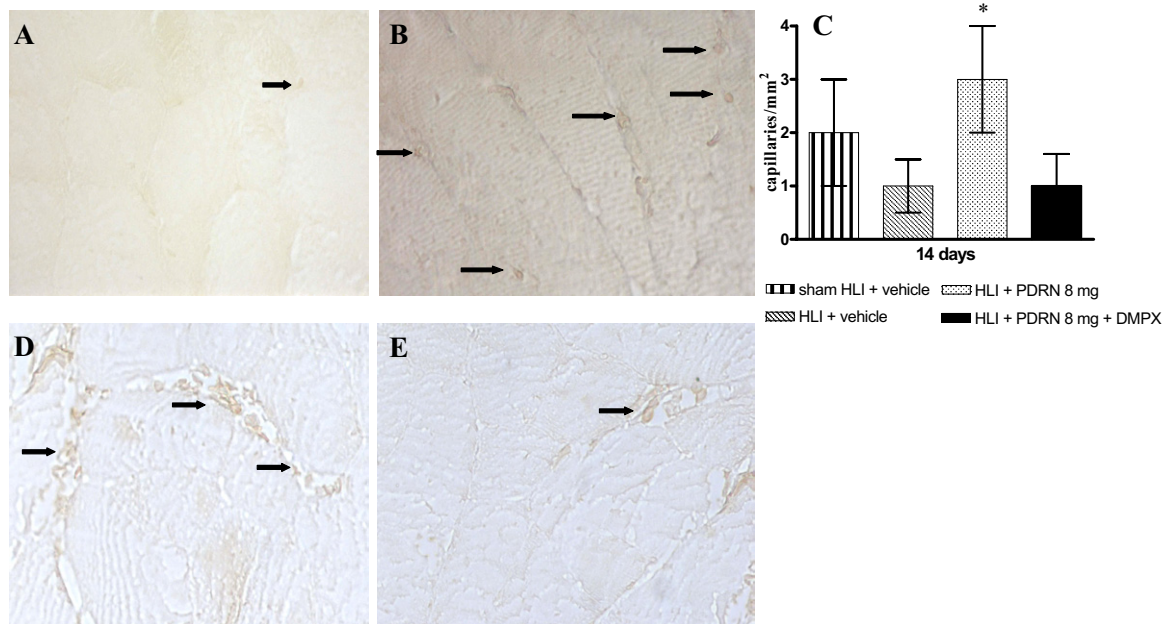


Fig 5. Immunohistologic evaluation of adductor muscles after hind limb ischemia (HLI) at day 14 (original magnification $\times 40$). **A**, Adductor muscle from HLI animal stained with platelet-endothelial cell adhesion molecule 1 (PECAM-1). *Arrowhead* shows very slight positive capillary between muscle fibers. **B**, Adductor muscle from HLI animal treated with polydeoxyribonucleotide (PDRN; 8 mg/kg) stained with PECAM-1. *Arrowheads* show positive capillaries between muscle fibers. $*P < .005$ vs HLI + vehicle. **C**, The mean \pm SD number of capillaries/mm² are presented; each bar is the mean of seven animals. $*P < .005$ vs HLI + vehicle. **D**, Adductor muscle from HLI animal stained with vascular endothelial growth factor (VEGF). *Arrowheads* show positive cells between muscle fibers. **E**, Adductor muscle from HLI animal treated with PDRN (8 mg/kg) stained with VEGF. *Arrowheads* show positive cells between muscle fibers.

PDRN in restoring blood flow by promoting angiogenesis in an experimental model of PAOD.

Femoral artery ligation and excision provoked an immediate impairment in perfusion, as revealed by laser Doppler flowmetry that persisted after 21 days after the surgical procedures. The inflammatory reaction consequent to the observed muscular damage is also responsible for the marked edema found in the ischemic limbs. The administration of PDRN, likely through the stimulation of the adenosine A_{2A} receptor, induced VEGF synthesis and release with a consequent neovascularization, as demonstrated by the incremented perfusion and number of capillaries in the ischemic limb as well as by the ameliorated histologic pattern. Indeed, the concomitant administration of DMPX, a potent and selective adenosine A_{2A} receptor antagonist,²⁸ abolished the effects of PDRN on blood flow restoration and VEGF production, as we previously observed in conditions of altered skin flow.^{11,12}

Ischemia causes also a defective perfusion of the contralateral limb as a consequence of the augmented oxidative stress. In fact, when ischemia occurs in a system that is strictly connected with another one, as is the case of limbs, is not surprising that a compensatory mechanism is activated in the contralateral apparatus that may be stressed and in turn results ischemia even if in a lower extent. The

ischemia we showed in the contralateral limb was present in all the HLI animals and we strongly believe that this cannot confound the results of PDRN, but the amelioration seen also in the contralateral limb may be a further proof of PDRN efficacy, as confirmed by the reduced MDA levels observed in both the ischemic and the contralateral limb. Besides the local injury that occurred within the muscle subjected to ischemia, the contralateral muscles also sustained remote injury, which was confirmed in our experimental model by a reduction in blood flow in the contralateral limb, and PDRN succeeded also in ameliorating this alteration.

These data collectively suggest that PDRN may favorably alter the longitudinal profile of critical limb ischemia towards revascularization, rather than simply sustain the process, and may have important clinical relevance for the safe and efficacious profile of this drug. PDRN has no antigenic properties since it consists of low-molecular-weight DNA fractions that can be defined as deoxyribonucleotide linear polymers. It is obtained by extraction from trout sperm and is then purified and sterilized to obtain >95% of DNA without pharmacologically active proteins and peptides.

Moreover, in previous unpublished observations, PDRN did not show any toxicity in primary cultures of rat

macrophages challenged with PDRN (up to 640 µg/mL). Furthermore, acute and chronic toxicity studies have been performed in mice and rats, and systemic administration of PDRN caused neither death, nor toxic effects on liver, lungs, brain, skeletal muscle, and heart evaluated macroscopically and by means of histologic analysis.

Because PDRN is approved in Italy for both parenteral and topical use (Registration Dossier, Ministry of Health), we hypothesized that in the future it might replace other systemic treatments that are usually difficult to manage and can be responsible for serious adverse effects; on the other hand, further studies are still needed to fully understand the benefits and limits of this compound.

CONCLUSION

This study used a novel approach to increase endogenous expression of vascular endothelial growth factor in a preclinical model of PAOD, where the capacity to induce therapeutic angiogenesis is known to be very difficult. To date, this is at least one of the most relevant studies showing beneficial effects of a drug with a high safety profile in critical limb ischemia. This approach will serve as a valuable link to advance this and related approaches, toward human investigation.

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AUTHOR CONTRIBUTIONS

Conception and design: AB, FS
Analysis and interpretation: AB, FP
Data collection: FP, LM, AM
Writing the article: AB, FS
Critical revision of the article: AB, FS
Final approval of the article: AB, FS
Statistical analysis: AB, FP
Obtained funding: FS, DA
Overall responsibility: FS
AB and FP contributed equally to this work

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